County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:09 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph 1 of 9
Attachments: Octinoxate HEL Monograph.pdf; 1Tsui Seasonal Occurence Removal Efficiences.pdf; 2 Tsui Occurence Distribution and Ecological Risk Assessment 2.pdf; 3 Tsui Occurence Distribution and Ecological Risk Assessment 1.pdf

Aloha Chair and Maui County Council,

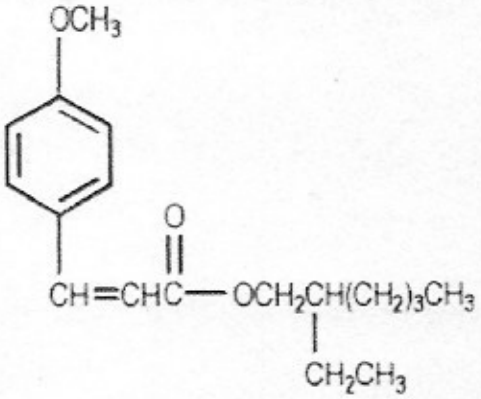
Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

Octinoxate



Chemical Identity

Chemical Abstract Service (CAS) Registry Number: 5466-77-3

Molecular Weight (MW) – 290.40 (A molecular weight below 500 Daltons allows for easy absorption of the chemical through animal and human membranes – e.g.; cells, skin, placenta ... etc).

United Nations Global Harmonized System (GHS) – Hazard Statements: H413 – May cause long lasting harmful effects to aquatic life [Hazardous to the aquatic environment, long-term hazard]

Technical Name(s): 2-Ethylhexyl Methoxycinnamate; 2-Ethylhexyl 4-Methoxycinnamate;

p-Methoxycinnamic Acid, 2-Ethylhexyl Ester; 3-(4-Methoxyphenyl)-2-Propenoic Acid, 2-Ethylhexyl Ester; Octyl Methoxycinnamate; 2-Propenoic Acid, 3-(4-Methoxyphenyl)-, 2-Ethylhexyl Ester.

Trade Name/Supplier: AEC Ethylhexyl Methoxycinnamate (Á & E Connock Perfumery & Cosmetics) Ltd.); Custoscreen OMC (Custom Ingredients, Inc.); Escalol 557 (Ashland Inc.); Heliosol 3 (Laboratoires Prod'Hyg); Jeescreen OMC (Jeen International Corporation); Neo Heliopan AV (Symrise); Nomcort TAB (The Nisshin OilliO Group, Ltd.); OriStar OMC (Orient Stars LLC); Parsol MCX (DSM Nutritional Products, Inc.); Uvinul MC 80 (BASF Corporation); Uvinul MC 80 N (BASF Corporation).

FDA Voluntary Cosmetic Registration Program (VCRP): Use as of 01/2015 = 4,783 products.

Use Level: Up to 7.5% in Sunscreens in the United States; Up to 10% in other countries.

Reported Product Categories:

Aftershave Lotions; Baby Shampoos; Basecoats and Undercoats; Bath Capsules; Bath Oils, Tablets, and Salts; Bath Preparations, Misc.; Bath Soaps and Detergents; Blushers (All types); Body and Hand Preparations (Excluding Shaving Preparations); Bubble Baths; Cleansing Products (Cold Creams, Cleansing Lotions, Liquids and Pads); Colognes and Toilet Waters; Cuticle Softeners; Deodorants (Underarm); Eye Lotions; Eye Makeup Preparations, Misc.; Eye Shadows; Eyebrow Pencils; Eyeliners; Face Powders; Face and Neck Preparations (Excluding Shaving Preparations); Foundations; Fragrance Preparations, Misc.; Hair Coloring Preparations, Misc.; Hair Conditioners; Hair Dyes and Colors (All Types Requiring Caution Statements and Patch Tests); Hair Preparations (Non-coloring), Misc.; Hair Rinses (Coloring); Hair Shampoos (Coloring); Hair Sprays (Aerosol Fixatives); Hair Wave Sets; Indoor Tanning Preparations; Lipsticks; Makeup Bases; Makeup Fixatives; Makeup Preparations (Not eye), Misc.; Manicuring Preparations, Misc.; Moisturizing Preparations; Nail Creams and Lotions; Nail Polish and Enamel Removers; Nail Polish and Enamels; Night Skin Care Preparations; Paste Masks (Mud Packs); Perfumes; Personal Cleanliness Products, Misc.; Powders (Dusting and Talcum, Excluding Aftershave Talcs); Rouges; Shampoos (Non-coloring); Shaving Preparations, Misc.; Skin Care Preparations, Misc.; Skin Fresheners; Suntan Gels, Creams, and Liquids; Suntan Preparations, Misc.; Tonics, Dressings, and Other Hair Grooming Aids.

Octinoxate: Human and Environmental Contamination

Octinoxate is a ubiquitous environmental contaminant – it is found in streams, rivers, lakes and in marine environments from the Arctic Circle (Barrow, Alaska) to the beaches and coral reefs along the equator [1-7, 82]. It is considered an environmental hazard in many locations [6-9, 86, 87], and is one of 10 chemicals listed on the European watch list of substances that may pose a significant risk to the aquatic environment [10]. Octinoxate can be found in both municipal treated and desalinated drinking water [11-13, 24]. Sewage sludge can be heavily contaminated by Octinoxate and other Personal Care Product Chemical, further expanding the types of sources contaminating the environment (e.g., biosolids)[14]. Swimmers directly contaminate water sources, but point and non-point sewage and treated waste-water effluent discharges maybe the largest source of contamination [14-16]. The United Nations Global Harmonized System (GHS) is used in the United States (US) by OSHA, EPA, DOT and CPSC as well as European and Asian countries, and identifies Octinoxate as an environmental hazard and carries the following warning label "H413 – May cause long lasting harmful effects to aquatic life" (see diamond logo at top of page)[17]. As sunscreen usage increases worldwide (projected global sales of $11 billion by 2020), so can the levels of environmental contamination that impact human and aquatic life. As of 2015, the U.S. Food & Drug Administration Voluntary Cosmetic Registration Program identifies 4,753 sunscreen and cosmetic formulas that contain Octinoxate [18].

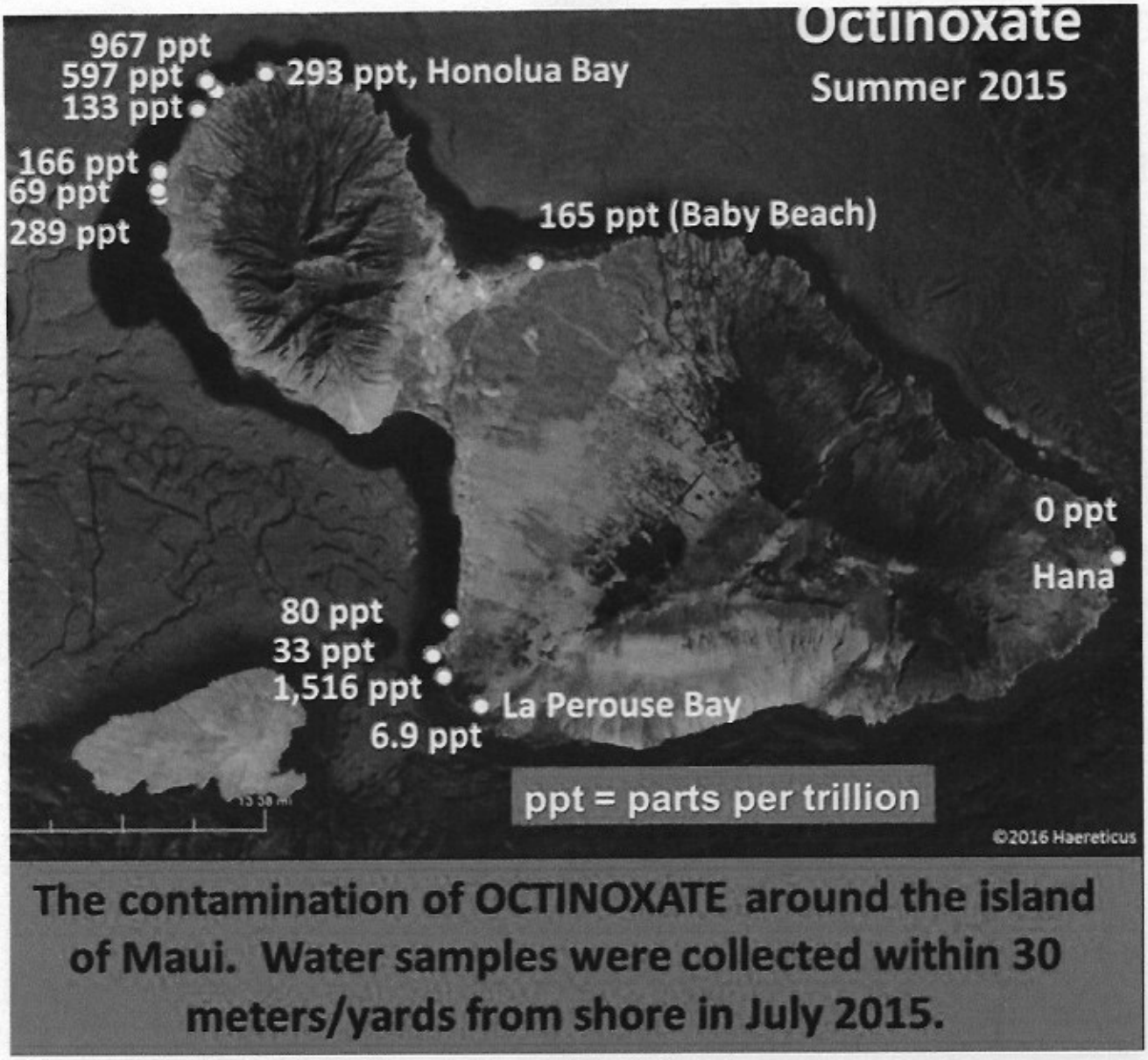
Octinoxate is absorbed directly through the skin, via the application of sunscreens and other personal care products. Depending on the topical vehicle used, relatively little chemical (less than 1% to 6%) is absorbed into the skin and excreted in the urine, leaving 94% – 99% on the skin that can be washed off into various water sources [19-23]. Octinoxate is a fat-soluble chemical, which means that some of it that absorbed by the body will be metabolized and excreted in urine, but much of it will be stored either in fat tissue or lipid-rich tissue such as the placenta [26, 27].

In aquatic and marine environments, water depth, light intensity and the amount of dissolved organic carbon content in the location determine the fate of Octinoxate [28]. Additionally, photo-degradation in the presence of titanium dioxide leads to the formation of more toxic by-products [34]. The increased toxicity is partially accounted for by the formation of 4-methoxybenzaldehyde, which is toxic to algae and aquatic invertebrates, such as *Daphnia*[29]. In sunscreen formulations, Octinoxate can react with Avobenzone reducing the overall sun protection factor of the product, leading to photo-instability and an increase risk of

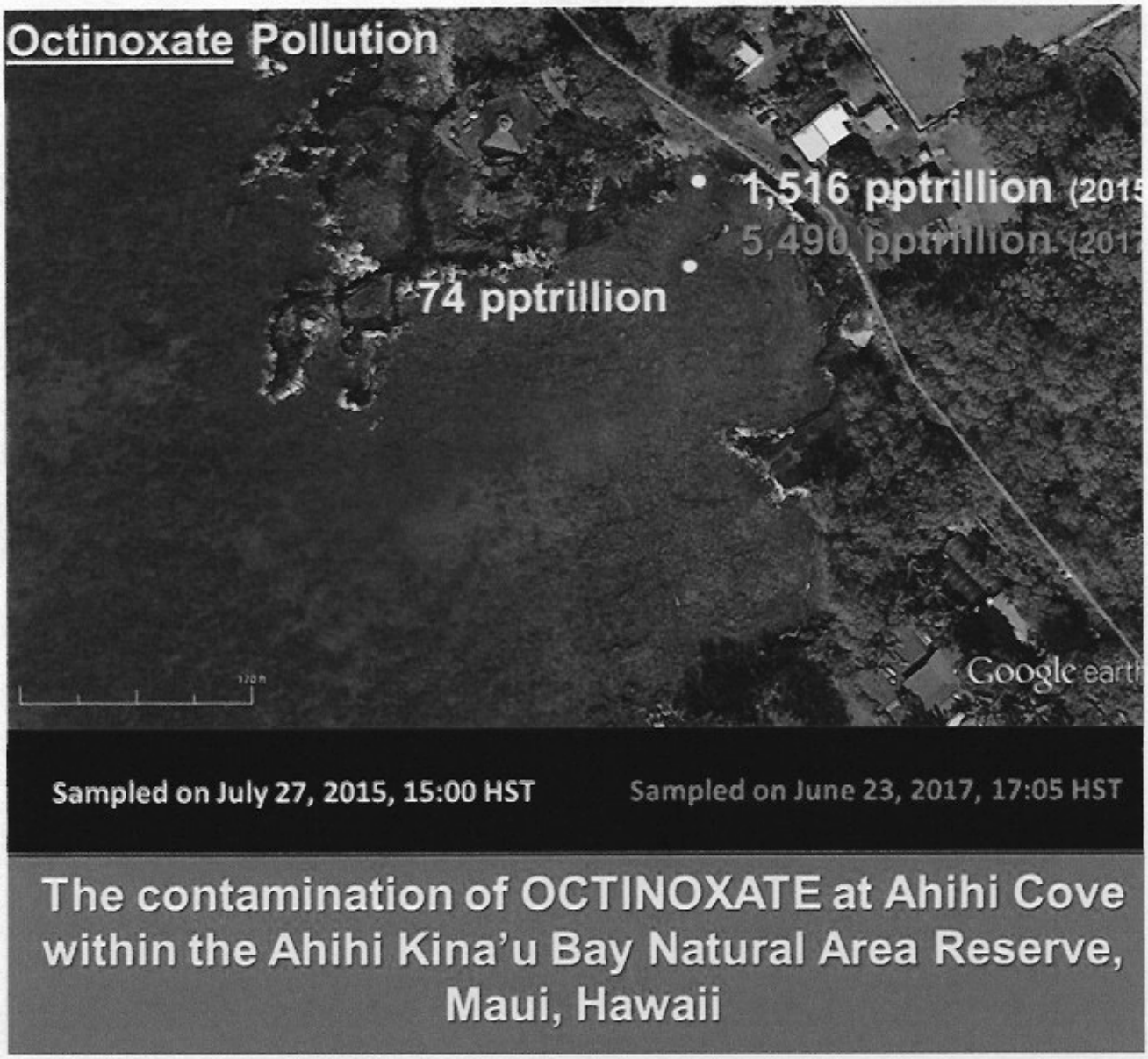
sunburn[30].

Octinoxate may also bioaccumulate and be biomagnified in organisms [28,87]. Biomagnification means Octinoxate may increase in concentration in the tissues of organisms as it travels up the food chain. A number of aquatic and marine species have been discovered to be contaminated, from carp, catfish, eel, white fish, trout, barb, chub, perch and mussels to coral, mahi-mahi, dolphins, sea turtle eggs, and migratory bird eggs [24,28, 86].

In coral reef environments, Octinoxate can reach more than 10 parts per billion. Along the west coast of Maui in 2015, Hawaii, 11 coral reefs sites that were sampled had octinoxate concentrations from 6.9 parts per trillion to 1,516 parts per trillion.



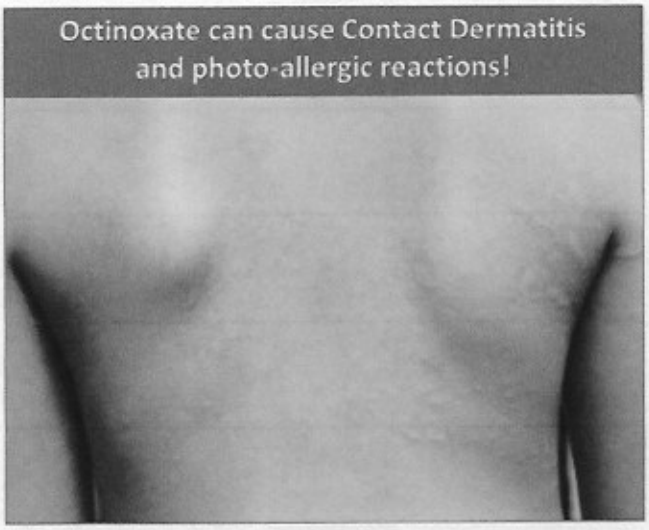
In the Ahihi Kina'u Bay marine protected areas, Octinoxate concentrations increased 3.6 times from 2015 to 2017.



Several scientific groups in different countries have done a formal environmental risk assessment of octinoxate to their marine ecosystems and have demonstrated that Octinoxate was a threat to the health of these ecosystems [6, 82, 86, 87].

Octinoxate Ecotoxicology

For humans and mammals, the most common pathological reaction to Octinoxate is contact dermatitis and photoallergic reactions [31-38].



Octinoxate on top of the skin or in the epidermal layer can be degraded by sunlight (called photodegradation), and those breakdown products can be especially toxic [39].

Once in the body, Octinoxate can cause toxicity to a number of different organ systems. Developing fetuses, babies, pre-adolescents, and even the pregnant mother are especially susceptible. In pregnant rats exposed to Octinoxate, there was a significant decrease in thyroid hormone levels (Thyroxine) [51]. Young male rats whose mothers were exposed to octinoxate had smaller testicals and lower semen quality, and a dose-dependent reduction in testosterone levels – meaning the more Octinoxate the mother was exposed to, the lower the level of testosterone in the offspring male. Young female rats from the same Octinoxate-exposed mothers exhibited reduced motor activity levels [51]. Like Oxybenzone, Octinoxate can impair both neurological and reproductive abilities [52-57, 73].

Another study that focused on two generations of rats exposed to Octinoxate also exhibited liver/blood disease, occurrence of ulcers in the stomach, and a higher risk to miscarriage [60]. Furthermore, the offspring had reduced organ weights, increased difficulty gaining weight during breast feeding, and a significant delay in sexual maturation [60]. It is by reasonable argument that Octinoxate should be classified as a **reproductive endocrine disruptor** [58-59]. A number of studies demonstrate that Octinoxate is a multi-system or multi-axis endocrine disruptor – meaning it can disrupt more than one type of endocrine system. Octinoxate can adversely affect estrogen receptors, androgen receptors, progesterone receptors, and thyroid hormone receptors [40-43, 48, 75, 80]. This mimicking of estrogen by Octinoxate was also shown to be able to adversely impact the immune system [67].

There have been a number of strong scientific studies on the impact of Octinoxate to the mammalian Thyroid gland and its function [53, 61]. Octinoxate affects both adult and juvenile mammals.

Danish scientists publicized in 2016 the impact of 29 different UV filters on sperm function and viability. Octinoxate was one of the UV filters that had an adverse effect on sperm function [85].

Genotoxicity of a chemical is a critical factor for its regulation and use in consumer products. The trend in the scientific literature indicates that Octinoxate is a genotoxin – meaning it damages DNA and the genetic material, and can give rise to genetic mutations, further resulting in the potential manifestation of reduced reproductive viability, adverse embryonic development, and cancer. One study provided data, using the Ames Test, that Octinoxate was mutagenic, as well as in a Fruit Fly genetic test [76]. The authors, in their paper, stated that "A trace contaminant may be implicated because many samples were obtained from several sources and the results were batch-related." This begs the question of why this study was allowed to be published by the journal, or were such statements in the paper a result of pressure from outside forces on the Journal's editorial staff. Other studies on Octinoxate's genotoxicity using bacterial models demonstrated positive mutagenicity [66, 77, 78]. One relatively recent study showed that Octinoxate does prevent one type of DNA damage by UV radiation, but it does not prevent DNA damage caused by oxidative stress [68].

There have been some *in vitro* cell culture studies, showing the toxicity of Octinoxate to neuroblastoma cells, liver stem cells, and human white blood cells [69, 70]. Some of these cell types exhibited a DNA-damage gene response exhibited, further arguing that Octinoxate is genotoxic [70, 72].

By 1994, over a million pounds of Octinoxate is manufactured each year (10). If historical evidence indicates the propensity of Octinoxate to be genotoxic, better studies by independent laboratories characterizing its genotoxicity and threat to human and ecological receptors is a necessity.

A "sister" compound of Octinoxate, called Cinoxate, supports this call for further investigation. Cinoxate was found to cause an increase in chromosome aberrations (type of genotoxicity) in mammalian cells using an industry-accepted method [79].

Carcinogenicity arises out of the interaction between genetic damage and cellular/tissue environmental instability. A recent paper by Alamer and Darbre shows that Octinoxate, Oxybenzone, Benzophenone-1 (breakdown product of oxybenzone), homosalate, and 4-MB-Camphor increased the metastatic behavior of breast cancer cells [80].

Toxicity to Wildlife – Most of the research has focused on the toxicity of Octinoxate to fish. Exposure to non-lethal concentrations radically alters the activation of genes in fish, altering the expression of over 1130 different gene transcripts [47]. Many of the altered genes play a role in hormonal regulation, including enzymes and proteins regulating estrogen and testosterone, as well as DNA damage and lipid synthesis.

At least three other studies in fish demonstrate that Octinoxate is an endocrine disruptor and causing reproductive disease at relevant environmental concentrations [44, 48-50]. A team of Dutch scientists were one of the first to show that Octinoxate induced estrogenic disruption in fish (Zebrafish) [48]. Scientists from Japan showed that male fish (Medaka) exposed to Octinoxate caused a reproductive endocrine disruption by having these male fish produce egg proteins [49]. Scientists in Switzerland confirmed these results using a different species of fish (fathead minnows); and that Octinoxate impacted multiple hormonal systems [50].

A team of Korean scientists did some amazing work showing that exposure to Octinoxate during embryonic development results in organ and body axis deformities in Zebrafish [71]. Octinoxate exposure had a statistically significant effect to induce liver defects. In this same study, a mixture of Oxybenzone and Octinoxate induced synergistic deformities in embryonic development of Zebrafish.

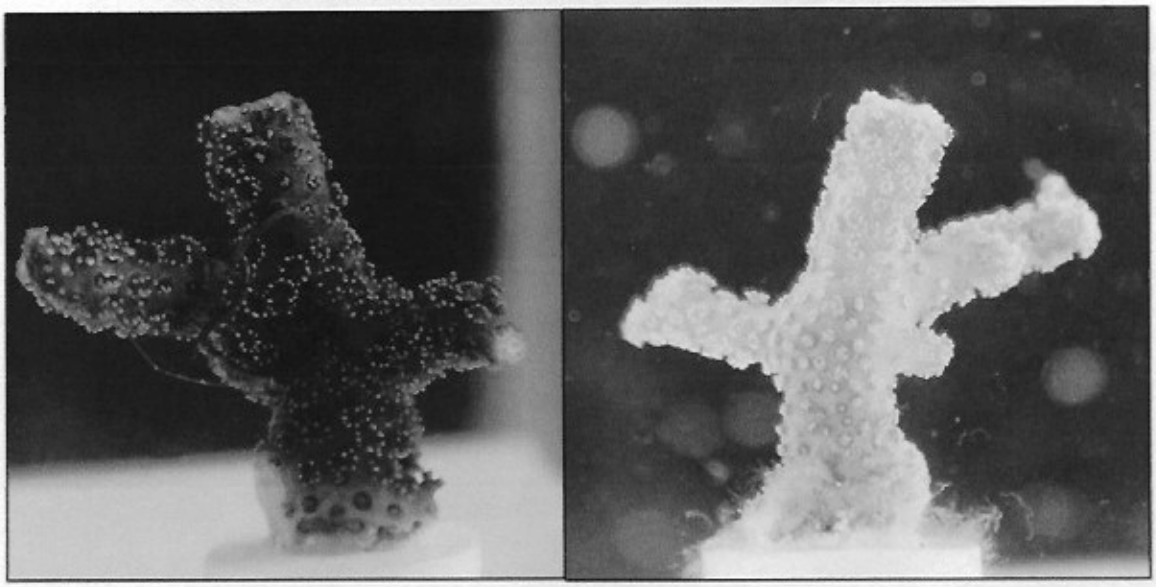
For invertebrates, such as a crustacean species of Daphnids, the same authors demonstrated that exposure of Octinoxate caused immobilization of *Daphnia*, as well as deformities [71, 45]. These results were consistent with an earlier study done by German Scientists on Octinoxate toxicity and Daphnia [83], which saw growth inhibition of Octinoxate at 240 parts per billion and as low as >40 parts per billion.

Studies on other invertebrates, such as the larvae of the aquatic midge, *Chironomus riparius*, indicated that Octinoxate induced the Stress Protein response in midges, as well as induced the overexpression of an insect hormone receptor (ecdysone receptor), indicating that it acts as an endocrine disruptor to insects [45].

One of the best scientific papers to examine the ecotoxicity of Octinoxate on the different trophic levels of a marine ecosystem was the work done by group of Spanish scientists [81]. In this study, the researchers looked at the toxicity of Octinoxate to an algae, a mussel, a sea urchin, and a shrimp (carnivore). They saw toxic effects of Octinoxate of these four organisms as low as 52 parts per billion and concluded that Octinoxate (and Oxybenzone) "could pose significant risks to marine aquatic ecosystem."

Future work from the Haereticus laboratory will be demonstrating the toxicity of Octinoxate to coral, including the inducing corals to undergo bleaching. Coral exposed to pollutants that causes them to bleach, makes these corals more susceptible when a climate event occurs, such as an El Nino-induced mass bleaching event.

Octinoxate Induces Coral Bleaching



Time 0

1 part per billion Octinoxate 14 days

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Seasonal occurrence, removal efficiencies and preliminary risk assessment of multiple classes of organic UV filters in wastewater treatment plants

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ABSTRACT

Organic ultraviolet (UV) filters are applied widely in personal care products (PCPs), but the distribution and risks of these compounds in the marine environment are not well known. In this study, the occurrence and removal efficiencies of 12 organic UV filters in five wastewater treatment plants (WWTPs) equipped with different treatment levels in Hong Kong, South China, were investigated during one year and a preliminary environmental risk assessment was carried out. Using a newly developed simultaneous multiclass quantification liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, butyl methoxydibenzoylmethane (BMDM), 2,4-dihydroxybenzophenone (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4) and 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) were frequently (≥80%) detected in both influent and effluent with mean concentrations ranging from 23 to 1290 ng/L and 18–1018 ng/L, respectively; less than 2% of samples contained levels greater than 1000 ng/L. Higher concentrations of these frequently detected compounds were found during the wet/summer season, except for BP-4, which was the most abundant compound detected in all samples in terms of total mass. The target compounds behaved differently depending on the treatment level in WWTPs; overall, removal efficiencies were greater after secondary treatment when compared to primary treatment with >55% and <20% of compounds showing high removal (defined as >70% removal), respectively. Reverse osmosis was found to effectively eliminate UV filters from effluent (>99% removal). A preliminary risk assessment indicated that BP-3 and EHMC discharged from WWTPs may pose high risk to fishes in the local environment.

1. Introduction

Organic ultraviolet (UV) filters are important components of sunscreen agents and other personal care products (PCPs) to protect human skin against sunburn and cancer by absorbing UV radiation (Giokas et al., 2007) and to prevent UV degradation of fragrances and dyes (Zenker et al., 2008). Organic UV filters can enter the aquatic environment (i) indirectly from wastewater treatment plants (WWTPs) and (ii) directly during

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recreational activities (Giokas et al., 2007). As WWTPs are not specifically designed to attenuate these micropollutants, incomplete removal of some UV filters including benzophenone-3 (BP-3), 4-methylbenzylidene camphor (4-MBC), octocrylene (OC) and 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) have been reported (Balmer et al., 2005; Kupper et al., 2006; Liu et al., 2012b). Organic UV filters have also been detected ubiquitously in various environmental samples including surface water, sediment (Kameda et al., 2011), and soil (Jeon et al., 2006), generally at ng/L to sub-ug/L levels for aqueous matrices and sub-ng/g levels for solid matrices.

With octanol–water partition coefficients (log K_{ow}) values higher than 3, bioaccumulation of organic UV filters in humans and other organisms is a concern because these compounds and their metabolites can act as environmental estrogens both *in vitro* and *in vivo* (Schreurs et al., 2002; Schlumpf et al., 2001); they also induced coral bleaching in both a lab and field study (Danovaro et al., 2008). To date, UV filters have been detected in organisms such as brown trout (*Salmo trutta fario*) up to 2400 (OC) ng/g lipid weight (lw) (Buser et al., 2006), Franciscana dolphins (*Pontoporia blainvillei*) at 89–792 ng/g lw (OC) (Gago-Ferrero et al., 2013), as well as in human breast milk and urine at maximum levels of 120 ng/g lw and 5900 ng/mL (BP-3), respectively (Schlumpf et al., 2008; Kunisue et al., 2012).

Authorized contents of organic UV filters and product formulations of PCPs vary according to legislation in force in the countries/regions of manufacture (Santos et al., 2012), resulting in spatial variation in the occurrence of these chemicals. Currently, data on the occurrence and effectiveness of wastewater treatment of UV filters in Asian countries are lacking. Hong Kong imports a wide variety of PCPs from several countries/regions and has no local regulations for organic UV filter content in products. The local environment is thus anticipated to be impacted by a greater diversity of UV filters than other locations. In light of these considerations, the objectives of this study were to (i) develop a quantitative analytical method for simultaneous multiclass determination of twelve globally consumed UV filters, (ii) determine their occurrence and removal efficiencies in wastewater samples collected from five WWTPs featuring different treatment levels including both conventional (e.g. primary sedimentation, biological treatment) and advanced treatment (e.g. chlorination, UV-disinfection, and reverse osmosis), and (iii) conduct a preliminary environmental risk assessment of these compounds.

2. Material and methods

2.1. Chemicals and materials

Information on chemical standards and preparation of standard solutions can be found in the Supporting Information (Section 1.1.). Standard purities were all ≥97%. As there is no list of UV filters approved for use in Hong Kong, the 12 analytes (butyl methoxydibenzoylmethane (BMDM), benzophenone-1 (BP-1), BP-3, benzophenone-4 (BP-4), benzophenone-8 (BP-8), EHMC, ethylhexyl salicylate (EHS), homosalate (HMS), isoamyl p-methoxycinnamate (IAMC), 4-MBC, OC and octyl dimethyl-p-aminobenzoic acid (OD-PABA)) were chosen based on a simple local survey of PCPs in shops in Hong Kong. Detailed properties of the targeted UV filters are shown in Table A1.

2.2. Sampling

Samples were obtained from five WWTPs equipped with different treatment levels (Figure A1). The types of samples collected, catchment populations, daily flows, hydraulic (HRT) and solid retention time (SRT) as well as treatment processes of the sampled WWTPs are listed in Table A2 (i) and (ii). Plant A is the largest WWTP in Hong Kong and features chemically-enhanced primary treatment (CEPT) to facilitate sedimentation via the addition of ferric (III) chloride. Chlorination is accomplished by addition of 10% (w/w) sodium hypochlorite (NaOCl) solution to CEPT-treated wastewater with 13 min of contact time. Plants B and C are secondary WWTPs featuring a Modified Ludzack-Ettinger system with UV-disinfection. Plant C is also equipped with pilot-scale micro-filtration/reverse osmosis treatment. Plant D is a preliminary screening plant screening grit with diameter >6 mm. Plant E, located in a tourist area in Hong Kong, is the only WWTP utilizing tertiary treatment which filters fine suspended solids by a dual-media filter containing carbon and sand followed by UV-disinfection and chlorination in order to provide reclaimed water.

Time-proportional 24-hour samples were collected at Plants A, B and C using refrigerated automatic samplers, while grab samples were taken personally from Plants D and E and by plant staff for the pilot-scale treatment at Plant C. Successive-three-day sampling was conducted at Plants A to D in both dry (February, November) and wet seasons (May, August) in 2012 and at Plant E in May 2012; as plant E serves a small population (0.6% of the total population of Hong Kong), seasonal sampling was not conducted. Three-day composite samples were obtained by pooling samples collected from each WWTP across days in each sampling month. Wastewater could not be collected on Sunday at Plants D and E due to the lack of auto-sampler and WWTP access. A detailed sampling schedule is shown in Table A3. Samples were collected in glass bottles pre-rinsed with Milli-Q water and methanol and the bottles were wrapped with aluminum foil to avoid contamination and photo-degradation of the target compounds. All samples were transported on ice to the laboratory in an insulated polystyrene box where they were stored in the dark at 4 °C prior to analysis. Sludge was not collected as it is sent to landfill sites for disposal; removal calculations and risk assessment were only conducted for the aqueous phase in this study as it was most relevant to risk assessment of the marine environment.

2.3. Analytical procedures

A new extraction method was developed for the simultaneous quantification of the targeted 12 globally authorized and commonly used UV filters in wastewater. Samples were filtered through 0.45-μm glass fiber filters (GC-50, 47 mm, Advantec) before solid phase extraction. Each cartridge was preconditioned successively with 15 mL of 50:50 v/v methanol: ethyl acetate (MeOH: EA) and 15 mL of Milli-Q water. Isotope-

labeled ^{13}C-BP-3 was spiked into each filtered sample (250 mL) as a surrogate standard before loading to the cartridge at a flow rate of 2–3 mL/min. Each sample bottle was rinsed with 10 mL Milli-Q water and loaded to the cartridge at the same speed. All eluates were discarded. After loading, the cartridges were washed by 10 mL Milli-Q water and dried under a vacuum for 10 min and then subjected to centrifugation at 3000 rpm (1734 rcf) for 2 min twice for further drying the water inside the cartridges. The target compounds were eluted from the cartridges using 3 × 4 mL of 50:50 v/v MeOH: EA. The volume of extracts was reduced to less than 0.5 mL under a gentle stream of nitrogen and the final volume was adjusted to 0.5 mL with methanol. All sample extracts were subjected to centrifugation at 9500 rpm (9384 rcf) for 10 min to avoid any suspended particles entering the instrument and then transferred to amber sample vials for standard addition. Finally the extracts were analyzed by a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometer (HPLC-ESI-MS/MS). Analytical duplication was carried out for each sample.

2.4. *Instrumental analysis*

Separation of analytes was performed using an Agilent HP1200 LC (Agilent, Palo Alto, CA, USA) interfaced with an AB SCIEX API 3200 triple quadrupole tandem MS equipped with a Turbo V ion source (AB SCIEX, Framingham, MA, USA) operated in both negative and positive mode. The instrumental detection limits, ranging from 0.003 to 0.218 ng/mL, were calculated by adding the intensity obtained from methanol (baseline) to three times its standard deviation. The detailed LC-MS/MS experimental parameters for the determination of UV filters are listed in Table A4 and Section 1.2. in the Supporting Information. Target compounds were quantified by the standard addition method in order to obtain reliable quantification because of the lack of representative commercially available internal standards for the target compounds. Chromatograms for the influent matrix spike standard are shown in Figures A2 and 3 in the Supporting Information.

2.5. *Method validation*

Matrix-matched recoveries, method limits of detection (MLODs) were calculated and Milli-Q blank samples were analyzed to ensure the reliability of the method. The detailed validation procedures or each compound are provided in the Supporting Information. Generally, recoveries ranged from 64% to 103% in influent and effluent. MLODs ranged from 0.3 to 66.6 ng/L in influent and 0.11–11.6 ng/L in effluent. All of the target compounds were below MLODs in both field and procedural blanks. Detailed method validation information can be found in the Supporting Information Section 1.3. and Table A5.

3. Results and discussion

3.1.1. *Occurrence of organic UV filters at five wastewater treatment plants*

All targeted compounds were detected in influent samples from Plants A to D during at least one of the sampling months throughout the year, and BMDM (mean – maximum, ng/L: 250–1290), EHMC (438–1134), BP-3 (284–577), BP-4 (643–946) and BP-1 (163–281) were detected in all influent samples (Fig. 1). The high detection frequencies of these compounds were likely due to wide usage of PCPs containing these compounds as active ingredients, a finding which corresponds to the results of a small-scale survey of products on the local market (data not shown). All compounds, except OC, were detected in the effluent samples from Plants A to D during at least one of the sampling months throughout the year, while BMDM (140–1018), BP-3 (111–541) and BP-4 (384–497) were detected in all effluent samples (Fig. 2). The detection frequencies of EHMC (163–505), BP-1 (86–155) and OD-PABA (57–224) were higher than 75% throughout the year. These results indicated that the elimination of organic UV filters in WWTPs was incomplete and that more than half of the targeted compounds are discharged to local freshwater and

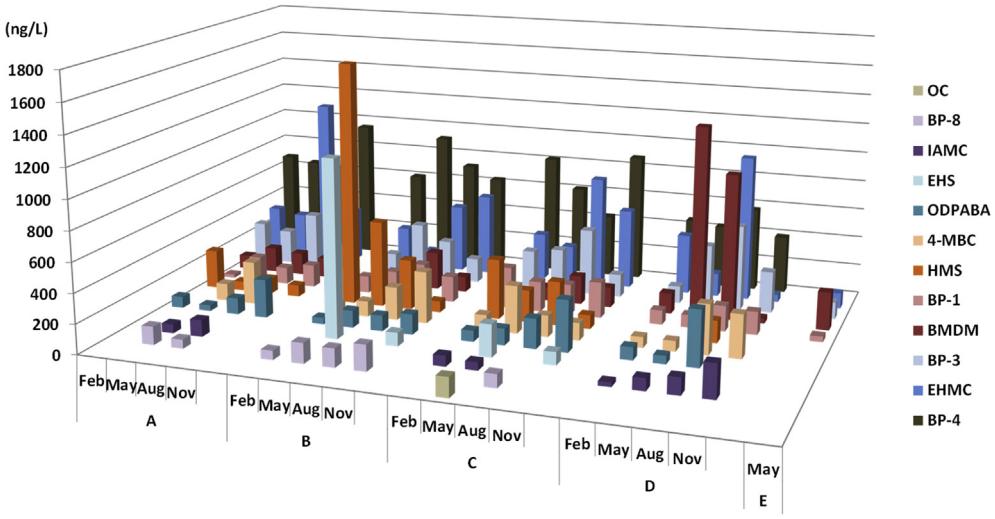

Fig. 1 – Influent concentrations of UV filters (ng/L) at the five sampled plants (A–D) in February, May, August and November 2012; (E) in May 2012. No bar indicates concentrations < MLODs. Detailed numerical data are shown in Table A6.

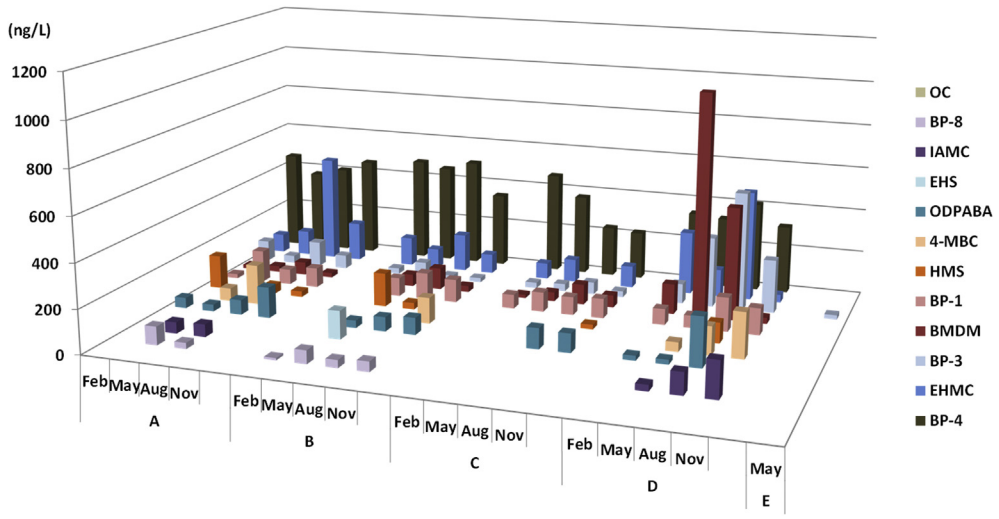

Fig. 2 – Effluent concentrations of UV filters (ng/L) at the five sampled plants (A–D) in February, May, August and November 2012; (E) in May 2012. No bar indicates concentrations < MLODs. Detailed numerical data are shown in Table A7.

marine habitats. Estimated consumption of organic UV filters was calculated by multiplying the maximum influent concentration detected in 24-hour composite samples from plants A and B by the daily flow rate and dividing this value by the catchment population served by the WWTP; the unit of this comparison is mg/day/1000 people. During the sampling period, BP-4 was the most abundant compound detected, with maximum concentrations of 946 ng/L and 497 ng/L in influent and effluent, respectively. These higher levels are probably due to its low octanol–water partition coefficient (log K_{ow} = 0.89) and tendency to distribute to the aqueous phase. The maximum daily consumption of BP-4 in Hong Kong was 378 mg/day/1000 people, which was comparable to that detected in Spanish WWTPs (438 mg/day/1000 people; Rodil et al., 2008). 4-MBC was found to have a lower daily consumption worldwide when compared with BP-4 and the maximum daily consumption levels of this compound—46.8, 46.4 and 36.1 mg/day/1000 people in Hong Kong, Spain and Australia, respectively—were comparable (Rodil et al., 2008; Liu et al., 2012b); as 4-MBC is not permitted for use as a cosmetic ingredient in Japan, it was not detected in wastewater or surface water there (Kameda et al., 2011). The average mass flow of BP-3 was 34.2 g/day and 37.1 g/day in the dry and wet seasons, respectively, which was comparable with that reported in Australia in October (36.9 g/day), but was eight times higher than that reported in April, 4.3 g/day (Liu et al., 2012b). In the same Australian study, the mass flow of 4-MBC was 12.2 g/day in October which was 2 and 8 times lower than that found in Hong Kong. The detailed mass flow and environmental loadings data are shown in Tables A8 and 9. IAMC was found occasionally in influent and effluent and was mainly detected in May and August, indicating greater use and release during the summer. The relatively higher MLODs and lipophilicity of OC and EHS likely explain their low detection frequencies (6% and 25% in influent, respectively) in the present study. It is predicted that these compounds would occur in the solid phase (e.g. particulate matter or sludge) due to their relatively higher log K_{ow} values (Table A1). Four compounds, BMDM, EHMC, BP-1 and BP-3, were detected in influent from Plant E at levels ranging from 37 to 257 ng/L; only BP-3 was detected in the final effluent at a concentration of 19 ng/L. The low occurrence and concentrations of UV filters found in this WWTP are likely due to a combination of a smaller serviced/resident population and the advanced treatment methods used. In order to compare the results among WWTPs, the results from Plant A were pooled across three days (inter-day RSD < 8%).

3.1.2. *Seasonal patterns*

Seasonal patterns were observed for BMDM, BP-1, BP-3, BP-8, EHS and EHMC during the sampling period in which they showed higher concentrations (by over 30%) in the wet season than the dry season. The results indicated that larger quantities of these compounds were used during the summer. As a result, these compounds may also pose higher risk for aquatic organisms during the wet season. Although BP-4 was found to be the most abundant compound in wastewater, it did not show a seasonal pattern. Based on our survey, this result is likely because it is used mostly in PCPs such as hand washes/soaps or shower gels that are not used seasonally. The concentrations of OD-PABA, 4-MBC, IAMC, OC and HMS were similar (<30% difference) in WWTP influent and effluent throughout the sampling period.

3.2. *Removal efficiencies of the targeted organic UV filters*

Removal efficiencies of organic UV filters from the aqueous phase were calculated as follows:

$$\text{Removal efficiency} = \frac{\text{Pre-treatment concentration} - \text{Post-treatment concentration}}{\text{Pre-treatment concentration}}$$

Removal efficiencies were defined as "low", "moderate" and "high" when the removal efficiencies were 0%–30%, 30.1%–70% and 70.1% to >99%, respectively.

The results from Plants A and D showed that the overall removal efficiencies of CEPT/chlorination and preliminary screening throughout the year ranged from 13 to 99% and 1–99%, respectively, with only 18% and 9% of the detectable compounds achieving high removal efficiencies, while those of biological secondary treatment/UV-disinfection at Plants B and C ranged from 20 to 99%, with 55% and 69% of the detectable compounds achieving high removal efficiencies, respectively (Fig. 3). Biological treatment with activated sludge and reverse osmosis was therefore an effective way to remove most of the UV filters compared to preliminary treatment and CEPT. In Plant E, removal efficiency of the target UV filters was >99% except for BP-3 (83%). In this study, benzophenone derivatives showed the best removal during secondary treatment with activated sludge, while compounds with relatively higher log K_{ow} values showed better removal during sedimentation and tertiary treatment. The removal efficiencies of 4-MBC, BP-3, OC and EHMC have been investigated previously: their removal ranged from 18 to 99% in WWTPs equipped with mechanical, biological, chemical treatment and sand filtration (Balmer et al., 2005); and from 92 to 99% in a WWTP equipped with conventional activated sludge treatment (Kupper et al., 2006).

3.3. Preliminary treatment

Plant D applies preliminary treatment to screen grit with diameter exceeding 6 mm with a HRT of less than 1 h. Except for OC, EHS and BP-8, all target compounds were detected in Plant D samples at concentrations ranging between 29 and 1290 ng/L and 21–1020 ng/L in influent and effluent, respectively. The removal efficiencies of detectable UV filters after preliminary treatment were low to moderate, except for OD-PABA (75%) during the dry season. This result showed that physical screening is not an effective way to remove most organic UV filters. Approximately 294 million m^3 of wastewater, or 30% of the annual wastewater treatment volume, is treated by preliminary treatment in Hong Kong (Drainage Services Department (DSD), 2011); in mainland China, 22% of annual wastewater receives only preliminary treatment (NBSC, 2011) and thus continuous release of these compounds to the aquatic environment is likely to occur, which raises concerns about potential ecotoxicological effects (Kaiser et al., 2012).

3.4. Primary sedimentation

During primary sedimentation, almost 50% of the suspended solids in the preliminarily-treated sewage are settled out and removed as primary sludge, with a HRT of 3 h. All detectable compounds showed low to moderate removal efficiencies during this treatment while the differences in removal of each compound between the dry and wet seasons were lower than 12% except for EHS (37%). The removal efficiencies for the benzophenone group UV filters (BP-1, BP-3, BP-4 and BP-8) in both plants were 10–26% and 7–28% in the dry and wet seasons, respectively. Removal efficiencies of primary sedimentation generally depend on the sorption coefficient of compounds and the amount of suspended solids in different batches of wastewater. Log K_{ow} values of the four benzophenone derivatives range from 0.89 to 3.82 (Table A1), which are lower than those of other organic UV filters, likely resulting in less sorption of the benzophenones to primary sludge. In contrast, moderate removal was observed for BMDM, HMS, 4-MBC, OD-PABA and EHMC (log K_{ow} > 4), indicating that sorption to primary sludge removed organic UV filters with higher log K_{ow} but still did not achieve high removal efficiency.

3.5. Chemically-enhanced primary treatment

Compared with primary sedimentation, the removal efficiencies of some of the UV filters improved, from 26% to 75% (BMDM), 23%–52% (BP-3) and 32%–52% (EHMC) during the dry season, and from 26% to 69% (BMDM) and 28%–67% (BP-3) during the wet season As a coagulant, ferric (III) chloride neutralizes the surface charges on suspended particles and colloidal material, and thus allows their aggregation into larger and heavier flocs for sedimentation. UV filters associated with the surface of suspended particles were likely further removed by coagulation and flocculation processes, thus achieving better removal efficiencies than sole primary sedimentation. Previous studies reported that coagulation-flocculation provided a high degree of removal (>70%) of another group of organic micro-pollutants, polycyclic musks, due to their strong lipophilicity (log K_{ow} ~6) (Carballa et al., 2005; Suarez et al., 2009). Similar to primary treatment, the removal efficiencies of UV filters with lower log K_{ow} values were still insufficient (e.g. BP-1 and BP-4, <35%), likely because of their lower tendency to adsorb to flocs. CEPT is applied to treat over 50% of wastewater in Hong Kong, but these results show that it is not an effective way to remove organic UV filters from wastewater.

3.6. Secondary treatment

Modified Ludzack-Ettinger (MLE) systems with an initial anoxic zone for nitrogen removal followed by aerobic digestion are applied in Plants B and C. The removal efficiencies of EHS, HMS, BP-1, BP-3 and BP-8 reached >70% (dry) and 60% (wet). The high attenuation of BP-3 could be due to effective biodegradation, which has been demonstrated in a laboratory study (Liu et al., 2012a) and in WWTPs (Kupper et al., 2006; Liu et al., 2012b). The present study showed that benzophenone- and salicylate-derivatives were effectively removed by secondary treatment. Kupper et al. (2006) and Liu et al. (2012b) reported that biological removal efficiencies of BP-3, EHMC, 4-MBC and OC were ≥60%. In the present study, removal efficiencies were moderate to high for 4-MBC and EHMC: 38% and 55% (dry season); 77% and 30% (wet season). BMDM, IAMC and OD-PABA showed low to moderate removal throughout the year, likely because they were less biodegradable in the given aeration time.

In Plant E, a Sequencing Batch Reactor is utilized rather than the MLE system. Four compounds, BMDM, EHMC, BP-1 and BP-3, were detected in influent at Plant E at concentrations ranging from 37 to 257 ng/L. The removal efficiencies of

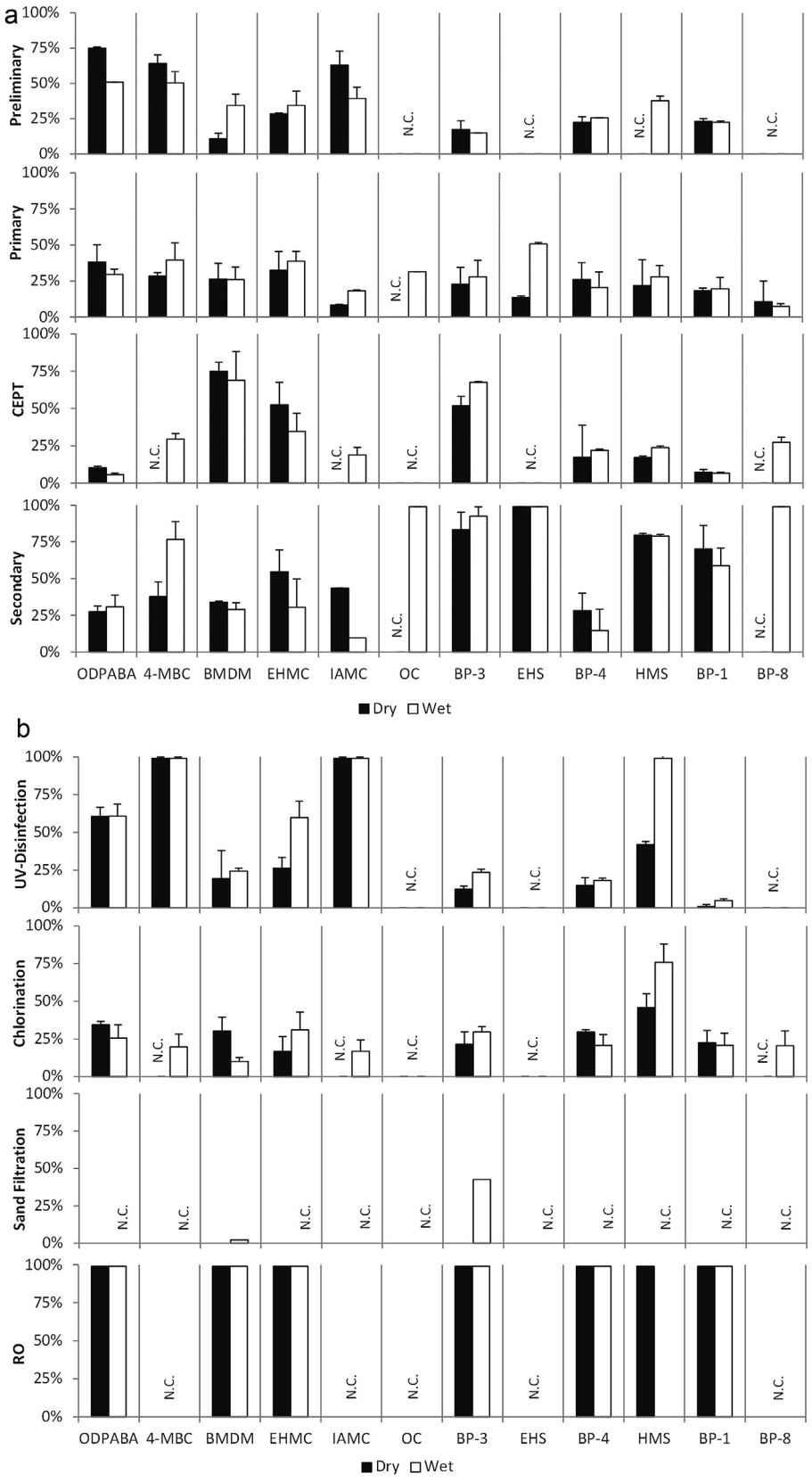

Fig. 3 – Mean removal efficiencies of UV filters in the dry and wet seasons by (a) preliminary screening, primary sedimentation, CEPT and secondary treatment and; (b) UV-disinfection, chlorination, sand filtration and reverse osmosis ($n = 4$). Detailed numerical data are shown in Table A10. N.C.: Not calculated, levels < MLODs.

BMDM, EHMC and BP-1 were high at 77%–99% while that of BP-3 was moderate (54%). The removal efficiencies of EHMC were consistent with those observed in previous studies (Kupper et al., 2006; Liu et al., 2012b), which showed that it was biodegraded during secondary treatment.

3.7. Chlorination

During chlorination, NaOCl reacts with organic compounds through (i) oxidation reactions, (ii) addition reactions to unsaturated bonds and (iii) electrophilic substitution reactions at nucleophilic sites (Deborde and von Gunten, 2008). In general, the removal efficiencies of chlorination treatment for detectable UV filters in Plant A in both seasons were low to moderate except for HMS, which showed 76% removal in the wet season. The removal efficiency of BMDM in Plant E was >99%, which was much higher than that in Plant A (lower than 30%), likely due to a longer chlorination time at Plant E compared to Plant A (30 and 13 min, respectively).

The most likely removal mechanism for UV filters was transformation by electrophilic substitution reactions. Negreira et al. (2008) detected chlorinated byproducts of OD-PABA and BP-3 in which chlorine atoms were substituted on the aromatic ring on the *ortho*- carbon to the amino moiety of OD-PABA as well as on the *ortho*- and *para*- carbons to the hydroxyl group of BP-3. A recent study also proposed transformation pathway of BP-4 in chlorine-containing water samples (e.g. tap water, swimming pool water and wastewater) via electrophilic substitution and oxidation (Negreira et al., 2012). As there is currently a lack of occurrence and toxicity information for UV filter chlorination by-products in the environment and in humans, development of analytical standards and further assessment of their environmental fate and behavior should be carried out.

3.8. UV-disinfection

UV irradiation can also oxidize organic contaminants in water either through direct photolysis or formation of reactive free radical from water or inorganic constituents to attack other organic compounds (Snyder et al., 2007). The UV irradiation parameters applied at Plant C were wavelength = 254 nm, dose = 47 mJ cm^{-2} and retention time = 0.426 s. The removal efficiencies of BP-1, BP-3, BP-4 and BMDM were <30% after UV-disinfection in both seasons; EHMC removal efficiencies were <30% in the dry season and 60% in the wet season (Fig. 3). The observed photostability of BP-3 towards UV irradiation was consistent with previous reports in which UV light intensity was 350 μmol photons m^{-2} and 2.3 W m^{-2} (Rodil et al., 2009; Liu et al., 2011). A laboratory study reported that irradiation with artificial sunlight resulted in *cis-trans* isomerization of EHMC and IAMC, keto-enol tautomerization of BMDM and dealkylation and hydroxylation of OD-PABA (Santos et al., 2012). However, removal of BMDM in both Plants C and E was low (<30%) and could be due to photostability enhancement by the presence of other UV filters creating a photoprotective effect (Kockler et al., 2012) and/or the presence of suspended particles that scattered and absorbed the UV irradiation during the treatment (Qualls et al., 1983). Photolysis of OD-PABA and EHMC were examined in previous studies (Sakkas et al., 2003; MacManus-Spencer et al., 2011) and the authors concluded that the photo-degradation kinetics of UV filters differed depending on the aqueous matrix tested (i.e. distilled water, swimming pool water and seawater) as well as dissolved organic matter, nitrate and chloride levels (Giokas and Vlessidis, 2007). Although one study showed that the photolysis by-products of IAMC, OD-PABA and EHMC were less toxic to algal reproduction rate than their parent substances after 14 h of irradiation (Rodil et al., 2009), it is important to note that UV irradiation of chlorine-containing wastewater is also a concern due to potential UV-mediated transformation of chlorinated by-products of UV filters (Sakkas et al., 2003). The UV-irradiation used in Hong Kong WWTPs for disinfection purposes employs a short irradiation time, which is likely ineffective for photo-degradation of organic UV filters having half-lives >20 h (Rodil et al., 2009).

3.9. Reverse osmosis

Microfiltration coupled with reverse osmosis (RO) is currently used at a pilot scale at Plant C for the investigation of wastewater reclamation and reuse purposes. In this treatment, pressure (210 psi) was applied to a polyamide membrane with a pore size of 1 nm. Studies showed that wastewater treated by this system can meet the water quality requirements for both potable and non-potable reuse applications by reducing the concentrations of pollutants such as hormones and disinfection by-products as well as microbial concentrations (Tam et al., 2007). The efficient removal of organic compounds during reverse osmosis is due to size exclusion, charge exclusion and/or interactions with the membrane (Radjenović et al., 2008). Under this advanced treatment, >99% of BMDM, HMS, OD-PABA, EHMC and the benzophenone derivatives (BP-1, BP-3 and BP-4) were eliminated. These high removal efficiencies can be attributed to the molecular weights of these compounds (ranging between 100 and 300), which are in the range of values for membrane exclusion (Verliefde, 2008). There is increasing application of reverse osmosis to water treatment (e.g. desalination, drinking water purification and wastewater treatment), though it is a comparatively expensive treatment method that has not yet been applied widely in Hong Kong. In 2011, less than 7% of wastewater was reused or recycled in China (NBSC, 2011), making it one of the world's largest industrial markets for RO application.

3.10. Sand filtration

In Plant E, a dual-media filter including carbon and sand is utilized as tertiary treatment. The removal of organic contaminants by activated carbon is due to its high surface area and different surface chemistry properties while its adsorption capacity depends on the pore size distribution, hydrophobicity of contaminants, and the pH of the aqueous media, among other factors (Delgado et al., 2012). In the present study, the removal efficiencies of sand filtration for both BP-3 and BMDM were low (43% and 2%, respectively) while that of EHMC was >99%. The log K_{ow} value of EHMC (5.8) is higher than those of BP-3 and BMDM (3.5 and 2.41, respectively). Previous studies reported that the removal efficiencies of selected pharmaceuticals and endocrine-disrupting chemicals were highly

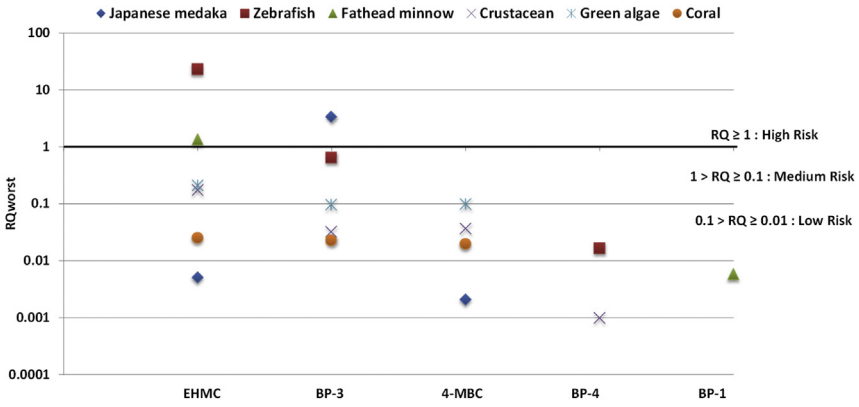

Fig. 4 – Environmental risk assessment of UV filters discharged into receiving waters in Hong Kong.

influenced by their hydrophobicity during the activated carbon adsorption process and sand filtration. Compounds with low hydrophobicities were inefficiently removed by sand filtration and breakthrough in activated carbon adsorption occurred (Nakada et al., 2007; Delgado et al., 2012).

4. Preliminary environmental risk assessment

Risk quotients (RQs) of UV filters were obtained by dividing predicted environmental concentrations (PECs) by predicted-no-effect concentrations (PNECs). PECs of UV filters in receiving surface waters were calculated by dividing effluent concentrations obtained in this study by a dilution factor of 10, while PNECs of UV filters were calculated by dividing the effect concentrations (ECs) by a standard assessment factor, 1000, to account for intra- (factor = 10) and inter-species variability (10) and chronic exposure conditions (10) (European Commission, 2003). Toxicity data were obtained from literature focusing on aquatic organisms at different trophic level including algae, crustaceans and fish. In order to assess the worst-case scenario (RQ_{worst}), maximum PECs of each compound and minimum PNECs were applied in the risk assessment (Tables A11, A12). The risk classification was based on the risk ranking criteria in which RQ < 0.01: "Unlikely to pose risk"; 0.01 ≤ RQ < 0.1: "Low risk"; 0.1 ≤ RQ < 1: "Medium risk" and RQ ≥ 1: "High risk" (Hernando et al., 2006). Because of the lack of toxicological literature, the risk assessment was only conducted for five UV filters: BP-1, BP-3, BP-4, EHMC and 4-MBC.

Generally, fish were relatively more susceptible to UV filters than organisms at lower trophic levels (e.g. algae, crustaceans). The RQ_{worst} of UV filters ranged from 0.001 to 23.0. 4-MBC and two benzophenone derivatives (BP-1 and BP-4) posed low to medium risk to aquatic organisms at different trophic levels while BP-3 and EHMC posed high risk to fish based on transcriptional changes observed in two fish species, fathead minnow (RQ_{worst} for EHMC: 1.35; Christen et al., 2011) and zebrafish (*Danio rerio*; EHMC: 23.0; Zucchi et al., 2011) and effects on egg development in Japanese medaka (*Oryzias latipes*; BP-3: 3.38; Coronado et al., 2008). Medium risk in terms of growth inhibition of green algae was also observed for these two compounds (EHMC: 0.21, BP-3: 0.10) (Fig. 4). Moreover, BP-3, EHMC and 4-MBC also posed low risk of bleaching to one kind of hard coral (*Acropora* sp.) (0.02–0.03) (Danovaro et al., 2008).

BP-3 and EHMC were detected in over 80% of wastewater effluent samples collected in Hong Kong and showed higher concentrations during the wet season, which is the breeding season for many local aquatic species. The results of this preliminary assessment show that organic UV filters may pose a risk to the local environment. More toxicological information for different trophic levels is needed for compounds (e.g. BMDM, BP-1 and BP-4) which showed high detection frequencies in effluent. Organic UV filters are used in combination in products and occur in mixtures in the environment, and exposure to some compounds (e.g. BP-1, 3-benzylidene camphor and benzophenone-2) has been observed to interfere with estrogenic activities in fish (*Pimephales promelas*) in an additive manner (Kunz and Fent, 2009), and therefore mixture toxicity should also be considered in future risk assessments.

5. Conclusions

In this study, the effectiveness of different wastewater treatment methods was investigated and most of the targeted 12 organic UV filters showed incomplete removal by these processes. Reverse osmosis treatment resulted in the highest removal efficiencies for these compounds. However, the operational costs of large-scale reverse osmosis treatment are likely to be prohibitive, particularly in developing countries. Seasonal variation in usage and release for some compounds has implications for their ecological impacts on sensitive species, especially if higher levels occur during breeding periods. Future work should focus on measuring organic UV filters and their chlorinated by-products in surface water and sediments and on assessing their environmental partitioning and potential ecological impacts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.01.014.

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Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in surface waters from different countries

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ABSTRACT

Organic UV filters are common ingredients of personal care products (PCPs), but little is known about their distribution in and potential impacts to the marine environment. This study reports the occurrence and risk assessment of twelve widely used organic UV filters in surface water collected in eight cities in four countries (China, the United States, Japan, and Thailand) and the North American Arctic. The number of compounds detected, Hong Kong (12), Tokyo (9), Bangkok (9), New York (8), Los Angeles (8), Arctic (6), Shantou (5) and Chaozhou (5), generally increased with population density. Median concentrations of all detectable UV filters were <250 ng/L. The presence of these compounds in the Arctic is likely due to a combination of inadequate wastewater treatment and long-range oceanic transport. Principal component analysis (PCA) and two-way analysis of variance (ANOVA) were conducted to explore spatiotemporal patterns and difference in organic UV filter levels in Hong Kong. In general, spatial patterns varied with sampling month and all compounds showed higher concentrations in the wet season except benzophenone-4 (BP-4). Probabilistic risk assessment showed that 4-methylbenzylidene camphor (4-MBC) posed greater risk to algae, while benzophenone-3 (BP-3) and ethylhexyl methoxycinnamate (EHMC) were more likely to pose a risk to fishes and also posed high risk of bleaching in hard corals in aquatic recreational areas in Hong Kong. This study is the first to report the occurrence of organic UV filters in the Arctic and provides a wider assessment of their potential negative impacts in the marine environment.

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1. Introduction

Organic ultraviolet (UV) filters are widely used as UV radiation-absorbing substances in personal care products (PCPs) to protect human skin from the negative effects of sunlight as well as in materials and paints to prevent product photodegradation. Authorized contents of organic UV filters in PCPs vary according to regulations in the countries/regions of their manufacture, where they may comprise up to 20% of product mass (Chisvert and Salvador, 2007). Owing to their large annual production quantities and widespread usage, particularly because of greater awareness of skin cancer risks in recent decades, organic UV filters can enter the aquatic environment (i) indirectly from wastewater treatment plants (WWTPs) after entering sewage systems following bathing or from industrial discharge due to incomplete removal as well as surface runoff and (ii) directly from recreational activities (e.g. swimming) (Giokas et al., 2007).

As a result of their extensive application and continuous release into aquatic systems, organic UV filters are regarded as pseudo-persistent environmental contaminants, and their ubiquity has raised concerns about their potential environmental impacts (Giokas et al., 2007). They have been found in various environmental samples including surface water, wastewater and sediment (e.g. Tsui et al., 2014; Kameda et al., 2011) generally at ng/L to sub-ug/L levels for aqueous matrices and sub-ng/g levels for solid matrices. However, only a few studies have reported the occurrence of UV filters in the marine environment, and only a limited number of globally authorized compounds have been investigated; for example, benzophenone-3 and -4 (BP-3 and BP-4), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) were detected in surface waters in some European countries and Japan (Tashiro and Kameda, 2013; Tovar-Sánchez et al., 2013; Rodil et al., 2008).

Many organic UV filters have high lipophilicity, with octanol-water partition coefficients (log K_{ow}) values generally greater than 3. They have been detected in various aquatic organisms such as brown trout (*Salmo trutta fario*) up to 1800 ng/g (4-methylbenzylidene camphor, 4-MBC) and 2400 ng/g (OC) lipid weight (lw) in Swiss rivers (Buser et al., 2006) and in marine mussels (*Mytilus edulis*) up to 256 ng/g (EHMC) and 7112 ng/g (OC) dry weight (dw) along the French Atlantic and Mediterranean coasts (Bachelot et al., 2012). Moreover, Fent et al. (2010b) suggested food chain accumulation of EHMC, reporting its concentrations in fish and cormorants (*Phalacrocorax* sp.) from six Swiss rivers up to 337 and 701 ng/g lw, respectively. Accumulation of these compounds in organisms is a concern because organic UV filters and their metabolites have been shown to interfere with endocrine function by acting as environmental estrogens both *in vitro* and *in vivo* (Schlumpf et al., 2001; Kunz and Fent, 2006). Moreover, they have been shown to induce bleaching in corals by promoting viral infections (Danovaro et al., 2008).

Data on the occurrence of organic UV filters in fresh surface waters are available for several developed countries (e.g. Kameda et al., 2011; Fent et al., 2010b), but relevant information is lacking for the marine environment in countries outside of Europe or Japan for certain uniformly approved and widely consumed UV filters (e.g. butyl methoxydibenzoylmethane (BMDM) and homosalate (HMS)). Moreover, previous studies have reported the occurrence of UV filters at beaches, but little information is known about coastal waters. In contrast to other organic contaminants (e.g. perfluoroalkyl substances (PFAS) and pharmaceuticals) which have been studied in detail (Richardson and Ternes, 2014), information on the occurrence, distribution, transport pathways and risks of organic UV filters in the aquatic environment is lacking. Therefore, it is of crucial importance to study the environmental distribution and concentrations of these emerging contaminants in order to evaluate their ecological risks.

In light of these considerations, the objectives of this study were to (i) determine the concentrations and spatial occurrence of twelve commonly consumed UV filters, including benzophenone-1, -3, -4 and -8 (BP-1, -3, -4 and -8), ethylhexyl salicylate (EHS), isoamyl p-methoxycinnamate (IAMC), octyl dimethyl-p-aminobenzoic acid (ODPABA), BMDM, EHMC, HMS, 4-MBC and OC in surface water samples collected from different countries including China (Hong Kong, Shantou and Chaozhou), the United States (New York City and Los Angeles), Japan (Tokyo Bay), Thailand (Bangkok) and the Arctic region, as well as their seasonal variation in Hong Kong over the course of one year; and (ii) conduct an ecological risk assessment by using the measured environmental concentrations and available toxicity data.

2. Materials and methods

2.1. Chemicals and materials

Information on chemical standards and preparation of standard solutions can be found in the Supplementary material. Standard purities were all ≥97%. Detailed information on the targeted UV filters is shown in Table A1.

2.2. Sampling

Surface water samples were collected from eight locations (Hong Kong, $n = 60$; Tokyo, $n = 8$; New York, $n = 6$; Los Angeles, $n = 4$; Shantou, $n = 4$; Chaozhou, $n = 3$; Bangkok, $n = 2$) and the Arctic ($n = 14$) from 2012 to 2013 using plastic or stainless steel buckets or glass bottles which were pre-cleaned by rinsing (in sequence) with methanol, Milli-Q water, and water from the specific location. All samples were marine surface water samples except those collected from Bangkok which were freshwater samples. Most of the selected cities are metropolitan areas featuring both commercial and industrial development. Temporal and spatial samples were collected in Hong Kong in both the wet and dry seasons; spatial samples were collected from Tokyo Bay, Los Angeles, New York City and the Arctic, while only a single location was sampled in Bangkok. Detailed information on the sampling locations is shown in Supplementary material Table A2 and Figs A1–5.

Surface water samples were collected from 20 points in Hong Kong in August 2012, February and June 2013; June and August samples represented the wet season, while the February samples represented the dry season. The sampled

points were expected to reflect WWTPs, beaches and aquatic recreational activities (further details are given in the Supplementary material). The Tokyo samples were collected in Tokyo Bay, while the New York samples were collected in Jamaica Bay, Upper New York Bay and the East River near WWTP discharge points. In Los Angeles, Shantou and Chaozhou, surface water samples were collected at beaches and near WWTP discharge points while only river water receiving municipal wastewater was collected in Bangkok. Arctic samples were collected in the Arctic Ocean and Chukchi Sea between 65 and 75 °N.

Water samples were stored in glass bottles pre-rinsed with Milli-Q water and methanol. All glass bottles were wrapped with aluminum foil to avoid contamination and photodegradation of the target compounds. Samples were stored in the dark at 4 °C prior to analysis.

2.3. Analytical procedures

Chemical analysis of the 12 target compounds was modified from a previously reported method (Tsui et al., 2014). Briefly, the analytical procedures consisted of addition of 5% (w/v) Na_2EDTA to each sample, solid phase extraction (SPE) with Bond Elut C18 cartridges, elution by 3 × 4 mL of 50:50 v/v methanol: ethyl acetate (MeOH: EA), concentration under nitrogen flow to less than 0.5 mL, reconstitution to 0.5 mL by MeOH and analysis by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). Detailed analytical procedures and optimal parameters of HPLC-MS/MS for quantification are shown in the Supplementary material.

2.4. Method validation

Details on method validation are given in Table A4 in the Supplementary material. Recoveries ranged from 63% to 106%, while the relative standard deviations (RSD) of target compounds ranged from 1.5% to 7.9%. The results presented in this study were not corrected by recoveries. The method limit of detection (MLOD) was defined as three times the standard deviation of procedural blank peak areas plus their mean value and then corrected by a matrix-induced interference factor which was the slope difference (ratio) of two calibration curves separately constructed in methanol and in water sample extracts (Leung et al., 2012). MLODs ranged from 0.03 to 1.38 ng/L. Field and procedural blanks were analyzed for each sampling trip and for each batch of samples in the laboratory by using Milli-Q water. All of the target compounds were below MLODs in both field and procedural blanks.

2.5. Statistical analyses

Normality tests (Kolmogorov–Smirnov) were performed before statistical analyses. Parametric Pearson correlation analysis was used for the examination of significant correlations among concentrations of different UV filters in surface water from different sampling cities/regions. Log_{10}-transformed values were used to perform the Pearson correlations in all locations except Hong Kong, for which principal component analysis (PCA) and permutational analysis of variance (PERMANOVA) were conducted to explore spatio-temporal patterns in organic UV filter levels because of the larger sample size for this city. Two-way analysis of variance (ANOVA) and *post hoc* Student-Newman-Keuls (SNK) tests were carried out to test spatiotemporal differences in compound concentrations in Hong Kong samples. Samples with concentrations < LOD were treated as zero in the analysis. The significance level was set at $\alpha = 0.05$. Univariate statistical analyses were carried out using SigmaStat 3.5 (Systat Software Inc, Chicago, USA) or SPSS 17 (SPSS Inc.). Multivariate analyses were carried out using PRIMER 6 & PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK).

2.6. Environmental risk assessment (ERA)

Hazard quotients (HQs) for individual UV filters were obtained by dividing measured environmental concentrations (MECs) obtained in this study by predicted-no-effect concentrations (PNECs) calculated by dividing the effect concentrations (ECs) by a standard assessment factor, 1000, to account for intra- (factor = 10) and inter-species variability (10) and chronic exposure conditions (10) (European Commission, 2003). Toxicity data were obtained from the literature focusing on aquatic organisms at different trophic levels including protozoa, algae, crustaceans, invertebrates and fishes. Because of the lack of toxicological literature on many organic UV filters, the risk assessment was only conducted for six compounds: BP-1, BP-3, BP-4, EHMC, 4-MBC and ODPABA.

Preliminary screening of the potential ecological risks of organic UV filters was carried out using the worst-case scenario, HQ_{worst}, in which the maximum MECs of each compound and minimum PNECs were applied in the hazard assessment (Table 1 and A5). The risk classification was based on risk ranking criteria in which HQ < 0.01: "Unlikely to pose risk"; 0.01 ≤ HQ < 0.1: "Low risk"; 0.1 ≤ HQ < 1: "Medium risk" and HQ ≥ 1: "High risk" (Hernando et al., 2006). Probabilistic risk assessment was conducted if the HQ_{worst} of UV filters exceeded 1 by plotting cumulative probability on a log scale. Risk probabilities (p) were calculated by substituting the log PNECs of each species in the linear equations for each sampled city, in which (100-p)% would be the percentage of samples containing concentrations of that compound exceeding the PNEC of a particular species and thus posing risk based on the assessed endpoint.

3. Results and discussion

3.1. Occurrence and composition of UV filters in surface waters

A total of 101 surface water samples collected from August 2012 to October 2013 were analyzed, and median concentrations in the samples ranged from <LOD to 230 ng/L (Table 1). The number of compounds detected was Hong Kong (12), Tokyo (9), Bangkok (9), New York, Los Angeles (8), Arctic (6), Shantou (5) and Chaozhou (5).

BP-3, EHMC and OC were detected in all cities and in the Arctic with detection frequencies ≥30% in each location (calculated by dividing the number of positive detections by

Table 1 – Concentrations (ng/L) and detection frequencies of 12 UV filters in surface water samples from different cities. Conc.: (median-maximum); DF: detection frequencies (calculated by dividing the number of positive detections by the total number of samples from each location); LOD: limit of detection.

Compounds	Hong Kong ($n = 60$)		Tokyo ($n = 8$)		New York ($n = 6$)		Los Angeles ($n = 4$)		Shantou ($n = 4$)		Chaozhou ($n = 3$)		Bangkok ($n = 2$)		Arctic ($n = 14$)	
	Conc.	DF	Conc.	DF	Conc.	DF	Conc.	DF	Conc.	DF	Conc.	DF	Conc.	DF	Conc.	DF
ODPABA	95–182	17	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0
4-MBC	173–379	12	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0
BMDM	24–721	97	78–104	100	70–87	100	67–109	100	53–100	75	<LOD	0	36–38	100	18–70	57
EHMC	89–4043	93	46–95	63	89–150	83	91–138	75	52–78	75	<LOD-79	33	88–95	100	25–66	71
IAMC	63–173	27	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0	<LOD	0
OC	103–6812	100	87–108	75	117–128	83	145–377	100	75–107	75	36–102	67	153–205	100	26–31	43
BP-3	39–5429	95	24–86	100	23–178	100	227–601	100	55–188	100	37–49	100	86–116	100	17–33	71
EHS	61–1030	59	71–95	88	<LOD	0	53–120	50	<LOD	0	121–128	67	28–56	50	<LOD	0
BP-4	54–389	49	71–136	100	89–574	100	<LOD	0	<LOD	0	<LOD-49	33	80–95	100	<LOD	0
HMS	66–2812	76	65–110	100	91–114	67	142–270	100	<LOD	0	<LOD	0	29–59	50	<LOD	0
BP-1	82–135	76	52–95	63	<LOD-74	33	100–117	75	22–58	50	<LOD	0	127–166	100	2.5–5	36
BP-8	64–117	88	76–96	100	72–92	100	29–96	50	<LOD	0	<LOD	0	63–71	100	2–3.3	50

the total number of samples in each place), showing their widespread distribution in the marine environment. Among these three frequently detected UV filters, the highest median concentrations were found in Los Angeles (227 ng/L; BP-3), New York (100 ng/L; EHMC) and Bangkok (153 ng/L; OC), while the lowest median concentrations were found in the Arctic (16.6, 25.4 and 25.8 ng/L for BP-3, EHMC and OC, respectively). The concentrations of five compounds including BP-3, EHMC, EHS, HMS and OC exceeded 1000 ng/L in surface water samples collected on hot summer days with strong UV radiation in June and August 2013 at a popular beach in Hong Kong. Apart from recreational activities and surface runoff, the incomplete removal of organic UV filters in WWTPs is a major contributor to their ubiquitous occurrence in the environment; in Hong Kong, their environmental loading can reach 200 g/day (Tsui et al., 2014). BP-4, with median concentrations lower than 100 ng/L at all locations, was only detected in indirect sources (i.e. through WWTP discharge) because it is used primarily in PCPs such as hand washes/soaps, shower gels and shampoo rather than sunscreen products and it is poorly removed in WWTPs (Tsui et al., 2014). In contrast, 4-MBC (173–378 ng/L), IAMC (62.7–173 ng/L) and ODPABA (95.1–182 ng/L) were only detected at snorkeling hot spots and other recreational beaches in Hong Kong, indicating that recreational activities would be the main sources of these three compounds instead of wastewater effluent discharge. The low detection frequencies of these compounds in WWTP-influenced samples are likely due to their relatively lower use (used in less than 2% of commercially available PCPs in Swiss and British markets; Manová et al., 2013; Kerr, 2011) together with the stronger dilution effects of ocean currents in Victoria Harbour.

Generally, the occurrence of individual UV filters at each location (calculated by dividing the total concentration of each UV filter by the total concentration of UV filters at that location) was <30%, except for EHS in Chaozhou which was >40% (Fig. A6). The composition profiles of UV filters in surface waters from Hong Kong, Tokyo, New York, Los Angeles and Bangkok were similar while those in samples from Shantou, Chaozhou and the Arctic showed higher percentages of each detectable compound due to the relatively lower number of positive detections. BP-3, EHMC and OC were the dominant compounds detected in all samples. Hong Kong imports a wide variety of PCPs from several countries/regions and has no local regulations for organic UV filter content in products, and therefore more compounds from several chemical classes were detected in these samples. The number of compounds detected in Shantou and Chaozhou were the lowest among all sampled cities, likely because of their lower population densities (2655/km^2 and 849/km^2, respectively; Shantou and Chaozhou Government, 2010) and development level. Liao and Kannan (2014) reported overall geometric mean levels of BP-3 in PCPs purchased in China and the United States, which were 20.1 and 1200 ng/g, respectively. Moreover, the maximum authorized concentrations of some of the targeted compounds in China are lower than those in other countries (e.g. up to 20% of product mass can consist of EHMC in Japan, but only 10% of product mass is permitted in China) (MoH, 2007), indicating comparatively lower application of these UV filters in PCPs in China. Sediments with high organic

carbon content are an environmental sink for contaminants with high log K_{ow} and UV filters can also be detected in sediment (e.g. Kameda et al., 2011).

3.2. Distribution and source determination of UV filters in surface water

Correlation analyses were performed among individual UV filters from Tokyo, the Arctic and the United States for source determination (Table A6). Significant positive correlations ($p < 0.05$) were observed between BP-3 and BP-8 ($r = 0.879$) and BP-3 and EHMC ($r = 0.774$) in the Arctic samples. Higher detection frequencies of UV filters were found in samples collected near Alaska (<72°N) than in those from the open ocean (>72°N). The overall detection frequencies of BMDM, BP-3 and EHMC were >50%, while concentrations of all detectable compounds were <70 ng/L (Table 1). This is the first report of the occurrence and distribution of organic UV filters in the Arctic, for which there are two possible pathways: (i) oceanic transport via ocean currents or (ii) atmospheric transport; these pathways may be either long-range or short-range. Inadequate wastewater treatment facilities could result in the direct release of untreated or undertreated wastewater to the marine environment via oceanic currents (Gunnarsdóttir et al., 2013) and thus wastewater runoff could be one of the local contamination sources of UV filters as some of the sampling points are located offshore of Point Hope and Point Barrow in Alaska (population: 674 and 4212, respectively; United States Census, 2010), both of which employ sewage lagoons as the major wastewater treatment method (BUECI and URS Corporation, 2005). Some compounds such as BP-3 and OC have been reported to be highly photostable towards UV irradiation (half-lives >72 h; Rodil et al., 2009), and they may undergo long-range or local transport via oceanic currents. However, information about the environmental half-lives of organic UV filters is limited. Though some of these compounds have similar Henry's Law constants (ranging from 10^{-5} to 10^{-15} atm·m^3/mol, Table A1) as other organic contaminants known to undergo long-range atmospheric transport such as PFAS and endosulfan (Butt et al., 2010; Weber et al., 2010), there is currently not enough evidence to conclude that they partition into the gas phase as no studies have reported the occurrence of UV filters in air samples or wet or dry deposition, and their atmospheric half-lives are also unknown. More work should be conducted to investigate the fate on these compounds in order to understand their occurrence in aquatic environments.

In Tokyo Bay, significant positive correlations ($p < 0.05$) were observed between EHMC and OC ($r = 0.957$); EHS and HMS ($r = 0.795$) and BP-1 and BP-8 ($r = 0.885$), suggesting that these compounds likely share contamination sources such as wastewater effluents from urban and industrial areas in Tokyo. Moreover, Tokyo Bay receives fresh water from the Tama, Arakawa and Edo Rivers, which flow through densely populated areas with WWTPs at different treatment levels. All wastewater collected from public sewers in Japan is treated with secondary treatment, while 15% is further treated with tertiary methods, including sewage discharged to Tokyo Bay (Ueda and Benouahi, 2009). Chemicals used in PCPs (e.g. UV filters and synthetic musks) were detected in these rivers in previous studies (Kameda et al., 2011; Yamagishi et al., 1983). Kameda (2007) reported the occurrence of EHMC in sediment core samples collected in Tokyo Bay (1977–1997) showing the long history of the occurrence of this compound in the Bay, and the present study showed its wide distribution in surface water in Tokyo Bay as well.

Significant positive correlations were observed between BMDM and OC ($r = 0.971$), BP-3 and BP-4 ($r = 0.903$) as well as HMS and BP-8 ($r = 0.985$) in the samples from New York City, suggesting that they shared similar contamination sources (WWTP effluents). There are 14 WWTPs in New York City serving 7.8 million people (New York City Department of Environmental Protection), and wastewater is treated by secondary treatment and chlorination disinfection, neither of which completely remove organic UV filters from effluents (Tsui et al., 2014). In Los Angeles, the highest concentrations of UV filters were detected at a popular beach, Huntington Beach.

No significant correlations were observed among UV filters in surface water from the two Chinese cities, suggesting that UV filters in the marine environment in these locations have distinct contamination sources such as both municipal and industrial wastewater discharge.

3.3. Seasonal variation of UV filter concentrations in surface waters in Hong Kong

The occurrence of organic UV filters in the Hong Kong samples was investigated in greater depth using PCA. Significant seasonal and zone differences (all $p < 0.0001$, PERMANOVA) were observed. The sample patterns were considered in monthly chronological order without considering the sampling year, and monthly data are presented individually to illustrate spatial patterns (Fig. 1a–c). In all sampling months, the concentrations measured at the sampled points largely conformed to expectations about the major sources of organic UV filters in each zone. February is the end of the dry season, when water temperatures are colder (Table A7) and there is little marine or coastal recreational activity, and thus it was assumed that there would be no or small inputs of organic UV filters at sites representing direct sources and at beaches; these sites cluster together in the PCA (Fig. 1a, I and III) and apart from sites representing indirect sources (WWTPs; Fig. 1a, IV). In contrast, samples collected at the beginning of the wet summer season in June from indirect and direct sources showed less separation, likely due to marine recreational activities increasing with warmer temperatures (Fig. 1b, V and VII), while beach samples (Fig. 1b, VI) were dissimilar to both of these groups. The collected samples were most similar to one another in August, when temperatures were highest (Fig. 1c), and increased usage of organic UV filters at the sampled beaches (Fig. 1c, IX) was evident. Surface water samples collected from Point-16 and -17 were dissimilar to the other beach sampling locations, grouping apart from all other points in February (Fig. 1a, II) and grouping with samples representing direct sources in June and August (Fig. 1b, V and 1c, VIII, respectively). These two points were located on a small beach adjacent to a village community where snorkeling and diving are the major recreational activities, and where there may also be some local wastewater release by

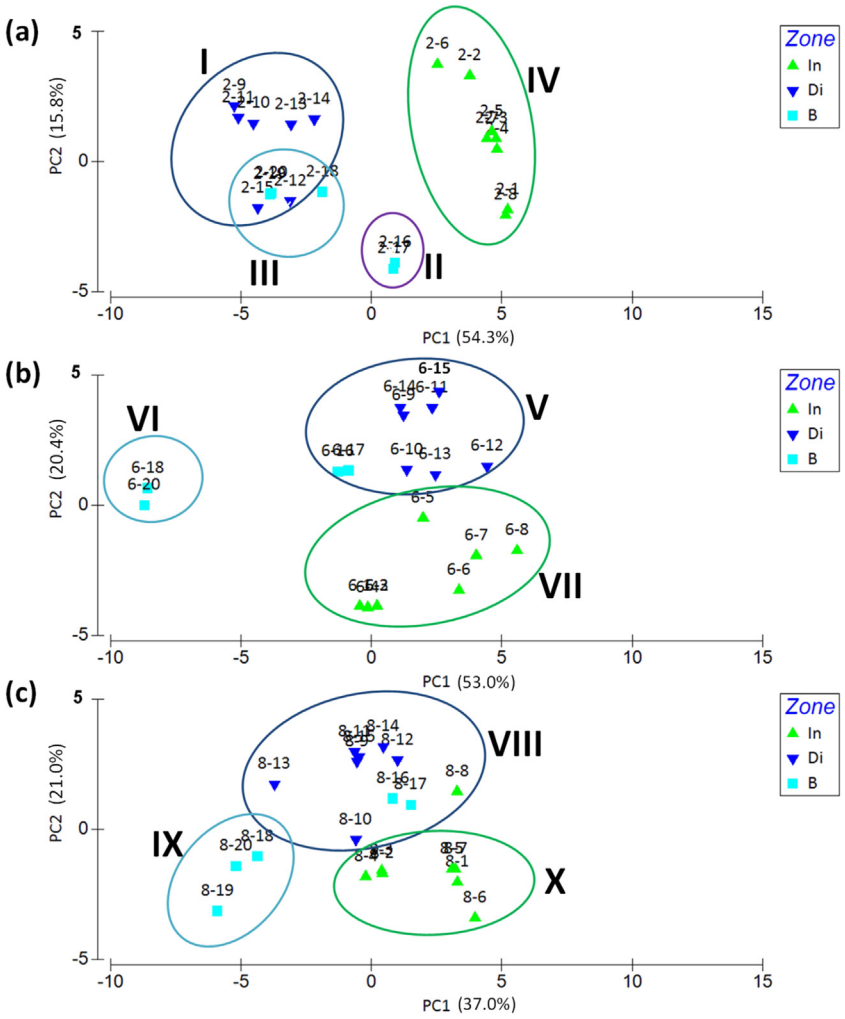

Fig. 1 – Principal Component Analysis (PCA) plots for surface water samples collected in (a) February 2013, (b) June 2013, and (c) August 2012 from Hong Kong (In: Indirect sources (WWTPs), Di: Direct sources (water sports & marine recreational activities), B: Beaches).

residents or tourists. These points therefore showed a distinct pattern compared to the other locations.

Concentrations of all the tested compounds varied according to season and/or sampling zone (ANOVA, Tables A8a & b). The results of *post hoc* SNK tests illustrated that all compounds showed significantly higher concentrations in the wet season in at least one sampling zone with the exception of BP-4, generally reflecting greater usage of PCPs containing organic UV filters during the sunnier and hotter wet season (Tables A7 and A8). BP-4 concentrations were greater in the dry season perhaps because it is increasingly used as a photodegradation retardant and shelf life extension ingredient in many types of PCPs (Hughes and Stone, 2007) that are not used seasonally. Moreover, it is the most hydrophilic of the target compounds (log K_{ow}: 0.89), and therefore this disparity may also be due to differences in precipitation in the dry and wet seasons. IAMC, 4-MBC and ODPABA were mainly detected in the wet season and at locations reflecting direct sources and beaches. This finding is consistent with our previous study of wastewater in Hong Kong which reported low detection frequencies of these compounds in effluent (Tsui et al., 2014).

The detected levels of EHMC showed no significant spatial differences in both seasons and its high detection frequency indicated continuous release of this compound to the marine environment throughout the year. On the other hand, the significantly higher concentration of BP-4 found in locations representing indirect sources in both seasons confirmed that WWTP effluent is a major source of this compound in the aquatic environment. However, the lack of information on product composition/formulations and usage by consumers in Hong Kong makes it difficult to understand how the occurrence patterns of organic UV filters in the environment are related to their use and release.

3.4. *Global comparison of UV filters in surface water*

3.4.1. *Marine environment*

To date, few studies have reported the occurrence of UV filters in the marine environment, and published reports have focused primarily on European countries and reported levels of a small number of compounds. One recent study reported that the maximum concentration of UV filters

detected at coral reef sites in Okinawa Island, Japan was 8.1 ng/L (OC) (Tashiro and Kameda, 2013), which was 28 times less than that detected in coral reef areas in Hong Kong (OC, 231 ng/L). A global comparison of median-to-maximum levels of the target compounds in the present study is shown in Fig. 2. This study is the first report of BMDM, BP-8 and IAMC in the marine environment. Maximum concentrations of BMDM were <200 ng/L in all sampled locations except Hong Kong (BMDM: 721 ng/L), while detection frequencies for BMDM and BP-8 were >60% and IAMC was only detected in Hong Kong. The maximum concentrations of OC, BP-3 and EHMC found in this study were 6810, 5420 and 4040 ng/L, respectively, which were comparable, 1.5 and 10 times higher than those detected in Norway and Spain (7300, 3300 and 390 ng/L, respectively; Langford and Thomas, 2008; Tarazona et al., 2010). The maximum concentration of BP-4 (574 ng/L) was found in a sampling point near the effluent discharge of a WWTP in Jamaica Bay in New York, but it was <LOD in all samples from beaches in this study; in contrast, BP-4 was detected at a Spanish beach at a maximum concentration of 138 ng/L (Rodil et al., 2008). 4-MBC is not permitted for use as a cosmetic ingredient in Japan and the United States, and thus it was not detected in seawater there, but it was detected in Europe and Hong Kong at maximum concentrations of 799 (Langford and Thomas, 2008) and 379 ng/L (present study), respectively.

3.4.2. *Freshwater environment*

The organic UV filter concentrations reported for river surface water samples from Bangkok in the present study are the first reported in Southeast Asia. The maximum concentration of BP-4 detected in Bangkok was 95 ng/L, which was 1.5 and 9 times lower than that in rivers in the United Kingdom and Spain (Kasprzyk-Hordern et al., 2008; Rodil et al., 2008), suggesting lower consumption of BP-4 in Bangkok. The median concentration of EHMC (88 ng/L) in Bangkok falls between those measured in heavily and moderately polluted rivers in Japan (266 and 26 ng/L, respectively) receiving industrial and domestic wastewater discharge (Kameda et al., 2011). Only 23% of wastewater in Bangkok is treated in WWTPs using conventional secondary treatment with activated sludge, and thus a high percentage of untreated wastewater is discharged directly to rivers (Tsuzuki et al., 2009), which likely explains the high number of UV filters detected in these samples. Moreover, incomplete removal of UV filters by secondary treatment with activated sludge in WWTPs may also contribute to their high detection (Tsui et al., 2014).

3.5. *Ecological risk assessment*

A preliminary screening of the worst-case scenario was conducted to assess the potential hazards of UV filters to the aquatic environment (Table A9). In the worst case scenario, BP-1 and BP-4 posed low to medium risk to crustaceans based

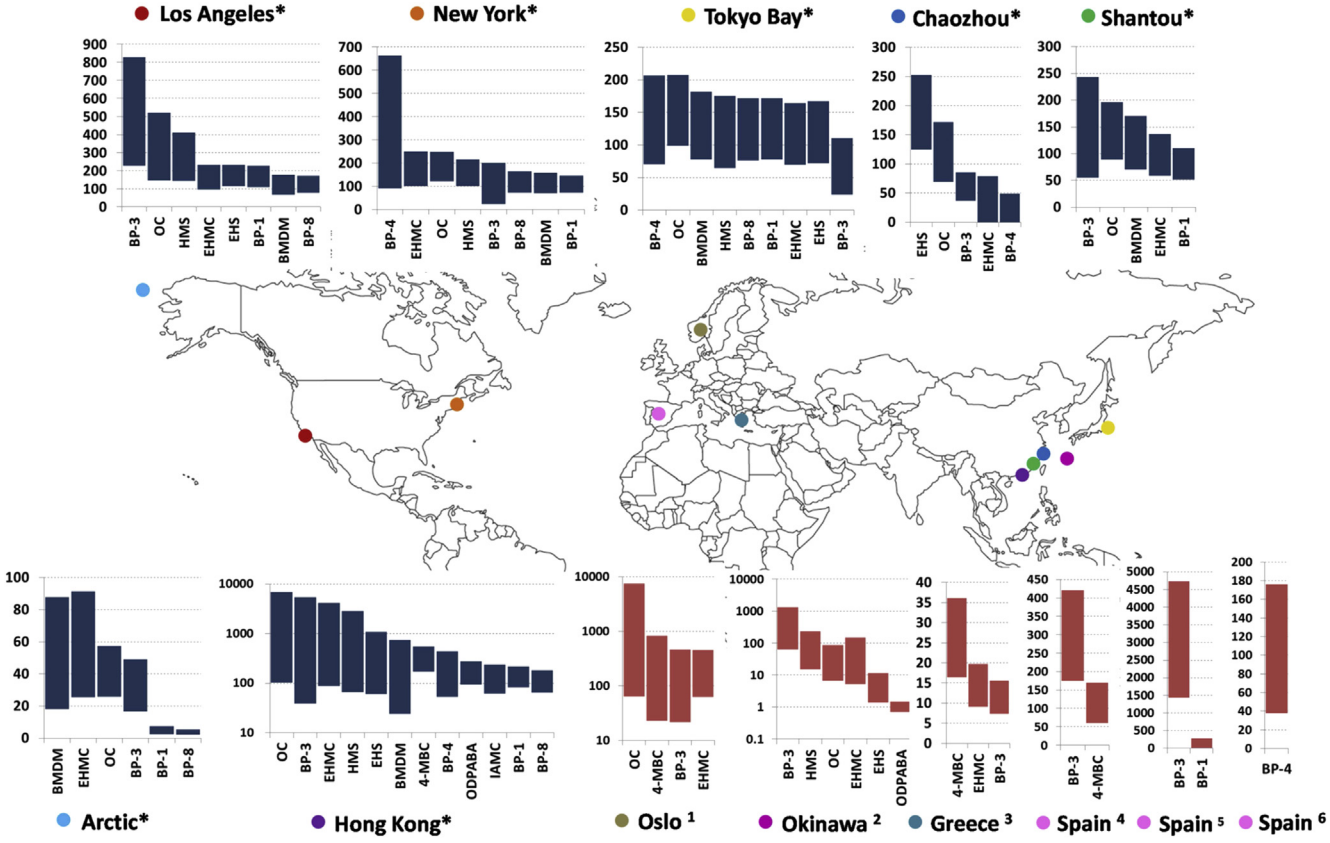

Fig. 2 – Global comparison of UV filters concentration (median-maximum, ng/L) in marine surface waters (*: this study; 1: Langford and Thomas, 2008; 2: Tashiro and Kameda, 2013; 3: Giokas et al., 2005; 4: Tovar-Sánchez et al., 2013; 5: Tarazona et al., 2010; 6: Rodil et al., 2008).

on the endpoints of immobilization and lethality in *Daphnia magna*, as well as in fish based on changes in the expression of endocrine genes in fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) with HQ_{worst} ranging from 0.001 to 0.06 for BP-1 and 0.001 to 0.19 for BP-4 (Sieratowicz et al., 2011; Fent et al., 2010a; Zucchi et al., 2011). The medium risk posed by BP-4 for zebrafish occurred in Hong Kong and New York, with HQ_{worst} at 0.13 and 0.19, respectively. ODPABA posed medium risk (HQ_{worst}: 0.18) to invertebrates based on changes in endocrine-related genes in *Chironomus riparius* as the endpoint (Ozáez et al., 2013).

As some of the HQ_{worst} values for BP-3, EHMC and 4-MBC exceeded 1, probabilistic plots were constructed for their concentrations in surface water samples (Fig. 3a and b, A7). Because of the small number of sampling points in Bangkok, Shantou and Chaozhou, probabilistic risk assessment was not performed for these cities. As a distinct pattern was observed for the concentrations of UV filters in the samples collected at beaches in Hong Kong, these data were considered separately. Detailed information on regression coefficients is shown in Table A10. Multiple threshold values were available for freshwater fish, and all of these were used for the risk assessment to include a range of sensitivities among species (shown as thresholds F_1–F_4 on Fig. 3a and b). The probabilities of 4-MBC causing growth inhibition in algae (based on the inhibitory concentration-10% (IC_{10}) for *Desmodesmus*

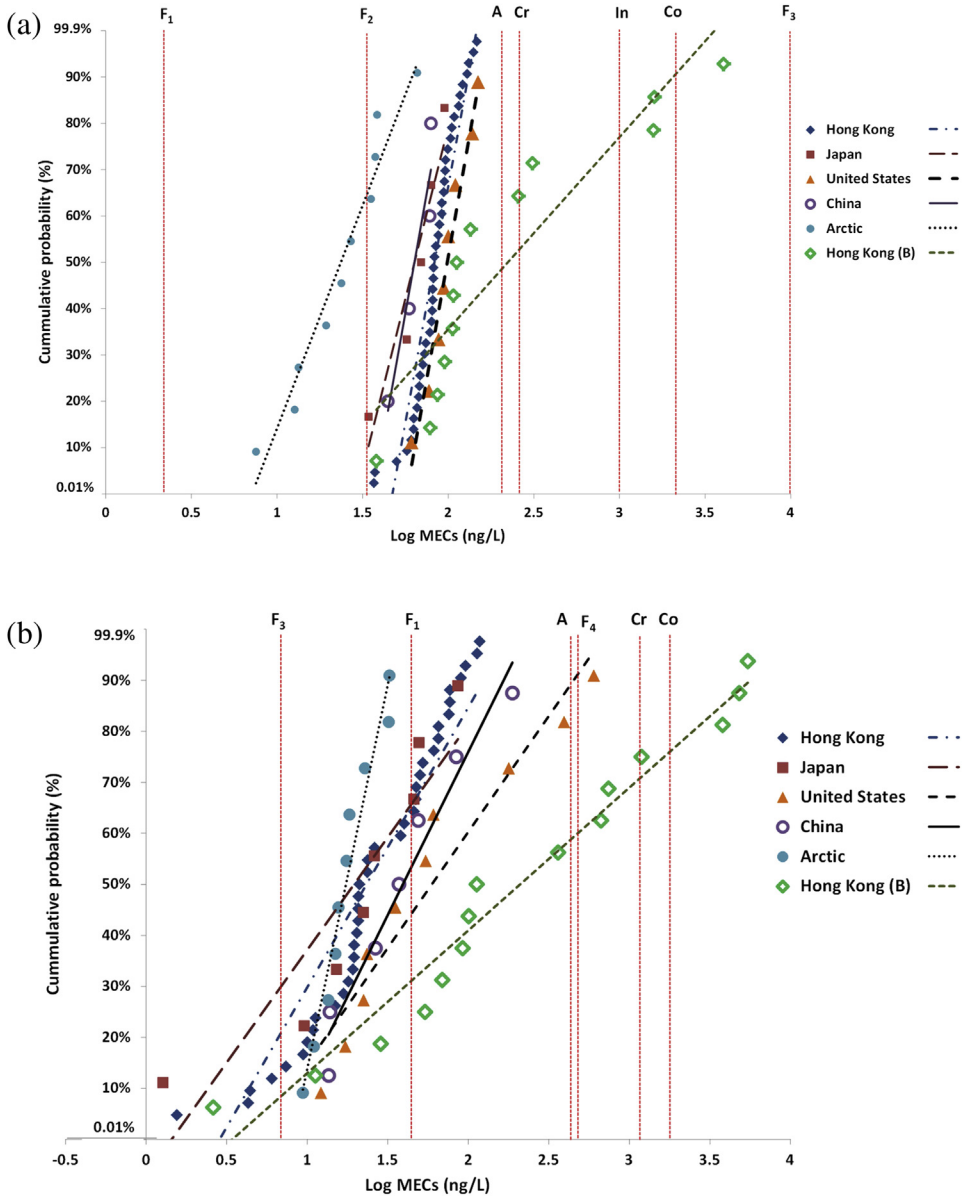

Fig. 3 – a. Probabilistic risk assessment of EHMC in marine surface waters from different locations. "F" thresholds are those derived from toxicity data for different fish species (F_1: Zebrafish; F_2: Fathead minnow; A: Algae; Cr: Crustacean; In: Insect; Co: Coral; F_3: Japanese medaka). Toxicity thresholds and endpoints are given in Table A5. 3b. Probabilistic risk assessment of BP-3 in marine surface water from different locations. "F" thresholds are those derived from toxicity data for different fish species (F_3: Japanese medaka; F_1: Zebrafish; A: Algae; F_4: Rainbow trout; Cr: Crustacean; Co: Coral). Toxicity thresholds and endpoints are given in Table A5.

subspicatus), altering endocrine genes in mosquito larvae (*C. riparius*) and inducing oxidative stress in a protozoan (*Tetrahymena thermophila*) were 34%, 68% and 99.9%, respectively (Gao et al., 2013; Ozáez et al., 2013; Sieratowicz et al., 2011). This result showed that organisms at lower trophic levels were more susceptible to 4-MBC. The probability of risk to fish based on transcriptional changes of endocrine genes in zebrafish was 99.9% for EHMC for all samples with positive detections in all locations, while that based on toxicity in fathead minnow was over 75% in all places except the Arctic, for which the risk was 31%. Moreover, EHMC posed high risk to both cladocerans and algae based on immobilization and growth inhibition as the endpoints (24 and 29%, respectively). For BP-3, the probability of effects on induction of vitellogenin in fish based on data from rainbow trout (*Oncorhynchus mykiss*), egg development in fish based on data from Japanese medaka (*Oryzias latipes*) and induction of oxidative stress in protozoans were over 34%, 50% and 99.9% in all locations, respectively (Coronado et al., 2008; Gao et al., 2013).

It should be noted that the majority of the toxicity values used for the assessment were derived from tests using freshwater organisms, as little information is available in the literature for marine species. The sensitivities of freshwater and saltwater species to different organic contaminants (e.g. pesticides, trace elements) are known to vary, and species sensitivity distributions have been used to understand whether freshwater datasets are protective enough; in some cases, saltwater species have been found to be less sensitive to contaminant effects than freshwater species, though the information available, both in terms of number of species and number of chemicals, is far from comprehensive (Wheeler et al., 2002). Because of the scarcity of information for marine species for UV filters, an inter-species safety factor of 10 was used in this study. Both BP-3 and EHMC posed 21% and 11% risk, respectively, of causing bleaching of hard corals (*Acropora* sp. and *A. pulchra*) at some beaches in Hong Kong located near snorkeling hotspots. It should be noted that these two compounds were detected widely and frequently at high concentrations at the majority of the sampled locations, and therefore their ecological risks and negative impacts should be investigated further.

4. Conclusions

Data on the international distribution and possible negative impacts of organic UV filters in the aquatic environment and the first report of their occurrence in the Arctic have been presented in this study. BP-3 and EHMC showed high detection frequencies at all sampled locations as well as high concentrations in recreational areas; probabilistic risk assessment indicated that these compounds posed various ecological risks to marine ecosystems, including causing coral bleaching and affecting reproduction in fish, though toxicity data for several compounds were not available. The pathways by which organic UV filters are transported to remote Arctic areas remain to be elucidated. These findings indicate that there is a need for greater understanding of the toxicities of these chemicals, both singly and in mixtures, and to consider the current extent of their use, particularly in potentially sensitive ecosystems such as coral reefs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.09.013.

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Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in marine sediments in Hong Kong and Japan

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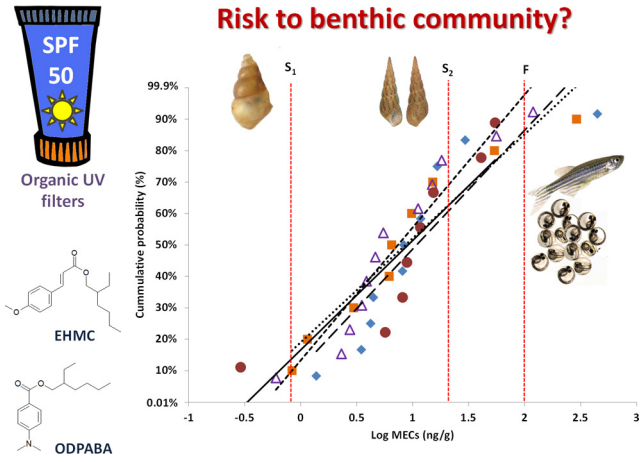
HIGHLIGHTS

- The occurrence of 11 organic UV filters in marine sediments was investigated.
- Sediments collected from different sources showed distinct patterns based on PCA.
- Compounds showed higher detection frequencies from direct sources except BP-1 and -8.
- Detection frequencies of UV filters increased with partition coefficient (K_{oc}).
- UV filters could pose high risks to benthic and sediment-dwelling organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic ultraviolet (UV) filters are used widely in various personal care products and their ubiquitous occurrence in the aquatic environment has been reported in recent years. However, data on their fate and potential impacts in marine sediments is limited. This study reports the occurrence and risk assessment of eleven widely used organic UV filters in marine sediment collected in Hong Kong and Tokyo Bay. Seven of the 11 target UV filters were detected in all sediment samples (median concentrations: <MLOD-21 ng/g dry weight) with detection frequencies higher in the wet season than in the dry season. Composition profiles showed that BMDM, EHMC and ODPABA were the predominant compounds, accounting for more than 60% of the total UV filter occurrence; this was likely due to their relatively higher octanol–water partition coefficients. Probabilistic ecological risk assessment showed that the likelihood of EHMC causing toxic effects on reproduction in snails was over 84% and 32% based on toxicity data for two species, respectively, suggesting potential risks of UV filters to benthic organisms and possible wider effects on the marine food web. However, more toxicity data for sediment organisms is necessary for better risk assessment of these compounds in benthic communities.

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1. Introduction

Organic ultraviolet (UV) filters are applied in various personal care products (PCPs) such as sunscreens, cosmetic products, body washes and hair sprays for protecting humans from harmful UV radiation by absorbing UVA (320–400 nm) and UVB (280–320 nm); they can also be used for photo-protective purposes in textiles or fragrances [1,2]. These compounds are produced and used extensively worldwide; while the authorized contents of organic UV filters in PCPs vary according to legislation in force (e.g., European Union Cosmetics Directive, United States Food and Drug Administration) in the countries/regions of their manufacture, they may comprise up to 20% of product mass [3]. As reported by different surveys, the usage frequencies of UV filters in PCPs available on the retail market vary among countries; for example, the percentage of PCPs containing ethylhexyl methoxycinnamate (EHMC) was 90% and 51%, and that for butyl methoxydibenzoylmethane (BMDM) was 30% and 71% in China and Switzerland, respectively [4,5]. As a result of their large annual production and widespread application, these compounds enter aquatic environments (i) indirectly, through wastewater treatment plant (WWTP) discharge and (ii) directly, through recreational activities [6]. Due to their incomplete removal in WWTPs and continuous release, organic UV filters are regarded as pseudo-persistent compounds; they have been found ubiquitously in wastewater and surface water at generally from ng/L to sub-μg/L levels, and seasonal variation has been observed for some compounds (e.g., benzophenone-3 (BP-3), EHMC) where higher concentrations were detected in samples collected in the summer [7,8] as well as in in groundwater (benzophenone-4 concentrations up to 36 ng/L [9] [9]).

Most organic UV filters have organic carbon partition coefficients ($\log K_{oc}$) generally greater than 3 (Table A1), making them likely to accumulate in environmental matrices with high organic carbon content such as sediment, which is considered to be a sink for organic contaminants in aquatic ecosystems. Organic UV filters have also been detected in aquatic organisms at different trophic levels such as EHMC in mussels (*Dreissena polymorpha*) at 22–150 ng/g lipid weight (lw) and octocrylene (OC) in La Plata dolphins (*Pontoporia blainvillei*) at 89–792 ng/g lw [1,10], while biomagnification was reported for EHMC by comparing concentrations in biota samples including macroinvertebrates, fishes and cormorants [1]. Moreover, organic UV filters have been reported to have multiple hormonal activities (estrogenic, antiestrogenic, androgenic and antiandrogenic activities) *in vitro* [11] as well as to cause growth inhibition of marine microalgae, affect gonad structure in fish and induce coral bleaching [12–14].

The uptake of organic contaminants from sediment into the food web is highly related to their concentrations in sediment; nevertheless, the occurrence and distribution of UV filters in sediment have only been reported by a limited number of studies and data is available for a small number of UV filters, with most studies carried out in freshwater ecosystems and reporting median concentrations ranging from 0.1 to 64 ng/g dry weight (dw) [15,16]. In light of the reported ubiquity and potential negative impacts and of organic UV filters in the aquatic environment [17], the objectives of this study were to (i) investigate the occurrence and spatial distribution of 11 commonly consumed UV filters including, benzophenone-1 (BP-1), benzophenone-8 (BP-8), ethylhexyl salicylate (EHS), homosalate (HMS), isoamyl *p*-methoxycinnamate (IAMC), 4-methylbenzylidene camphor (4-MBC), octyl dimethyl-*p*-aminobenzoic acid (ODPABA), BMDM, BP-3, EHMC and OC in marine sediment samples collected in Hong Kong and Tokyo Bay and (ii) conduct a preliminary environmental risk assessment to evaluate the potential consequences of these compounds in marine benthick communities.

2. Experimental

2.1. Chemicals and solvents

Standards for BP-3 (purity: 100%), BMDM (99.5%), HMS (99.4%), IAMC (99.3%) and OC (99%) were obtained from United States Pharmacopeia Reference Standards (Rockville, MD, United States). 4-MBC (98%), BP-1 (99%), BP-8 (98%), ODPABA (98%) and OMC (98%) were obtained from Sigma–Aldrich (St. Louis, MO, USA) and EHS (99%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Isotope-labeled ^{13}C-BP-3 (99%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Detailed physicochemical characteristics of the targeted UV filters are shown in Table A1. Individual solutions of each analyte were prepared in pure methanol. Mixed standard solutions were prepared at 10 μg/mL in methanol and subsequently diluted as necessary. All standard solutions were stored in amber glass bottles at −20 °C to avoid photodegradation.

Solvents including Milli-Q water (Millipore, Bedford, MA, USA), HPLC-grade methanol (MeOH; Sigma–Aldrich, St. Louis, MO, USA) and HPLC-grade ethyl acetate (EA; Merck, Darmstadt, Germany) were utilized. ASE® Prep diatomaceous earth and cellulose filter paper (19.8 mm) were purchased from Dionex (Sunnyvale, CA, United States); sodium sulphate (99%) and graphitized carbon black were obtained from Sigma–Aldrich (St. Louis, MO, USA). Bond Elut C18 (500 mg, 6 mL) cartridges (Agilent Technologies, Hong Kong) were used for sample purification.

2.2. Sampling

Sediment samples were collected from 13 locations in Hong Kong in both the wet and dry weather seasons in August 2012 (wet), February 2013 (dry) and June 2013 (wet) (n = 39), and eight locations in Tokyo Bay Japan in July 2013 using a stainless steel grab in a manner similar to that used in previous studies of organic UV filters [18,19]. Detailed information on sampling locations is shown in Table A2 and Figs. A1 and A2.

Victoria Harbor (sampling points 1–5 in Fig. A1) is a major tidal channel in Hong Kong receiving wastewater effluent with a catchment population of 3.5–4 million people, and is influenced by industrial and municipal discharge from the heavily industrialized and urbanized Pearl River Delta region located west of the harbor. Sediment samples were collected in the harbor near wastewater treatment plant (WWTP) discharge points representing indirect sources of UV filters [7]. Sai Kung (sampling points 7–13 in Fig. A1) is located in the eastern marine waters of the city and features generally good water quality [20,21] with a diversity of marine life such as corals. Therefore, recreational activities (*e.g.*, snorkeling and scuba diving) are frequently conducted in these areas, especially in the summer, representing direct sources of UV filters. Point 6 was selected as a reference site because it is far from both indirect and direct sources of UV filters. The Tokyo sediment samples were collected in Tokyo Bay, a semi-enclosed sea surrounded by three prefectures (Kanagawa, Tokyo and Chiba) comprising a population greater than 28 million people [22] and a receiving body for industrial, domestic and agricultural wastewater [23].

In Hong Kong, offshore waters are influenced by two seasonal currents, the northeast-flowing Hainan Current in June and July and southwest-flowing Taiwan and Kuroshio Currents from October to March; the transition between these currents is observed in other months [24]. Based on previously reported seasonal variation in organic UV filter concentrations in wastewater and surface water collected from this area [7,17], as well as potential seasonal differences in the distribution of suspended sediments due to changes in water currents, surface sediment samples were sampled in different months. All sediment samples were freeze-dried and

homogenized using a mortar and pestle and stored in a dessicator in the dark at −20 °C prior to analysis.

2.3. Analytical procedures

Sediment sample extractions were performed with an automatic pressurized liquid extractor, ASE 200 (Dionex Corporation, Sunnyvale, CA, USA), equipped with 22-mL stainless steel cells. For the in-cell packing (bottom to top), two cellulose filter papers were placed at the bottom of each cell, followed by 2 g of sodium sulphate, 0.3 g of graphitized carbon and 2 g of each freeze-dried non-sieved sediment sample, after which the cell was filled with diatomaceous earth. ^{13}C-BP-3 was spiked into each sample as a surrogate standard for determination of procedural recoveries before analysis. Analytes were extracted with MeOH/EA (30:70, v/v), at 160 °C and 1500 psi with 5 min static time for two cycles. The extraction cell was flushed with 60% extraction solvent with a purge time of 60 s. The extracts, *ca.* 40 mL, were transferred to amber pear-shaped flasks and concentrated to less than 1 mL by rotary evaporation. The concentrated extracts were subjected to further purification by transferring them to C18 SPE cartridges that were previously preconditioned with 15 mL of MeOH/EA (50:50 v/v). Six milliliter (2 mL × 3) of MeOH/EA (50:50 v/v) was used to rinse the amber pear-shaped flasks and loaded to the cartridge, and the target compounds were eluted by gravity using 6 mL of MeOH/EA (50:50 v/v). The extract volume (*ca.* 12 mL) was reduced to less than 0.5 mL under a gentle stream of nitrogen and the final volume was adjusted to 0.5 mL with methanol. All sample extracts were subjected to centrifugation at 9000 × *g* for 10 min to remove suspended particles that could obstruct the instrument and then transferred to amber sample vials for standard addition. Finally the extracts were analyzed by a high-performance liquid chromatography–electrospray ionization-tandem mass spectrometer (HPLC–ESI-MS/MS). Analytical duplication was carried out for each sample. Total organic carbon (TOC) was determined by the wet dichromate oxidation method according to the United States Environmental Protection Agency [25].

2.4. Instrumental analysis

Separation and quantification of analytes was performed using an Agilent HP1200 LC (Agilent, Palo Alto, CA, USA) interfaced with an AB SCIEX API 3200 triple quadrupole tandem MS equipped with a Turbo V ion source (AB SCIEX, Framingham, MA, USA) operated in both negative and positive mode. A 10 μL aliquot of extract was injected onto an XBridge™ C18 column (Waters Corporation, 5 μm, 2.1 mm × i.d. 50 mm length) equipped with a guard column at a flow rate of 0.3 mL min^{-1} using pure Milli-Q water (A) and pure methanol (B) in a gradient elution (0 min, 5% B; 15 min, 100% B; 20 min, 100% B; 20.1 min, 5% B; 30 min 5% B). Analytes were determined by ESI-MS/MS either in positive or negative mode by multiple reaction monitoring (MRM). Turbo V ion source and MS/MS parameters were as follows: curtain gas (CUR), 15 psi (positive mode) or 10 psi (negative mode); collision gas (CAD), medium; ion spray voltage, 5500 V (positive mode) or −4500 (negative mode); temperature, 400 °C (positive mode) or 600 °C (negative mode); ion source gas 1 (GS1), 40 psi; and ion source gas 2 (GS2), 55 psi. The instrumental detection limits, ranging from 0.003 to 0.218 ng/mL, were calculated by adding the intensity obtained from methanol (baseline) to three times its standard deviation. The detailed LC–MS/MS experimental parameters for the determination of UV filters are listed in Table A3.

Target compounds were quantified by the standard addition method using a 5-point curve for each sample in order to obtain reliable quantification and overcome matrix effects because of the lack of representative commercially available internal standards for the majority of the target compounds. The linear response range of the HPLC-MS/MS instrument was investigated with standards at six concentrations ranging from 0.1 to 800 ng/mL. Within this range, the system provided linear response plots (peak area versus standard concentration) with linearity (R^2) ranging from 0.990 to 0.999 (Table A3).

2.5. Quality assurance and quality control

Field blanks consisting of 10 g of diatomaceous earth were prepared to check for any contamination during sampling, transportation and storage, while procedural blanks (diatomaceous earth only in ASE cells) were included with each batch of samples during extraction and quantification to check for possible contamination during the analytical procedure. To avoid contamination during sample collection, sunscreens containing only inorganic UV filters and clothing without UV-protection were used by all participants. All of the target compounds were below method limits of detection (MLODs) in both field and procedural blanks. Table A4 shows the method recoveries, MLODs and relative standard deviations (RSD) of the analytical method.

Recoveries were calculated using the ratio of spiking 40 ng of the target compounds and 100 ng of the ^{13}C-BP-3 isotope-labeled surrogate standard into sediment samples collected from the reference site prior to ASE (pre-spiked samples) to a similar aliquot of samples spiked after extraction (post-spiked samples). Recoveries ranged from 75% to 110%. Analytical repeatability was examined by analyzing triplicate sediment samples over three days, and the RSD of the target compounds ranged from 4% to 15%. The MLOD was defined as three times the standard deviation of procedural blank peak areas plus their mean value corrected by a matrix-induced interference factor which was the slope difference of two calibration curves separately constructed in methanol and in sediment sample extracts [26]. MLODs ranged from 0.51 to 7.55 ng/g dw.

2.6. Statistical analyses

Normality tests (Kolmogorov–Smirnov) were performed before statistical analyses. Parametric Pearson correlation analysis was used for the examination of correlations among UV filter concentrations (data were $\log_{10}$-transformed) and TOC content in sediment using SigmaStat 3.5 (Systat Software Inc., Chicago, USA). The significance level was set at $\alpha = 0.05$. Principal component analysis (PCA) and permutational analysis of variance (PERMANOVA) were conducted to explore spatial patterns of organic UV filter levels in the Hong Kong samples. Samples with concentrations <MLOD were treated as zero in the analysis. All statistical analyses were carried out using PRIMER 6 & PERMANOVA+ (PRIMER-E Ltd., Plymouth, UK).

3. Results and discussion

3.1. Concentrations of organic UV filters in marine sediments

3.1.1. Hong Kong

A total of 39 surface sediment samples collected from 13 sampling points in Hong Kong from August 2012, and February and June 2013 were analyzed. The concentrations and detection frequencies of all UV filters are summarized in Table 1. Seven out of 11 UV filters were detected in the samples, with median concentrations ranging from <MLOD to 21 ng/g dw, while the maximum concentrations of EHMC and ODPABA were found to be >100 ng/g dw throughout the sampling period.

Generally, the detection frequencies of BMDM, BP-1, BP-8 and EHMC (calculated by dividing the number of positive detections by the total number of samples in each season) were >50% during the

Table 1
Concentrations (ng/g dw) and detection frequencies (D.F.) of 11 UV filters in marine sediment from Hong Kong and Tokyo Bay.

Compounds	August 2013 (HK)			February 2013 (HK)			June 2013 (HK)			July 2013 (Tokyo Bay)		
	(*n* = 13)			(*n* = 13)			(*n* = 13)			(*n* = 8)		
	Median	Range Min–Max	D.F.%	Median	Range Min–Max	D.F.%	Median	Range Min–Max	D.F.%	Median	Range Min–Max	D.F.%
EHMC	8.3	1.4–447	85	6.5	0.8–291	69	5.1	0.6–119	92	10.3	0.3–54.5	100
ODPABA	21.1	8.0–81.6	69	19.9	15.2–21.8	38	19.0	1.5–150	85	7.8	0.8–13.9	100
BMDM	8.0	4.3–42.9	77	10.8	5.5–18.6	54	8.6	4.3–16.0	69	17.0	2.5–64.5	100
BP-3	8.6	1.0–39.8	62	2.5	2.5–2.5	8	5.7	0.05–10.2	46	<MLOD		0
BP-1	1.8	0.6–6.1	62	2.3	0.9–5.0	54	1.6	0.8–8.2	69	11.2	2.7–14.6	88
BP-8	12.6	2.7–42.2	77	8.4	0.8–38.4	69	16.2	2.4–62.2	92	5.8	1.3–14.1	88
OC	1.3	0.04–15.2	23	8.7	5.0–12.4	15	10.7	0.7–15.6	31	<MLOD		0
4-MBC	<MLOD		0	<MLOD		0	<MLOD		0	<MLOD		0
IAMC	<MLOD		0	<MLOD		0	<MLOD		0	<MLOD		0
EHS	<MLOD		0	<MLOD		0	<MLOD		0	<MLOD		0
HMS	<MLOD		0	<MLOD		0	<MLOD		0	<MLOD		0

D.F.: calculated by dividing the number of positive detections by the total number of samples from each location; MLOD: method limit of detection.

sampling period, with maximum concentrations of 43, 8, 62 and 447 ng/g dw, respectively. These four compounds not only showed high detection frequencies in sediment, but also high occurrence in both wastewater effluent and surface water in Hong Kong [7,17], indicating their ubiquitous occurrence in the marine environment. The detection frequencies of ODPABA were >35% and >65% during the dry and wet weather seasons, respectively, with concentrations (median–maximum) ranging from 19 to 150 ng/g dw. This compound showed low detection frequency in surface water collected in the same sampling locations [17], suggesting that it readily partitions to sediments after being released into the environment, likely because of its relatively higher estimated log K_{oc} value, 5.28–6.23 ([27]; Table A1). 4-MBC, IAMC, EHS and HMS were not detected in the sediment samples even though EHS and HMS were detected in surface water in Hong Kong (detection frequencies in seawater: EHS, 59%; HMS, 76%) [17]. The absence of EHS and HMS in sediment could be due to the degradation of the salicylate molecule by microorganisms, as has been reported for *Halomonas campisalis* and *Pseudomonas putida* [28,29]. 4-MBC and IAMC were not frequently detected in surface water and sediment in Hong Kong, probably because of their relatively lower application rates in products [5]. TOC content in sediment ranged from 0.76 to 2.67%, and no significant correlation ($p < 0.05$) was found between TOC and concentrations of any of the UV filters measured in the present study. The correlation of organic contaminant concentrations with TOC in sediments depends on their physicochemical properties (*e.g.*, K_{oc}, pK_a) and sediment conditions (*e.g.*, microbial activities, pH) [30]. The lack of correlation between UV filters and organic carbon in sediment was consistent with a previous study on eight UV filters in riverine sediment [16], suggesting that these compounds are able to partition to other phases (*e.g.*, surface water [17]) in dynamic aquatic environments.

The total UV filter concentrations in sediment were 718, 1089 and 1247 ng/g dw in February 2012, June and August 2013, respectively. The relatively higher concentrations (1.5–1.7 times higher) detected in the wet season (June and August) could be due to (i) greater usage of PCPs containing UV filters during hot summer days with stronger UV radiation, and (ii) higher volumes of suspended sediments entering Hong Kong waters from the Pearl River Estuary, a heavily contaminated and densely populated and industrialized region of southern China [24], during the wet weather season. In terms of composition profile (calculated by dividing the total concentration of each UV filter by the total concentration of UV filters detected in that season), BMDM, EHMC and ODPABA were the predominant compounds in sediment samples accounting for more than 60% of the total UV filter occurrence in each season, probably due to their high usage in PCPs in Hong Kong based on their high detection frequencies in wastewater influent (>75%) [7] and accumulation in sediment. In contrast, although the three benzophenone derivatives (BP-1, -3 and -8) showed high detection frequencies (>75%) in wastewater effluent and surface water collected from similar sampling points in Hong Kong [7,17], they accounted for <35% of the total UV filter occurrence in sediment, likely because of their relatively lower estimated log K_{oc} values, 2.12–4.49 (Fig. A3). It should be noted that there is no routine monitoring of sedimentation rate in Hong Kong, and the sedimentation rate (and therefore organic UV filter concentrations) could be influenced by tidal variation or human activities (*e.g.*, trawling, wastewater discharge, dredging and reclamation) and is expected to vary among the sampling locations. The reported maximum marine sedimentation rate in Hong Kong has been reported to be less than 3 mm per year [24].

3.1.2. Tokyo Bay

Eight sediment samples collected from Tokyo Bay in July 2013 were analyzed and the results are summarized in Table 1. Five of 11 UV filters were detected in the samples; median concentrations ranged from <MLOD to 17 ng/g dw while the minimum detection frequency of all compounds was 88%. BMDM, EHMC and ODPABA showed 100% detection frequencies in Tokyo Bay with maximum concentrations <70 ng/g dw. As in the Hong Kong samples, BMDM, EHMC and ODPABA were the predominant compounds found in sediment while the benzophenone derivatives comprised <30% of the total organic UV filter concentrations in the samples. The high occurrence of EHMC was consistent with a previous study of a sediment core collected in Tokyo Bay which indicated the ubiquitous occurrence of EHMC over a 20-year period (1977–1997) [31]. The major source of these compounds is probably effluent discharge from WWTPs along several polluted rivers (*e.g.*, the Tama, Arakawa and Edo Rivers) which discharge into Tokyo Bay. More compounds (*e.g.*, OC and HMS) were detected in sediment samples collected from these rivers compared to Tokyo Bay in a previous study, perhaps due to a dilution effect in the Bay [32].

3.2. Spatial distribution of organic UV filters in sediment in Hong Kong

The median concentrations of detectable UV filters were 1.3–20.0 ng/g dw and 1.5–21.0 ng/g dw from indirect and direct sources, respectively. The highest concentration was observed in Kwun Tong (Fig. A1, sampling point 4) which is a typhoon shelter that receives effluent discharge *via* the Kai Tak Approach Channel from two WWTPs (*i.e.*, Shatin and Tai Po) serving up to 14% of the total population in Hong Kong. It is a semi-enclosed area with

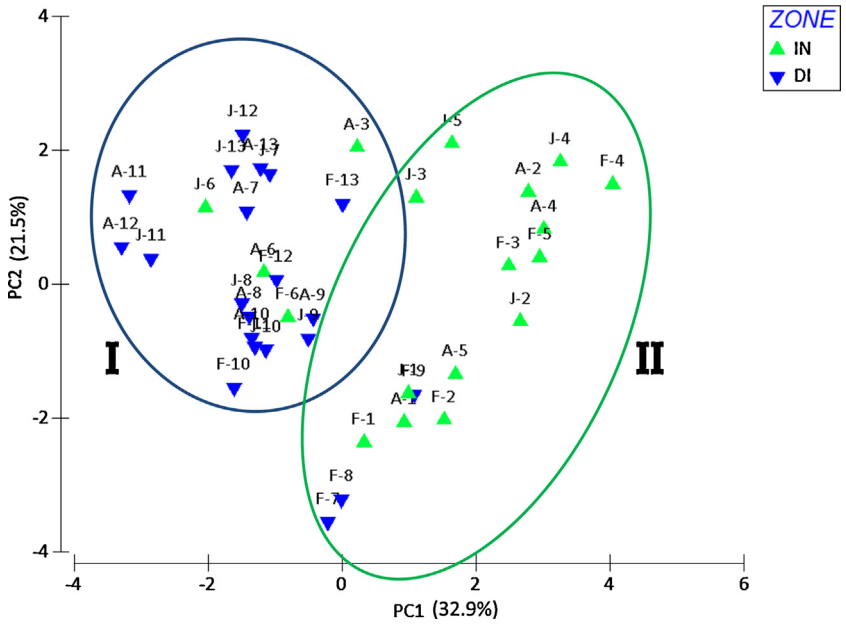

Fig. 1. Principal component analysis (PCA) plots for sediment samples collected in February 2013 ("F-" points), June 2013 ("J-" points), and August 2012 ("A-" points) in Hong Kong [In: Indirect sources (WWTPs; sampling points 1–6), Di: Direct sources (water sports & marine recreational activities; sampling points 7–13)].

little flushing, and therefore is known as a heavily contaminated area because of the high occurrence and concentration of organic contaminants in sediment there [33]. High concentrations of EHMC were measured at this sampling point, ranging from 119 to 447 ng/g dw over the sampling period.

The occurrence of organic UV filters in the Hong Kong sediment samples was investigated using PCA, in which significant zone differences ($p < 0.0001$, PERMANOVA) were observed among locations representing indirect and direct sources (Fig. 1). Sampling point 6 is located at the eastern end of Victoria Harbor and far from WWTP discharge locations and marine recreational areas. This site clustered together with locations representing direct sources (Fig. 1I), probably due to various human activities on the island (*e.g.*, camping, climbing). In February, sampling points 7–9 clustered together with locations representing indirect sources (Fig. 1II), likely due to wastewater discharge from a local secondary WWTP (serving 20,000 people) which is expected to the main source of organic UV filters in the winter when aquatic recreational activities are infrequent. All detectable UV filters showed higher detection frequencies from direct sources (8–62% higher than those from indirect sources), except for BP-1 and BP-8, which showed 51 and 17% higher detection frequencies from indirect sources than direct sources (Fig. A4). The presence of these two compounds in the environment could originate from PCPs (BP-1 is authorized for use at up to 10% of product volume in Japan, while BP-8 is allowed at up to 3% in the United States and Australia [3]), but also from biotransformation of BP-3 through demethylation [34]. Liu et al. [34] reported a faster biodegradation half-life of BP-3 under sulfate-reducing redox conditions (4.3 days) than oxic conditions (10.7 days). The total sulfide content and electrochemical potential of sediment samples influenced by wastewater discharge (indirect sources) in Victoria Harbor were reported to be higher and more negative, respectively, than those from Sai Kung (direct sources) (149 and 17 mg/kg; −260 and −197 mV, respectively; [35]; Fig. A1), suggesting a higher biodegradation rate of BP-3 to BP-1 and BP-8 in sediments representing indirect sources. It should be noted that higher estrogenic activities were observed for metabolites of BP-3 than the parent compound itself [15], and thus higher potential environmental risks might occur in sediments under low-oxygen conditions.

3.3. Global comparison of the occurrence of UV filters in riverine and marine sediment

There are limited reports in the literature about the occurrence of UV filters in sediment and most of the available studies have focused on freshwater sediments. Detailed occurrence data of organic UV filters in sediments is shown in Fig. 2. The occurrence of three UV filters was reported in marine sediments in Chile and Colombia with maximum concentrations of 17.8, 7.9 and 2.5 ng/g dw for EHMC, 4-MBC and BP-3, respectively; urban discharge and power generation stations were suggested to be the main sources of the detected compounds [38]. Levels of 4-MBC were found to be <MLOD for marine sediment samples collected in Tokyo Bay ([31]; present study) and Hong Kong (present study), probably due to (i) its relatively lower usage in PCPs [5] and (ii) the ban on its use in PCPs in Japan [3]. Comparable maximum concentrations of BMDM were reported in marine sediments in Hong Kong in the present study and in freshwater sediments in Germany (43 and 62 ng/g dw in rivers and lakes, respectively [19]). Because of the previous lack of analytical methods for analyzing this compound in sediment samples, the distribution of this commonly consumed compound in freshwater and marine sediments is still not well known. The widespread occurrence of OC was observed in sediments from Europe (Germany, Spain and the Mediterranean Sea) and Asia (present study), probably due to its relatively higher stability toward photodegradation [39]. Higher concentrations were detected in freshwater sediments (*i.e.*, rivers and lakes) than in marine sediments, with the maximum concentrations of OC in freshwater sediment reported as 2400 ng/g dw in a sample collected near a WWTP in Huerva Basin, Spain [16] and 635 ng/g dw in Japan [32]; the maximum OC concentration in sediments from the Mediterranean Sea was only 37 ng/g dw [18] and that in Hong Kong was only 16 ng/g dw in marine sediment (present study). This difference is likely due to the presence of WWTP point sources along rivers and stronger dilution effects in coastal areas due to the effects of tides and currents.

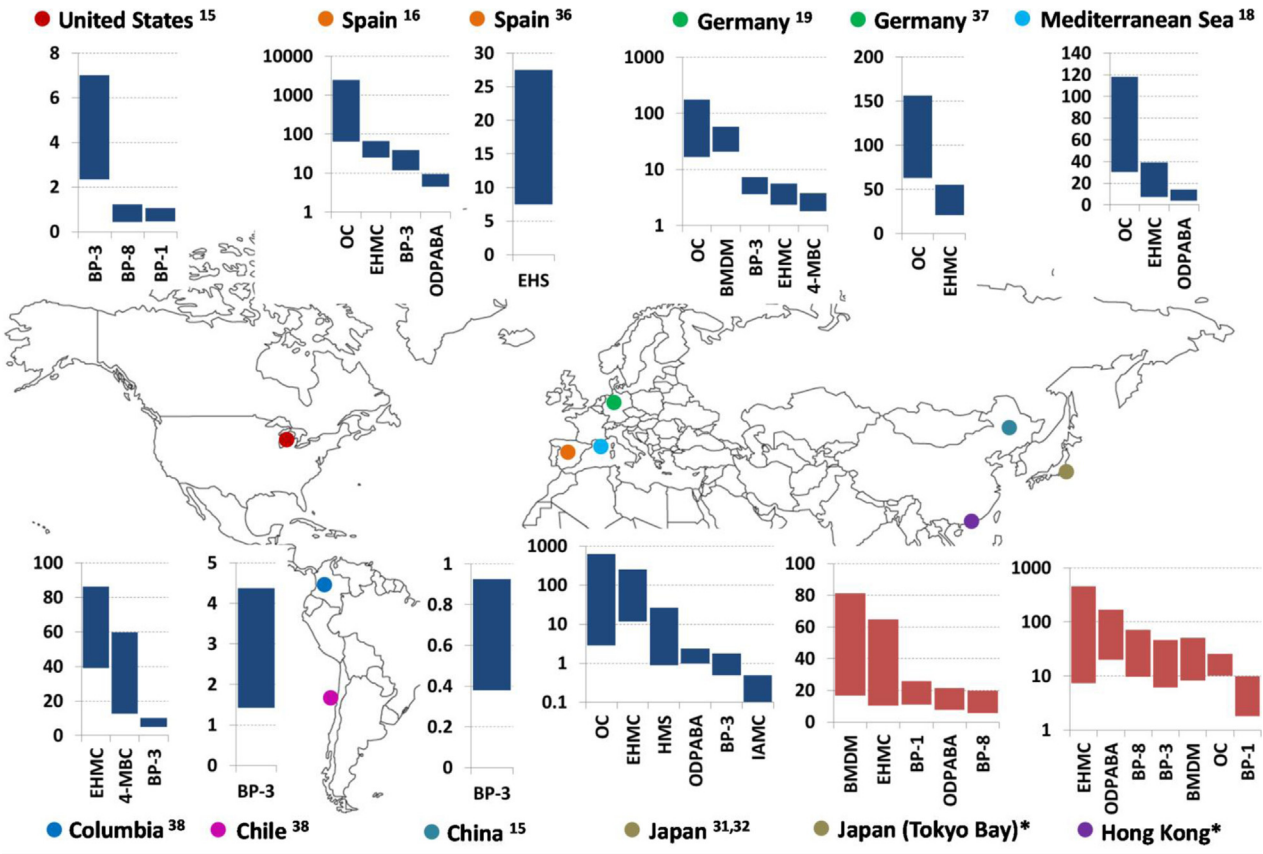

Fig. 2. Global comparison of organic UV filter concentrations (median–maximum, ng/g dw) in marine (M) and freshwater (F) sediment.
*: Present study (M); [15]: (F); [16]: (F); [36]: (M&F); [19]: (F); [37]: (F); [18]: (M&F); [38]: (M&F); [31,32]: (F).

3.4. Environmental risk assessment

Hazard quotients (HQs) for individual UV filters were obtained by dividing measured environmental concentrations (MECs) obtained in the present study by predicted-no-effect concentrations (PNECs) calculated by dividing the effect concentrations (ECs) by a standard assessment factor, 1000, to account for intra- (factor = 10) and inter-species variability (10) and chronic exposure conditions (10) [40]. Probabilistic plots were constructed for the measured environmental concentrations (MECs) in sediment samples from Hong Kong in three months, two seasons and Tokyo Bay. Risk probabilities (p) were calculated by substituting the log PNECs

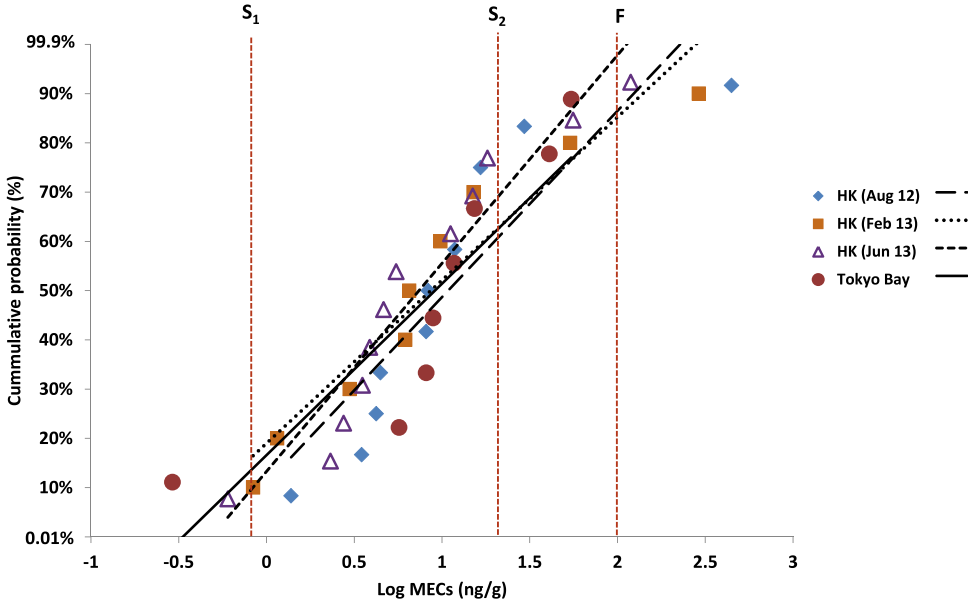

Fig. 3. Probabilistic risk assessment of EHMC in marine sediment from Hong Kong and Tokyo Bay. "S" thresholds are those derived from toxicity data for different snail species (S_1: Snail, *P. antipodarum*; S_2: Snail, *M. tuberculata*; F: Fish, *D. rerio*). Toxicity thresholds and endpoints are given in Table A5.

of each species in the linear equations for each sampled city, in which (100-p)% is the percentage of samples containing concentrations of that compound exceeding the PNEC of a particular species, and thus, posing risk based on the assessed endpoint. The toxicological information of UV filters for freshwater benthic organisms used in the ecological risk assessment is shown in Table A5. Only two studies have reported *in vivo* sediment toxicity values for EHMC and 4-MBC for invertebrates and fishes [41,42]. As 4-MBC levels were <MLOD in the marine sediment samples, probabilistic risk assessment was only carried out for EHMC. Two threshold values were available for freshwater snails, and both of these were used for the risk assessment (shown as thresholds S_1–S_2 on Fig. 3) and detailed information on regression coefficients is shown in Table A6. The probabilities of EHMC causing a reduction in fecundity in snails based on a no-observed-effect concentration (NOEC) in *Melanoides tuberculata* in Hong Kong sediment were 32–40% for the two sampling seasons, while that for Tokyo Bay was comparable at 38%. In contrast, higher sensitivity was observed for another snail (*Potamopyrgus antipodarum*) toward EHMC, where the probabilities of EHMC causing the same toxicity were 84–93% in Hong Kong sediment and 87% in Tokyo Bay sediment. Relatively lower risks were observed for EHMC in sediments in Hong Kong (2–15%) and Tokyo Bay (14%) based on a NOEC for developmental cardiovascular disorders in zebrafish embryos (*Danio rerio*). Though the probabilistic risk of sediment associated with UV filters to fish directly was found to be lower than 20%, these compounds may still pose a threat to aquatic organisms at higher trophic levels by entering the food chain due to their high lipophilicity.

As shown in our previous surface water study [17], some commonly used and frequently detected organic UV filters (*e.g.*, EHMC) could pose high risk to aquatic organisms at different trophic levels; however, there is insufficient information available for these compounds in the benthic environment. As a result, more ecotoxicological information and monitoring data are needed for comprehensive evaluation of the potential risks of UV filters in sediment in order to understand the degree of negative consequences posed to the benthic community. Moreover, it is of crucial importance to investigate the occurrence of these compounds in aquatic organisms at different trophic levels and their metabolic pathways in order to understand their bioaccumulation potential and possible risks to aquatic ecosystems.

4. Conclusion

The present study provides information on the occurrence, distribution and fate of 11 UV filters in marine sediments collected in Hong Kong and Tokyo Bay. Sediment concentrations reflected previous data obtained for the same locations in wastewater and surface water, with maximum concentrations detected in sediment samples in the wet season in Hong Kong, indicating higher usage of PCPs containing these compounds. Most of the detectable compounds showed higher detection frequencies in sediments representing direct sources (marine recreational activities) than indirect sources (WWTPs), except for BP-1 and BP-8. It should be noted that some UV filter metabolites have been shown to be more toxic than the parent compound [15] and hazards posed by these metabolites to the environment cannot be ruled out. The results of the present study also suggested that UV filters pose higher potential risks to benthic communities during periods with stronger UV radiation when usage is higher. There is a need for greater understanding of (1) the toxicities of these chemicals, both singly and in mixtures, on benthic and sediment-dwelling organisms, and (2) their potential bioaccumulation/biomagnification through the food chain and effects on higher-trophic-level organisms.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.03.025.

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:10 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 2 of 9
Attachments: 4 Balmer Occurrence of Some Organic UV Filters in Wastewater.docx; 5 Tashiro Concentrations of organic sun-blocking.docx; 6 Sang Environmental occurrence and ecological risk.pdf; 7 Bachelot Organic UV filter concentrations in marine.pdf; 8 Barosa Occurance and removal.pdf; 9 Dhanirama Cosmetics as a potential environental.pdf; 10 EU Watch List 2015.pdf; 11 Sankoda Seasonal and Diurnal Variation.pdf; 12 da Silvia Occurrence UVF Drinking Water Sao Paulo Brazil.pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

Occurrence of Some Organic UV Filters in Wastewater, in Surface Waters, and in Fish from Swiss Lakes

Marianne E. Balmer ,* Hans-Rudolf Buser , Markus D. Müller , and Thomas Poiger
Agroscope FAW, Swiss Federal Research Station, CH-8820 Wädenswil, Switzerland
Environ. Sci. Technol., 2005, 39 (4), pp 953–962

Abstract
Organic UV filters are used in personal care products such as sunscreen products, and in cosmetics, beauty creams, skin lotions, lipsticks, hair sprays, hair dyes, shampoos, and so forth. The compounds enter the aquatic environment from showering, wash-off, washing (laundering), and so forth via wastewater treatment plants (WWTPs) ("indirect inputs") and from recreational activities such as swimming and bathing in lakes and rivers ("direct inputs"). In this study, we investigated the occurrence of four important organic UV filter compounds (benzophenone-3, BP-3; 4-methylbenzylidene camphor, 4-MBC; ethylhexyl methoxy cinnamate, EHMC; octocrylene, OC) in wastewater, and in water and fish from various Swiss lakes, using gas chromatographic/mass spectrometric analyses. All four UV filters were present in untreated wastewater (WWTP influent) with a maximum concentration of 19 μg L-1 for EHMC. The data indicate a seasonal variation with influent loads higher in the warmer season (June 2002) than in the colder one (April 2002). The influent loads were in the order EHMC > 4-MBC ? BP-3 > OC. The concentrations in treated wastewater (WWTP effluent) were considerably lower, indicating substantial elimination in the plants. 4-MBC was usually the most prevalent compound (maximum concentration, 2.7 μg L-1), followed by BP-3, EHMC, and OC. UV filters were also detected in Swiss midland lakes and a river (Limmat) receiving inputs from WWTPs and recreational activities. However, all concentrations were low (<2–35 ng L-1); no UV filters (<2 ng L-1) were detected in a remote mountain lake. Data from passive sampling using semipermeable membrane devices (SPMDs) supported the presence of these UV filters in the lakes and the river and suggested some potential for accumulation of these compounds in biota. SPMD-derived water concentra tions increased in the order Greifensee < Zürichsee < Hüttnersee. This order is reversed from that observed for methyl triclosan, used as a chemical marker for WWTP-derived lipophilic contaminants in the lakes. This indicated inputs of UV filters from sources other than WWTPs to the lakes during summer, for example, inputs from recreational activities. Fish (white fish, Coregonus sp.; roach, Rutilus rutilus; perch, Perca fluviatilis) from these lakes contained low but detectable concentrations of UV filters, in particular, 4-MBC (up to 166 ng g-1 on a lipid basis). 4-MBC concentrations relative to methyl triclosan were lower in fish than in SPMDs exposed in the same lakes, suggesting that 4-MBC is less bioaccumulated than expected or metabolized in fish. The lipid-based bioconcentration factor (BCFL) estimated from the fish (roach) data and SPMD-derived water concentrations was about 1–2.3 × 104 and thus approximately 1 order of magnitude lower than expected from its Kow value.

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Concentration of organic sun-blocking agents in seawater of beaches and coral reefs of Okinawa Island, Japan.

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Abstract

The concentration of UV filters (UVFs) and UV light stabilizers (UVLSs) were measured in seawater and river water collected from sites at four beaches, two reefs, and one river on Okinawa Island, Japan. UVFs and/or UVLSs of 8-10 types were detected in beaches samples and 6-9 types were detected in reef samples. The total UVF concentrations at the beach sites were highest either in July or August with a maximum of 1.4 μg L(-1). The concentrations at the reef sites did not show peaks in summer and the maximum values were close to 10 ng L(-1). The detected UVF profiles reflected the ingredients of sunscreens used in each region. The highest UVLS concentrations at the reefs were observed not only in summer but also in June and September. The UVLS concentrations at the reefs were similar to or even higher than that at the beaches or in the river.

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Environmental occurrence and ecological risk assessment of organic UV filters in marine organisms from Hong Kong coastal waters

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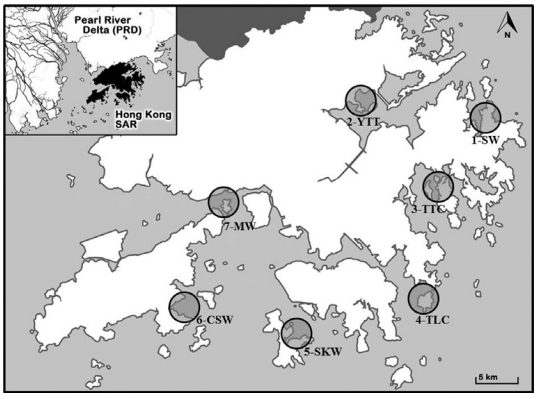
HIGHLIGHTS

- The occurrence of seven organic UV filters in marine organisms was investigated.
- UV filters showed higher detection frequencies and levels in mussels than in fish.
- Their spatial distribution implied a positive correlation with the direct sources.
- EHMC and BP-3 could pose significant risks to marine aquatic ecosystem.
- UV filters showed higher risks to marine aquatic ecosystem than freshwater.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic UV filters, now considered to be emerging contaminants in aquatic ecosystems, are being intensively tracked in environmental waters worldwide. However, their environmental fate and impact of these contaminants on marine organisms remains largely unknown, especially in Asia. This work elucidates the occurrence and the ecological risks of seven UV filters detected in farmed fish, wild mussels and some other wild organisms collected from local mariculture farms in Hong Kong. For all of the organisms, ethylhexyl methoxycinnamate (EHMC) and octyl dimethyl *p*-aminobenzoic acid (OD-PABA) were the predominant contaminants with the highest concentrations up to 51.3 and 24.1 ng/g (dw), respectively; lower levels were found for benzophenone-8 (BP-8), octocrylene (OC) and benzophenone-3 (BP-3) from <LOQ to <14.4 ng/g (dw); 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) were rarely detected. Additionally, the detection frequencies and measured concentrations of all targets were clearly higher in mussels than in fish. Spatial distribution of studied UV filters indicated a positive correlation between their measured concentrations and the anthropogenic activities responsible for their direct emission. The ecological risk assessment specific to the marine aquatic environment was carried out. The risk quotient (RQ) values of EHMC and BP-3 were calculated as 3.29 and 2.60, respectively, indicating these two UV filters may pose significant risks to the marine aquatic environment.

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1. Introduction

Organic ultraviolet (UV) filters, as the active ingredients in sunscreen products, block solar radiation by absorbing UV-A (320–400 nm) and UV-B (280–320 nm) (Nakata et al., 2009). Nowadays, the growing concern over the risks associated with UV radiation exposure have popularized their inclusion in a wide array of personal care products in addition to sunscreens, such as cosmetics, skin lotions, hair dye and shampoos as well as in some packing materials and plastics products (Li et al., 2007; Coltro et al., 2003; Zenker et al., 2008). As a consequence of their extensive consumption, these chemicals inevitably enter the aquatic ecosystem not only directly through recreational activities in water or by the shore but also indirectly via wastewater treatment plants (WWTPs) release (Groz et al., 2014) sourced from water used for showering, laundry and even excretion (Asimakopoulos et al., 2014).

Preliminary animal tests have indicated that at least a few commonly used UV filters such as BP-3, 4-MBC, 3-BC and EHMC (Inui et al., 2003; Kunz and Fent, 2006; Kunz et al., 2006a; Schlumpf et al., 2004) can disrupt endocrine function. Current toxicity information suggests widespread application of these substances could be risky to the aquatic ecosystem. Moreover, due to their high lipophilicity, UV filters are readily concentrated and accumulated in living aquatic organisms, such as fish and mussels, which has been documented by several research groups in Europe (Gago-Ferrero et al., 2015; Gomez et al., 2012; Fent et al., 2010). Bioaccumulation of EHMC and OC has been observed in Gomez's study (Gomez et al., 2012). With the exposure concentrations of around 11.0 μg/L, EHMC was accumulated in the soft tissue of marine mussels (*Mytilus galloprovincialis*) from 15 ng/g (dw) to 38 ng/g (dw) over a period of 48 h, and OC was accumulated from <2 ng/g (dw) (below LOD) to 60 ng/g (dw). Another study found that when *Pimephales promelas*, a type of minnow, was exposed to 3-BC for 21 days, an average bioconcentration factor was found up to 313 (Kunz et al., 2006b). In the long term, UV filters could bring even greater damage to aquatic ecosystem since their exceptional bioavailability may magnify their environmental impact as these xeno-estrogens accumulate along the aquatic food web (Fent et al., 2010; Gago-Ferrero et al., 2015).

As far as we know, knowledge of occurrence for UV filters in biota samples is still limited, especially in Asia. 4-MBC was firstly reported in the occurrence study focusing on various marine organisms collected from the Ariake Sea, in Japan, with an undetectable level in any tested samples (Nakata et al., 2009). Recently in South China, BP-3, 4-MBC and OP-PABA were detected in the marine fish and other wild organisms, which were collected from the Pearl River Estuary, within a concentration range of 0.1–41.5 ng/g (dw), while EHMC and OC were not detected in this study (Peng et al., 2015). Contrarily, relatively ampler studies and information of UV filters found in aquatic organisms were reported in Europe. BP-3, EHMC, OC and OD-PABA are the predominant sunscreen compounds that frequently detected in the marine organisms collected along the coasts of Norway, Portugal and France, with much higher concentration levels up to >800–1000 ng/g (dw); these organisms mainly include cod, shrimp, and mussels (Bachelot et al., 2012; Groz et al., 2014; Langford et al., 2015). Besides in marine organisms, EHMC, OC, BP-3 and 4-MBC have also been detected in aquatic organisms collected from rivers and lakes, mostly in Spain and Switzerland (Balmer et al., 2005; Buser et al., 2006; Fent et al., 2010; Gago-Ferrero et al., 2015), albeit with relatively lower concentrations (mainly below 700 ng/g (lipid weight)) compared to those in marine organisms. Additionally, OC was once detected in the liver tissue of Franciscana dolphins – a kind of aquatic mammals collected in Brazilian coast, within the concentration range of 89–782 ng/g (lipid weight) (Gago-Ferrero et al., 2013a). Combining the environmental risks of UV filters along with the limited information of their occurrence associated with aquatic organisms in Asia, a comprehensive study is necessary so as to support good environmental management, both in Hong Kong and worldwide.

The objectives of this study were to (i) make an investigation on the occurrence of seven UV filters in marine organisms, namely BP-3, BP-8, 4-MBC, 3-BC, OD-PABA, EHMC and OC; (ii) perform their spatial distribution in farmed fish, wild mussels and other organism samples collected in Hong Kong coastal environment; and (iii) evaluate the ecological risks of the present UV filters pose to the marine aquatic environment.

2. Materials and methods

2.1. Chemicals and reagents

Chemical standards for BP-3 (CRM), BP-8 (98%), 4-MBC (>99%), EHMC (CRM) and OC (CRM) were obtained from Sigma-Aldrich (St. Louis, MO, USA); OD-PABA (95%) was purchased from TCI (Shanghai, China); 3-BC (90–100%) was obtained from MP Biomedical (Irvine, CA, USA). 1000 mg/L individual stock solution of each UV filter was prepared in pure methanol and then stored in sealed amber glass vessels at 4 °C. A mixture of standard solutions of seven UV filters at the concentration of 10 mg/L was prepared daily and diluted to 1 mg/L for spiking when needed.

Solvents including Milli-Q water (Millipore, Bedford, MA, USA) LC-MS grade methanol, dichloromethane (DCM), ethyl acetate (AcEt) (Duksan Pure Chemicals, Seoul, Korea), absolute ethanol (Uni-Chem, Orientalab, China) and formic acid (98% purity, Merck, Rahway, NJ, USA) were employed in this study. Diatomaceous earth and cellulose filter papers (19.8 mm) were purchased from Dionex (Sunnyvale, CA, USA).

2.2. Sample collection and preparation

Farmed fish, wild mussels and some other wild organisms (including prawns, conch and sea urchin) were collected in June to July 2015, from seven local mariculture farms located around the coast of Hong Kong. The farms were selected such that they geographically represent the entire mariculture area of this region. Hong Kong is situated on the southern coast of China; it is surrounded by sea on three sides. Currently, there are 26 mariculture zones distributed on the eastern and southern coasts of the city (AFCD, 2006). In all of these zones, mariculture farms are usually and preferably placed in sheltered areas, far from pollution sources. The seven sampling locations are shown in Fig. 1, namely Sham Wan (1-SW), Yim Tin Tsai (2-YTT), Tai Tau Chau (3-TTC), Tung Lung Chau (4-TLC), Sok Kwu Wan (5-SKW), Cheung Sha Wan (6-CSW) and Ma Wan (7-MW).

Among these locations, 1-SW and 4-TLC are the most remote from urban areas; while 2-YTT and 3-TTC are located in Tai Mei Tuk and Sai Kung, respectively, which are two areas famous for seaside and water recreational activities. 5-SKW is close to the pier on Lamma Island, near a popular tourist fishing village with a population of >6000 residents. 7-MW is also near a small traditional fishing village on the shore near Tuen Mun but the fishery there is on the wane. 6-CSW is situated in a secluded bay on the southeastern corner of Lantau Island, the largest inland in Hong Kong.

The farmed fish samples collected from these seven locations were: mangrove snappers (*Lutjanus argentimaculatus*) from 1-SW, 2-YTT and 4-TLC; white blotched snappers (*Lutjanus stellatus*) from 5-SKW and 6-CSW; and Sabah giant groupers (*Epinephelus lanceolatus*) from 3-TTC and 7-MW. For each location, three samples of fish reared in different floating rafts were randomly collected (except 3-TTC and 7-MW, where only two fish from each could be caught) with the body length between 30 and 40 cm; triplicate measurements were carried out for each fish sample. Most of these cultured fish are mainly fed with moist or dry pellet feed; trash fish are also sometimes used as feed. The wild mollusk species including green mussels (*Perna viridis*) and clams (*Mactra antiquata* and *Corbicula* sp.) were collected in five locations (i.e., all except 5-SKW and 7-MW). A mixture of 15 individuals

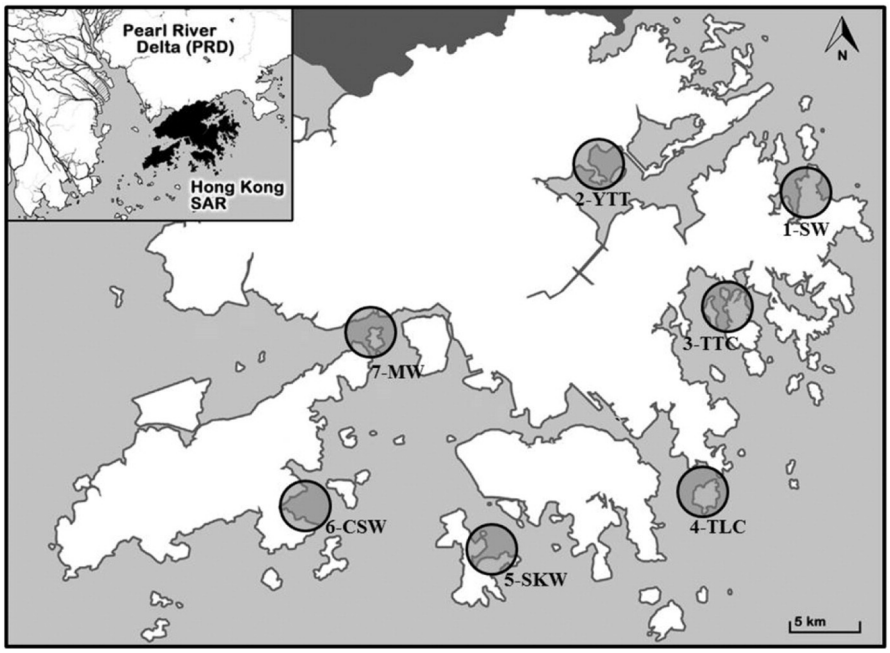


Fig. 1. Map of Hong Kong showing sampling locations of seven local mariculture farms.

with similar body length (5–8 cm) was regarded one complete sample, representative for one location. Triplicate analyses of one complete mussel sample (15 individuals) were carried out for each location as well. Other wild marine organisms were also collected occasionally such as tiger prawns (*Penaeus monodon*), conch (*Babylonia* sp.) and purple sea urchin (*Anthocidaris crassispina*), since it was not always possible to find them. Totally, there were 28 marine samples collected in this study.

Samples were individually packaged in single-used plastic bags and kept in a portable insulated container with ice packs to maintain the organisms alive and fresh during the one-hour transportation from the field to the laboratory. Treatment of samples was begun as soon as they arrived in the laboratory. For fish samples, evisceration and removal of skin, tail, head, fins and bones was carried out, such that only filets were used for testing. Mussels and other non-scaly marine organisms were cleaned of sedimentary materials and deshelled so that the soft tissues of the whole body were used. Filets and soft tissues were then homogenized individually and stored at −20 °C/−80 °C for no <24 h before lyophilization. After that, the totally dried samples were ground into fine powder that was individually packed in zip-lock bags and placed in an opaque desiccator for further use.

2.3. Analytical procedures

The extraction method for biota samples was modified from Gago-Ferrero et al. (2013b). After accelerated solvent extraction (ASE) (ASE 200, Dionex Corporation, Sunnyvale, CA, USA), the sample solution was further purified using Supelclean LC-18 solid-phase extraction (SPE) cartridges (500 mg/3 mL, Supelco, PA, USA). Each cartridge was conditioned with 3 mL × 3 of methanol follow by 3 mL × 3 of Milli-Q water. Then the sample solution was loaded onto the cartridge at the speed of 1 drop/s. After drying for 30 min under vacuum, the extractant was eluted with 3 mL methanol followed by two aliquots of 2.5 mL ethanol at a rate of 1 drop/2 s. Finally, the eluent was evaporated under a gentle nitrogen stream with purity of 99.9% until totally dried and then reconstituted with 1 mL methanol. Before instrumental analysis, the reconstituted eluent was syringe-filtered via 0.22 μm PTFE (Grace, IL, USA).

2.4. Instrumental analysis

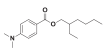
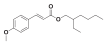
Instrumental analysis was performed using an Acquity Ultra Performance LC system hyphenated to a Quattro Ultima triple quadrupole mass spectrometer (Waters, MA., USA). The UPLC system comprises an auto-injector with a 10-mL sample loop and a temperature-controlled column compartment. Chromatographic analysis for UV filters was carried out with an Acquity BEH C18 column (2.1 i.d. × 50 mm, 1.7 μm, Waters); an injection volume of 4 μL was used for all samples. The mobile phases consisted of a mixture of milliQ water (A) and LC-MS grade methanol (B), both with 0.1% (v/v) formic acid added. The elution program protocol entailed a total run time of 5.5 min as follows: elution started with 65% of mobile phase B for 0.5 min, followed by 100% for 1.7 min; elution gradient was held at 100% for 1 min, then returned to 65% for 0.3 min, and initial conditions were reached in the final 2 min. The constant flow rate of 0.4 mL/min was applied throughout the elution program.

MS/MS detection was performed with electrospray interface operated in positive ionization mode (ESI+) at the capillary voltage of 1.6 kV. The source and desolvation temperatures were set at 150 °C and 500 °C, respectively. Nitrogen was used with a cone gas flow of 150 L/h nebulization and of 1000 L/h for desolvation. The quantitative analysis was performed in selected reaction monitor (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M + H]^+$ were monitored for each analyte to enhance the sensitivity and selectivity. The retention time (t_R), SRM transition pairs, cone voltages and collision energies are shown in Table 1. Instrument control, data acquisition and processing were performed using MassLynx software.

2.5. Analytical method validation

Five-point standard calibration curves were plotted within the concentration range of 0–100 ng/g, by spiking the standard solutions of UV filters into the sample-free blank solutions comprising 5 mL ethanol and 45 mL Milli-Q water before SPE. The linearities were calculated using the least square method. Recovery test was performed by spiking UV filter standard solutions at three concentrations, namely 8, 40 and 400 ng/g (dw), in the market samples, and the sample-free blanks as

Table 1
SRM conditions used in UPLC-MS/MS determination of UV filters.

UV filters	Chemical structure	Log Kow[a]	Retention time (min)	Molecular mass	SRM transition (*m/z*)	Cone voltage (V)	Collision energy (eV)
BP-8		4.31	0.89	244.24	245.08 → 121.00	24	18
					245.08 → 151.03		20
BP-3		3.52	1.34	228.25	229.09 → 151.03	26	20
					229.09 → 105.04		20
3-BC		4.49[b]	1.79	240.34	241.18 → 91.01	26	36
					241.18 → 97.01		18
4-MBC		5.47	2.01	254.37	255.15 → 105.02	26	30
					255.15 → 97.01		18
OC		6.88	2.25	361.47	362.25 → 250.13	24	8
					362.25 → 232.10		20
OD-PABA		5.77	2.33	277.40	278.18 → 150.97	30	30
					278.18 → 166.09		22
EHMC		5.80	2.38	290.40	291.18 → 179.11	12	8
					291.18 → 161.00		18

[a] Kameda et al. (2011).
[b] Zenker et al. (2008).

well as triplicate tests were carried out to test repeatability. Recoveries were expressed as the ratio of concentrations calculated from the spiked samples to spiked blanks. LOD and LOQ were determined by spiking the UV filter standard solutions into the sample-blanks and then using the signal-to-noise ratios of 3 times and 10 times for calculations.

3. Results and discussion

3.1. Method validation

3.1.1. Validation results

Validation results including linearity (R^2), recovery, repeatability (RSD), LOD and LOQ for each UV filter are summarized in Table 2. In previous studies, wide concentration ranges for UV filters detected in aquatic organisms have been reported. For example, Peng et al. reported 0.11–1.52 ng/g (dw) of BP-3 in wild marine organisms (Peng et al., 2015), while Fent et al. reported up to 151 ng/g lipid, also in marine organisms (Fent et al., 2010). OC was detected in mussels in a wide concentration range of <2–7112 ng/g (dw) (Bachelot et al., 2012). Therefore, in order to accommodate a wider working range, three standard calibration curves covering the low (0–100 ng/g (dw)), medium (0–500 ng/g (dw)) and high (0–1000 ng/g (dw)) concentration ranges were validated. In our study, most of the detected UV filters were in a low concentration, a five-point standard calibration curve with a linear range of 0–100 ng/g (dw). $R^2 > 0.995$ was obtained for all targets. Recovery was tested by spiking the standard solutions at three concentration levels which were 8 (low), 40 (medium) and 400 (high) ng/g (dw) so as to ensure a high accuracy of the method. For all of the analytes, recoveries were in the range of 80.4–114.8% and the variation was determined to be random, which means no bias was observed in any of the UV filters at the three concentrations. Also in this step, triplicate measurements were performed for these three concentration levels. Good repeatability of RSD ≤ 8% (n = 3) for all was obtained. LOD was calculated as 3 times the signal-to-noise ratio, and found to be in the range of 0.9–1.9 ng/g (dw). LOQ was calculated as 10 times the signal-to-noise ratio and found to be in the range of 2.9–6.2 ng/g (dw). These results are comparable to those reported in previous studies (Gago-Ferrero et al., 2013a, 2015; Groz et al., 2014), and it is reasonable to apply this method for the analysis of real samples.

3.1.2. Matrix effect study

Due to the complexity of the organisms' matrices, which may affect the analytical performance of the developed method, evaluation of the matrix effect was carried out using imported frozen aquatic products brought from the local market, namely fish of red bigeye (*Priacanthus macracanthus*), blue mussels (*Mytilus edulis*) and tiger prawns (*Penaeus*

Table 2
Summaries of the analytical method validation test results.

UV filters	Linearity (R^2) (range: 0–100 ng/g dw)	LOD (ng/g dw)	LOQ (ng/g dw)	Recoveries (RSD%)			
				Spiked levels (ng/g dw)	Fish	Mussels	Prawns
EHMC	0.9975	1.2	4.0	8	96.9% (1.7)	93.7% (6.7)	92.7% (4.3)
				40	94.6% (5.9)	80.4% (3.5)	112.3% (7.3)
				400	99.8% (5.5)	82.1% (2.5)	112.2% (2.4)
OD-PABA	0.9976	1.9	6.2	8	89.2% (5.9)	90.0% (3.1)	94.4% (5.6)
				40	108.2% (2.8)	88.8% (4.1)	111.4% (2.9)
				400	110.0% (8.0)	109.8% (3.0)	99.3% (0.5)
BP-8	0.9968	0.9	3.0	8	97.6% (1.8)	93.5% (2.3)	91.0% (5.3)
				40	88.1% (1.9)	89.9% (5.1)	89.7% (4.7)
				400	91.2% (2.4)	95.7% (0.7)	102.4% (0.7)
OC	0.9985	1.4	4.5	8	94.6% (6.3)	98.0% (4.7)	95.7% (7.4)
				40	110.3% (7.5)	86.6% (6.2)	106.8% (7.0)
				400	113.1% (7.4)	83.7% (8.0)	96.1% (6.9)
BP-3	0.9958	0.9	2.9	8	96.4% (1.1)	95.5% (1.0)	98.3% (2.0)
				40	113.0% (3.4)	102.9% (1.5)	92.2% (3.9)
				400	94.8% (4.2)	99.0% (3.2)	114.8% (2.4)
4-MBC	0.9989	1.8	6.0	8	88.6% (6.0)	95.0% (3.5)	94.8% (4.8)
				40	91.5% (7.2)	94.0% (4.9)	111.4% (7.5)
				400	96.4% (5.7)	90.9% (1.7)	113.8% (1.9)
3-BC	0.9975	1.8	6.0	8	91.4% (1.1)	90.9% (1.6)	96.6% (6.5)
				40	88.6% (5.2)	95.5% (6.3)	114.3% (7.9)
				400	86.6% (5.0)	89.6% (0.7)	94.5% (2.5)

monodon). The matrix effect of the marine organisms on each procedure throughout the whole analytical method was investigated step by step, including ASE extraction, SPE purification and instrumental analysis. Standard solution of UV filters at a concentration of 400 ng/g (dw) was pre- and post-spiked into the market samples and into the sample-free controls for each investigated analytical procedure. The matrix effects were expressed as the ratio of the pre-spiked signal abundance to relevant post-spiked signal abundance. Afterwards, the results were compared with those obtained from the sample-free controls (e.g. sample$_{[\text{pre-ASE spiked/post-ASE spiked}]}$ vs. sample-free$_{[\text{pre-ASE spiked/post-ASE spiked}]}$), and the calculated values <1 or >1 represented the signal suppression or enhancement, respectively, caused by the matrix effects corresponding to each analytical step.

The results indicated that the matrices of fish, mussels and prawns had no significant influence on the extraction efficiencies of ASE. At the same time, the signal suppression and enhancement in the instrumental analysis (UPLC-MS/MS) caused by the matrix effects of the three species was acceptable, ranging from 81.2% to 118.4%. However, with respect to the matrix effects on SPE purification efficiencies, the recoveries for three species calculated as pre-SPE spiking/post-SPE spiking of OD-PABA, EHMC and OC were <78% and generally lower than the values for the other UV filters. To solve this, the standard calibration curves were finally created by spiking the relevant concentrations into 50-mL solvent blanks (5 mL ethanol and 45 mL Milli-Q water) and subsequently loading those solutions onto the SPE cartridges using the same process for the sample solutions.

During the measurement of real samples, three groups of fishes including mangrove snappers, white blotched snappers and Sabah giant groupers were collected from seven local mariculture farms and were further analyzed to present the spatial distribution of seven UV filters in Hong Kong marine environment. Thus, it is necessary to evaluate the matrix effects sourced from three different fishes, since it is not always available to collect same species during the sampling. Standard solutions of UV filters were spiked into the fish samples and the sample-free controls before ASE. After analysis, the signal abundances for all seven UV filters obtained from each group of fish were compared to those from the sample-free controls. As the result, the variation ranged from 88 to 117% for three fishes collected from all seven locations, and no bias was found for any UV filters. Therefore, it is reasonable to use the measurement of our fish samples to present the distribution of target UV filters.

3.2. Environmental occurrence of UV filters in marine organisms

3.2.1. Concentrations in marine organisms

In this study, almost none of the seven targets UV filters were detected in the other wild organisms that occasionally collected (including prawns, conch and sea urchin), except EHMC, OD-PABA and BP-8 were only once detected in a conch, at the concentration levels below LOQ. For the fish and mussels, five sunscreen compounds namely EHMC, OD-PABA, BP-8, OC and BP-3 were commonly detected in the collected samples, whereas 4-MBC and 3-BC were rarely found (specifically, 4-MBC was only detected in one mussel sample at the concentration below LOQ and 3-BC was never observed in this study). Among all the UV filters studied, EHMC was determined at the highest concentrations both in fish and mussels, and it had the highest detection frequency of 75% in all samples (see Tables 3A and 3B). The concentration range of EHMC from the mean value to the maximum detected in fish was 4.2–12.7 ng/g (dw) and in mussels it was measured as 21.9–51.3 ng/g (dw) (detection frequency of 100% for mussels). OD-PABA was also measured at relatively higher concentrations compared to other UV filters; specifically, it was detected in a range of 14.2–24.1 ng/g (dw) in mussels, while from below LOQ to 10.3 ng/g (dw) in fish. Lower concentrations were similarly observed for BP-8, OC and BP-3, which were as high as 4.9, 5.4 and 3.1 ng/g (dw) in fish and 14.4, 11.6 and 12.4 ng/g (dw) in mussels, respectively. However, for these three UV filters, the

Table 3A
Mean concentrations ± S.D. of UV filters detected in marine organisms (ng/g dw), triplicate measurements (n = 3) with RSD ≤ 8%.

Sampling location	Species	EHMC			OD-PABA			BP-8			OC			BP-3			4-MBC			3-BC		
1-SW	Fish	n.d.	n.d.	N.Q.	n.d.	n.d.	N.Q.	N.Q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Mussel	N.Q.			N.Q.			N.Q.			n.d.			n.d.			n.d.			n.d.		
	Conch	N.Q.			N.Q.			N.Q.			n.d.			n.d.			n.d.			n.d.		
2-YTT	Fish	5.5 ± 0.2	6.2 ± 0.3	N.Q.	6.4 ± 0.2	N.Q.	N.Q.	4.2 ± 0.1	4.9 ± 0.1	N.Q.	5.4 ± 0.1	N.Q.	N.Q.	N.Q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Queen clam	24.6 ± 0.6			18.7 ± 0.4			10.7 ± 0.4			N.Q.			9.7 ± 0.3			n.d.			n.d.		
	Sand clam	33.1 ± 0.7			22.6 ± 0.6			13.5 ± 0.4			11.6 ± 0.5			12.4 ± 0.5			n.d.			n.d.		
3-TTC	Fish	9.3 ± 0.2	12.7 ± 0.2	-	7.2 ± 0.2	10.3 ± 0.2	-	4.8 ± 0.1	N.Q.	-	N.Q.	N.Q.	-	3.1 ± 0.1	N.Q.	-	n.d.	n.d.	-	n.d.	n.d.	-
	Mussel	51.3 ± 1.5			24.1 ± 0.8			14.4 ± 0.4			8.8 ± 0.3			10.3 ± 0.4			N.Q.			n.d.		
	Prawn	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
4-TLC	Fish	n.d.	n.d.	N.Q.	n.d.	N.Q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Mussel	N.Q.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
5-SKW	Fish	8.8 ± 0.2	6.4 ± 0.1	N.Q.	7.1 ± 0.2	9.7 ± 0.2	N.Q.	3.8 ± 0.1	4.7 ± 0.2	N.Q.	N.Q.	N.Q.	n.d.	n.d.	3.0 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Sea urchin	n.d.			n.d.			n.d.			n.d.			n.d.			n.d.			n.d.		
6-CSW	Fish	n.d.	4.1 ± 0.1	N.Q.	n.d.	N.Q.	N.Q.	N.Q.	N.Q.	3.2 ± 0.1	n.d.	n.d.	N.Q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Mussel	17.5 ± 0.3			16.2 ± 0.3			8.9 ± 0.2			N.Q.			N.Q.			n.d.			n.d.		
7-MA	Fish	N.Q.	5.1 ± 0.1	-	7.3 ± 0.2	6.8 ± 0.1	-	4.1 ± 0.2	N.Q.	-	n.d.	4.7 ± 0.1	-	n.d.	N.Q.	-	n.d.	n.d.	-	n.d.	n.d.	-

n.d.: <LOD.
N.Q.: <LOQ.
-: no sample collected.

Table 3B
Detection frequencies of detected UV filters in marine organisms.

Sample size	EHMC	OD-PABA	BP-8	OC	BP-3	4-MBC	3-BC
All samples (28)	75.0% (21/28)	71.4% (20/28)	71.4% (20/28)	46.4% (13/28)	32.1% (9/28)	3.6% (1/28)	0.0% (0/28)
Fish (19)	73.7% (14/19)	73.7% (14/19)	73.7% (14/19)	47.4% (9/19)	26.3% (5/19)	0.0% (0/19)	0.0% (0/19)
Mussels and clam (6)	100% (6/6)	83.3% (5/6)	83.3% (5/6)	66.7% (4/6)	66.7% (4/6)	16.7% (1/6)	0.0% (0/6)
Others organisms (3)	33.3% (1/3)	33.3% (1/3)	33.3% (1/3)	0.0% (0/3)	0.0% (0/3)	0.0% (0/3)	0.0% (0/3)

mean values were all measured below LOQ in fish, while they were calculated as 8.2, 5.1 and 5.9 ng/g (dw), respectively, in mussels. Beyond that, the detection frequencies for BP-8 in both fish and mussels were as high as those of OD-PABA, namely 73.4% and 83.3%, respectively, and they were much higher than the detection frequencies of OC and BP-3. In general, the concentrations of each UV filter in both fish and mussels showed the same descending order of concentration, as EHMC > OD-PABA > BP-8 > OC ≈ BP-3.

EHMC was the most frequently detected UV filter in environment samples (such as wastewater, surface water, seawater, sediment and biota) and was always found to be at high concentration levels (Zenker et al., 2008; Tsui et al., 2014a, 2014b; Tsui et al., 2015; Gago-Ferrero et al., 2015; Gago-Ferrero et al., 2013b). These high concentrations are likely due to its chemical properties—EHMC has good stability and high lipophilicity—as well as to the high consumption of products containing it and the failure of WWTPs to remove it (Li et al., 2007; Balmer et al., 2005). The concentration limits of EHMC contained in cosmetics and personal care products are as high as 10–20% depending on the different regulations worldwide, which are the highest limitations among all the organic UV filters (Sánchez-Quiles and Tovar-Sánchez, 2015). Moreover, the percentage of the personal care products containing EHMC reached 90% in China (Zhang et al., 2008; Tsui et al., 2015). At the same time, much higher loading of EHMC in WWTPs (as high as 2.3–119 g per 10,000 persons per day) was reported in Zurich, when compared to other commonly used UV filters (Balmer et al., 2005). Furthermore, it has been reported that the removal efficiencies of EHMC in most WWTPs in Hong Kong were mainly below 60% (Tsui et al., 2014b). Therefore, the large consumption in daily life along with the limited removal efficiencies in the WWTPs contribute to the massive amount of EHMC discharged into the environmental waters. In our previous study on the occurrence of UV filters in the seawaters in Hong Kong, EHMC was detected at the highest concentration of all filter studied, namely 191.67 ng/L in coastal water (Sang, 2016. Table A2). In addition, its relatively higher lipophilicity—with log Kow value of 5.80 (Kameda et al., 2011)—makes it much easier for biota to absorb and accumulate this UV filter from the aqueous phase. As a result, high concentrations of EHMC were observed in marine organisms.

Similar to EHMC, OD-PABA also has a high log Kow value (5.77) and is permitted at high contents in commercial products. Formulations with concentrations as high as 8% are permitted in Europe and China, while up to 10% are allowed in Japan. Moreover, the detection frequencies higher than 75% for OD-PABA along with a concentration up to 224 ng/L in the effluents of WWTPs in Hong Kong indicated the incomplete elimination of this compound and its inevitable emission into the local water environment (Tsui et al., 2014b). Thus, the relatively higher concentrations of OD-PABA measured in the marine organisms in this study may be related to its high usage and its higher log Kow value (Kameda et al., 2011) as well. It has been reported that EHMC and OD-PABA are the predominant UV filters in sediments in Hong Kong (Tsui et al., 2015). In contrast, OC, which is also frequently detected in environmental waters, also has high lipophilicity and good stability, and is also widely used in personal care products, was not found in high concentrations in marine organisms in this study. The reasons for this phenomenon remain unclear at this stage and further studies are still needed. Coincidentally, similar circumstance for OC was also observed in Tsui's study (Tsui et al., 2015), in which lower concentrations of OC was detected in the sediment samples collected in marine of Hong Kong when compared to the other UV filters with similar log Kow values. An observation of extremely low detection frequencies for OC in the influents and effluents of five local WWTPs (Tsui et al., 2014b) may imply a rather lower emission of this compound into the environmental water. Moreover, Bachelot has indicated that OC may be not as persistent as EHMC when investigated in mussels collected from French coastal regions (Bachelot et al., 2012). He reported that OC was detected up to >1000 ng/g (dw) in June to August in mussel samples, while dropping to as low as total absence in September and November; in contrast, EHMC remained present in mussels even after the summer. Therefore, to acquire better knowledge on the environmental behaviors of these sunscreen compounds in aquatic biota, further studies on their uptake, metabolism and excretion mechanisms in the organisms are highly demanded.

BP-3 is another UV filter, widely detected in environmental waters, widely consumed and with a high content in personal care products and cosmetics—up to 10% allowed by regulations in both Europe and China (Sánchez-Quiles and Tovar-Sánchez, 2015). However, due to its lower log Kow value of 3.52 (Kameda et al., 2011), meaning it is less lipophilic than other UV filters, the concentrations of BP-3 detected in the biota samples were not as high as those in environmental waters compared with EHMC and OD-PABA. Another aspect to consider is the metabolism of this UV filter. Many studies have proved that BP-3 is readily metabolized (Kunz and Fent, 2006). As a major metabolic product of BP-3, BP-8 has a higher log Kow value of 4.31 and higher bio-concentration factor (BCF = 524) compared to its parent compound (BCF = 502) (Kim and Choi, 2014). This high lipophilicity could explain why BP-8 is often found in the sediments. To the best of our knowledge, 3-BC has never been detected in marine organisms. 4-MBC was often reported as not detected or in extreme low concentrations in aquatic organisms for those previous studies in USA, Spain and Japan (Gago-Ferrero et al., 2015; Mottaleb et al., 2009; Nakata et al., 2009).

3.2.2. Spatial distribution in Hong Kong

Seven sampling locations along the coastline of Hong Kong were briefly introduced in Section 2.2. Fig. 2A and B show the spatial distributions of the UV filters detected in the fish and mussels, respectively, with regard to the local mariculture farms from which the samples were taken. The maximum total concentrations of seven investigated UV filters in the farmed fish were observed at the location 3-TTC, followed by 5-SKW and 2-YTT (Fig. 2A). The mussels showed the same concentration pattern (Fig. 2B), with the highest concentrations of all detected UV filters being reported at 3-TTC. Relatively higher levels were also detected in sand clams and queen clams both collected in 2-YTT. Relevant information for 5-SKW was absent since no mollusk species was collected there. In contrast, the lowest concentration levels of the target compounds were found at 4-TLC and 1-SW for both fish and mussels. Especially at 4-TLC, only EHMC and OD-PABA were occasionally detected there at concentrations even lower than LOQ. Additionally, the median levels of UV filters measured in the marine organisms were observed at 7-MW and 6-CSW.

Since all the sampling locations in this study were at official mariculture farms in Hong Kong, they were as far away from sewage discharge outlets as possible in order to avoid pollution via the effluent of WWTP. Thus, any indirect input of UV filters from incomplete treatment in the WWTP may not be a focus of concern in this study. Even so, there is always some chance of indirect input, and this should not be ignored.

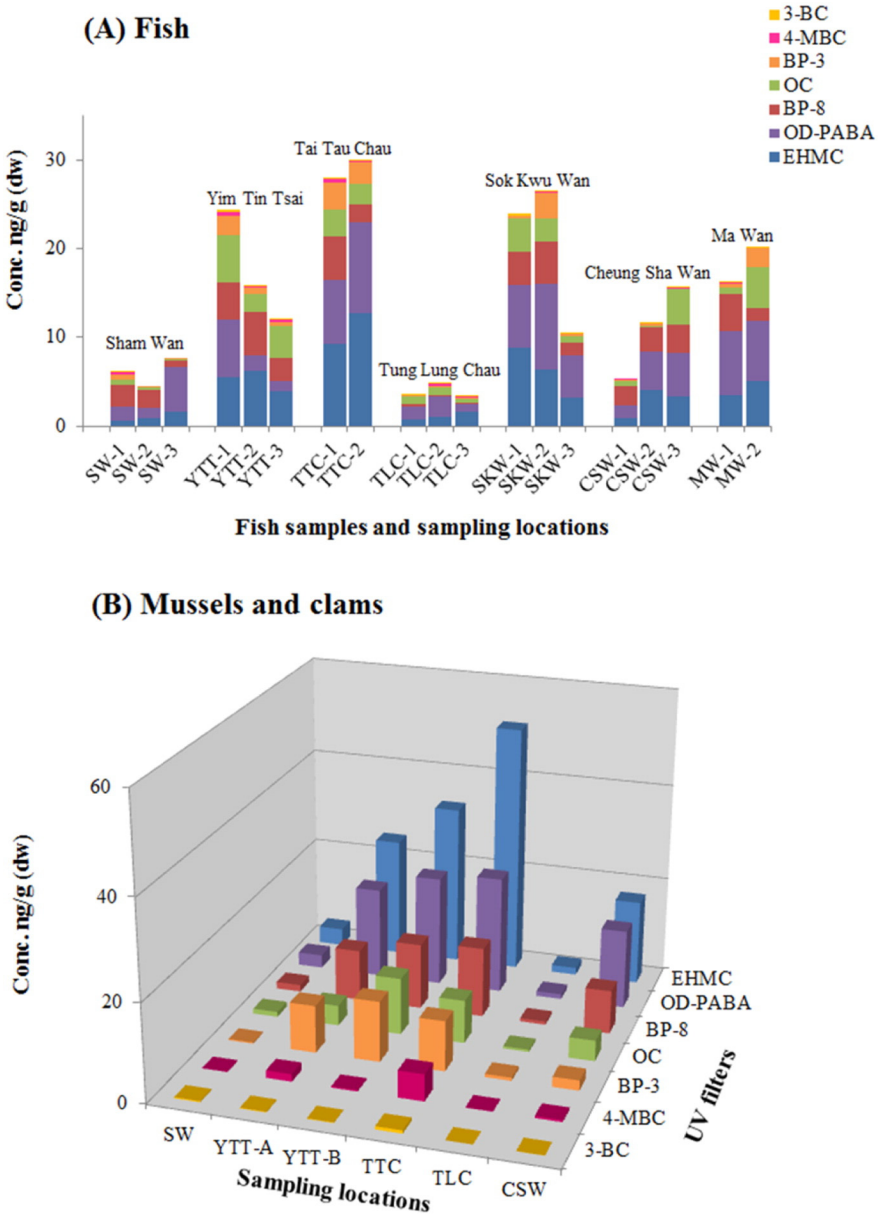


Fig. 2. Distribution of detected UV filters in (A) fish and (B) mussels and clams along the coast of Hong Kong. SW, TTC, TLC and CSW: Mussels. YTT-A: Queen clams. YTT-B: Sand clams.

Nevertheless, assuming that input is negligible, further discussion here will focus on the direct input of UV filters into the ecosystem. As previously described, 4-TLC and 1-SW, where the minimum concentrations of UV filters were detected, are remote from urban areas and sparsely populated. As a result, the anthropogenic activities and influence there is less intensive compared to the other locations and thus the pollution caused by the directly input of UV filters should be limited. Conversely, 3-TTC, 2-YTT and 5-SKW, where the relatively larger amounts of UV filters were measured, are famous places for intensive recreational activities such as water sports, seaside leisure activities and tourism, not only in summer but all year round. These activities represent enormous opportunities for the substances contained in personal care products such as UV filters to enter the ecosystem directly and easily. We did indeed find a positive correlation between the measured concentrations and the anthropogenic activities; from this it can be inferred that the direct input of the UV filter compounds related to the anthropogenic activities significantly affects the their occurrence in marine organisms. Such direct input could result in rapid and even serious effects compared to indirect input way because the substances might directly and rapidly interact with the biota once entering the ecosystem.

3.2.3. Global comparison

So far, many studies have focused on the occurrence of UV filters in environmental waters especially coastal water, rivers, lakes, and WWTP effluent (Zenker et al., 2008; Tsui et al., 2014a, 2014b; Tsui et al., 2015). Studies of the occurrence of UV filters in marine organisms, however, are very few. Up to now, most of the studies have come from Europe, with only a few from Asia. In Europe, BP-3, EHMC, OC and OD-PABA are the predominant UV filters detected, and they were reportedly determined in coastal biota samples collected from Norway, France and Portugal at concentrations higher than thousands ng/g (dw) (Langford et al., 2015; Bachelot et al., 2012; Groz et al., 2014). In Oslo, Norway, the concentration levels and detection frequencies of BP-3, OC and EHMC were found to be higher in cod liver than in shrimp. BP-3 and OC were measured at levels as high as 1037 and 11,875 ng/g (ww), whereas EHMC was determined at comparatively lower concentrations, in the range of 30–36.9 ng/g (ww). None of the UV filters were

detected in any wild shore crab samples in this study (Langford et al., 2015). In France, in a study of mussels collected both from the coastal regions of the Atlantic and Mediterranean, 100% of the samples had quantifiable EHMC, ranging from 3 to 256 g/g (dw), whereas 55% of the samples had detectable amounts of EHMC, ranging from <2 to 7112 ng/g (dw). In summary, EHMC was the most frequently detected UV filter while OC was at its highest concentration in mussels. Interestingly, OD-PABA was never detected in this study (Bachelot et al., 2012). Also in mussels collected from the coasts of Portugal, OC was measured at the maximum concentration of 3992 ng/g (dw), while EHMC and OD-PABA reached levels up to 1765 and 833 ng/g (dw), respectively (Groz et al., 2014). Viewing the studies in freshwater biota, all the UV filters mentioned above (BP-3, EHMC, OC and OD-PABA) together with 4-MBC were detected at comparable or a little lower concentration levels in aquatic organisms sampled from the rivers and lakes, mainly in Switzerland and Spain (Gago-Ferrero et al., 2015; Fent et al., 2010; Zenker et al., 2008; Buser et al., 2006). Another similar study on fish samples collected from rivers and creeks in the USA reported non-detectable levels of 4-MBC and OC (Mottaleb et al., 2009). Additionally, along the Brazilian coast, OC was detected at concentrations from 89 to 782 ng/g (lipid weight) in the liver tissue of dolphins (Gago-Ferrero et al., 2013a). Even less information on the occurrence of UV filters in marine biota is available for Asia. In the Ariake Sea of Japan, 4-MBC was not found in any of 55 marine organisms nor in sediments (Nakata et al., 2009). In south China, BP-3 and OD-PABA were detected in farmed fish and wild marine organisms collected from the Pearl River Estuary at extremely low levels, within ranges of 0.106–1.520 ng/g (dw) and 0.239–0.360 ng/g (dw), respectively. Surprisingly, the highest concentration of 4-MBC, up to 14.7 ng/g (dw), was measured in the filets of farmed fish. EHMC and OC were not detected in this study (Peng et al., 2015). In our study in Hong Kong, which is geographically located adjacent to the Pearl River Estuary, six UV filters were detected in marine fish and mussels with frequencies from 3.6% to 75% in the concentration range of <6.0–50 ng/g (dw).

Comparing the occurrence in marine organisms in Europe and Asia (Table A1), in general, the measured total concentrations of UV filters in East Asia are lower than those in Europe by at least 1–2 orders of magnitude; detection frequencies are also much lower. Probably the differences in habits associated with the use of skin care products between the two studied areas are the main reason for this observation. It has been reported that consumption of sunscreens is much higher in Europe than Asia. In 2012, the average regional consumption in Europe was reported as 52 mL, while the value in Asia Pacific was only 4 mL (Osterwalder et al., 2014). As a result of extremely high consumption, UV filters would inevitably enter the aquatic ecosystem as well as accumulate in aquatic organisms. Another factor contributing to regional differences could be the higher annual average temperature in East Asia, especially in South China, when compared with Europe. Higher temperature may accelerate the degradation and metabolism for the UV filters in the aquatic ecosystem and even inside organisms' bodies. More investigations are needed to determine the underlying reasons. Even so, there are some similarities between the two regions. The UV filters that predominate in both Europe and Asia are EHMC, BP-3 and OD-PABA. The occurrence of OC differs in these two areas. It was detected at relatively higher frequencies and concentrations in Europe whereas it was rarely detected, or detected only at limited levels, in East Asia.

3.3. Ecological risk assessment

According to the Technical Guidance Document (TGC) of European Commission on risk assessment (EC, 2003), the basic approaches to risk assessment for marine aquatic environments are very similar from those for freshwater environments; there are numerous common principles and objectives. Beyond that, the modification specific for the assessment of the marine environment is focusing on the assessment factor (AF) (10–10,000). However, the ecotoxicity data of compounds specific to the marine organisms are sometimes limited and not always available. Then, the ecotoxicity data derived from freshwater species are used (EC, 2003).

In this study, the measured environmental concentrations (MECs) of several UV filters detected in marine environment of Hong Kong (Sang, 2016; Table A2) are adopted to perform the risk assessment using approaches for both inland aquatic compartment and marine aquatic environment. The aim was to compare the differences in the assessment results derived from two series of AFs, and also in the ecological risks of UV filters posed to the freshwater and marine aquatic systems. The median and maximum MECs of BP-3, 4-MBC, EHMC and OC were used to calculate the $RQ_{general}$ and RQ_{worst}, respectively. For these four detected UV filters, the ecotoxicity information according to the aquatic species in one to three tropic levels both in freshwater and seawater are collected to get the predicted no effect concentrations (PNECs) via being divided by the AFs which are depended on the relevant data of ecotoxicity for each substance. Results and related information for the ecological risk assessment are given in Tables 3A and 3B.

Table 4A gives the results of ecological risk assessment for the marine aquatic environment. The ecotoxicity data with the highest sensitivity tested via the freshwater and saltwater organisms were used and the AFs of 1000 for BP-3, 4-MBC and EHMC while 10,000 for OC are used respectively for the risk assessment specific to marine system. The calculated $RQ_{general}$'s of four UV filters are as follows: (a) $RQ_{general}$ of OC < 0.001. This means, at the measured concentrations, OC poses an extremely low risk to the marine aquatic system. (b) $RQ_{general}$ of 4-MBC is 0.33. This is between 0.1 and 1, which means, at the measured concentrations, 4-MBC poses a medium potential risk to the marine environment. (c) $RQ_{general}$ of BP-3 is 2.60, while $RQ_{general}$ of EHMC is 3.29. Values > 1 indicate that both these UV filters are highly suspected of having an adverse effect on the marine aquatic environment (Hernando et al., 2006; Rodríguez et al., 2015). Considering its widespread occurrence and relatively high measured concentrations in the environmental waters as well as its stronger ecotoxicity, EHMC may present the highest environmental risk to the marine aquatic system compared with the other tested UV filters.

Table 4A
Ecotoxicological information from three trophic levels of freshwater and saltwater organisms of UV filters and the risk assessment for marine aquatic environment.

UV filters	Ecotoxicity data for freshwater organisms (mg/L)			Ecotoxicity data for marine organisms[d] (mg/L)				AF	NOEC (mg/L)	PNEC (ng/L)	MEC_{max} (ng/L)	MEC_{median} (ng/L)	RQ_{worst}	$RQ_{general}$
	Algae[a]	Invertebrates[a]	Fish	Autotrophs	Herbivores		Carnivores							
BP-3	0.560	1.670	0.191[b]	0.014	3.473	3.280	0.711	1000	0.014	13.87	82.35	36.07	5.94	2.60
4-MBC	0.210	0.100	–	0.171	0.587	0.854	0.193	1000	0.100	100	74.50	33.32	0.75	0.33
EHMC	0.240	0.040	10000[c]	0.075	3.118	0.284	0.199	1000	0.040	40	191.67	131.74	4.79	3.29
OC	–	–	10000[c]	–	–	–	–	10,000	10,000	1,000,000	63.63	36.64	<0.001	<0.001

[a] IC10 of *D. subspicatus*, EC50 and NOEC of *D. magna* (Sieratowicz et al., 2011).
[b] NOEC of *D. rerio* (Kinnberg et al., 2015).
[c] LC50 of *B. rerio* and *L. idus* (Brooke et al., 2008).
[d] EC50 of *I. galbana*, *M. galloprovincialis*, *P. lividus* and *S. armata* (Paredes et al., 2014).

Table 4B
Ecotoxicological information from three trophic levels of freshwater organisms for UV filters and the risk assessment for freshwater environment.

UV filters	Ecotoxicity data for freshwater organisms (mg/L)			AF	NOEC (mg/L)	PNEC (ng/L)	MEC_{max} (ng/L)	MEC_{median} (ng/L)	RQ_{worst}	$RQ_{general}$
	Algae[a]	Invertebrates[a]	Fish							
BP-3	0.560	1.670	0.191[b]	100	0.191	1910	82.35	36.07	0.04	0.02
4-MBC	0.210	0.100	–	100	0.100	1000	74.50	33.32	0.07	0.03
EHMC	0.240	0.040	10000[c]	100	0.040	400	191.67	131.74	0.48	0.33
OC	–	–	10000[c]	1000	10,000	10,000,000	63.63	36.64	<0.001	<0.001

[a] IC10 of *D. subspicatus*, EC50 and NOEC of *D. magna* (Sieratowicz et al., 2011).
[b] NOEC of *D. rerio* (Kinnberg et al., 2015).
[c] LC50 of *B. rerio* and *L. idus* (Brooke et al., 2008).

In Table 4B, the same ecotoxicity data from the freshwater organisms and the same MECs of four UV filters are used for the risk assessment on freshwater environment, in which the AFs of 100 for BP-3, 4-MBC and EHMC while 1000 for OC are employed respectively for calculation. As the result shows, all the $RQ_{general}$ values of the four UV filters are <1, which means all these tested UV filters do not show the significant potential risk to the freshwater environment according to the assessment methodologies generally used for the inland aquatic compartment. For BP-3, 4-MBC and OC, the $RQ_{general}$ values are even lower than 0.1 which indicate only slight potential risk to the inland aquatic system, whereas only for EHMC the $RQ_{general}$ value is between 0.1 and 1 which indicates only slightly higher potential risks to the freshwater environment.

These results are similar in both evaluation methodologies in that EHMC always shows relatively higher potential risks than the other three UV filters, and OC shows the lowest $RQ_{general}$ values (<0.001). However, comparing the evaluation criteria with those for marine aquatic environment, the $RQ_{general}$ values of four tested UV filters are one to two orders of magnitude lower when using inland aquatic environment assessing methods. For example, for BP-3 in particular the $RQ_{general}$ value in freshwater risk assessment is 0.02 (<1, no significant potential risk) while in marine risk assessment it is 2.60 (>1, adverse effect). In other words, the prediction is totally different based on the same MECs. Thus, the potential risks posed to the marine aquatic environment of the detected UV filters could be largely underestimated when simply using inland aquatic environment methodologies. Underestimating potential risks can mislead both scientists and legislative bodies seeking to protect water resources; further studies may not be done and appropriate safeguards may not be enacted. As a result, it may not be able to get sufficient attention and controls for the substances which are potentially harmful to the marine aquatic environment, such as the emerging contaminants contained in the personal care products with quite large consumptions.

Another difference in the ecotoxicity to each level of organisms may also reflect that UV filters pose the environmental risks to the marine aquatic system distinct from those to the freshwater environment. Comparing the ecotoxicity data of marine organisms and fresh water organisms, it can be seen that the lowest EC50 values for three UV filters tested via marine organisms are all reported from the alga *Isochrysis galbana* (Paredes et al., 2014), whereas in freshwater organisms this is not true. For 4-MBC and EHMC in freshwater, the lowest ecotoxicity data are the no observed effect concentrations (NOECs) tested in the freshwater flea, *Daphnia magna*, rather than in an alga (*Desmodesmus subspicatus*) (Sieratowicz et al., 2011), while for BP-3 in freshwater, the lowest ecotoxicity data are observed from the zebrafish (*Danio rerio*) (Kinnberg et al., 2015). This may indicate that organisms at lower trophic levels such as alga might be the most affected species due to the existence of UV filters in marine ecosystems, whereas in freshwater systems the organisms at higher trophic levels may be more sensitive to UV filters than the alga species. Therefore, UV filters are likely to express inconsistent ecotoxicities in different ecosystems, such as marine versus freshwater, and thus it is unreasonable to assess their ecological risks by simply using general criteria without any specific adjustment.

In previous reports, the RQ values for BP-3 and OC detected in surface waters in Norway were calculated as <1 and their risks were reported as low (Langford et al., 2015). Similarly, the occurrence of BP-3 and several other UV filters found in the urban groundwater in Barcelona posed no acute risk according to their risk assessment, in which the RQs were all below 4×10^{-3} (Molins-Delgado et al., 2016). For the study in Gran Canaria Island, Spain, the risk assessment was performed on BP-3, 4-MBC and EHMC with the RQ values of 0.0–6.6, 0.1–10.4 and 0.4–18.9, respectively (Rodríguez et al., 2015). Significant adverse effects were found for 4-MBC and EHMC, consistent with the fact that RQ values higher than 10 were reported for both compounds. Lower potential risk for BP-3 was obtained. The RQ values of BP-3, 4-MBC and EHMC were also calculated as higher than 1 using the MECs of the UV filters detected in the seawater samples collected in Hong Kong (Tsui et al., 2014a), indicating the potential risks to the aquatic ecosystem posed by UV filters are becoming both significant and ubiquitous.

4. Conclusion

The present report describes the occurrence study of seven UV filters in farmed and wild marine organisms. It is based on a study of organisms collected in Hong Kong coastal fish farms. The report not only provides information on the occurrence and regional distribution of these filters, but also provides a global comparison of these figures with similar figures from East Asia and Europe. Higher concentrations were universally detected in wild mussels than in farmed fish collected from the same locations. The highest total amount of UV filters were found in samples from Sai Kung, reflecting the direct impact of anthropogenic activities on their occurrence in aquatic environments. Comparison of these results with those from studies in Europe indicates that the predominant UV filters detected in aquatic organisms are almost same worldwide; these filters are EHMC, OD-PABA and BP-3. Compared with Europe, however, whereas the concentrations measured in Hong Kong are much lower. Additionally, risk assessment indicates that long-term persistence of these UV filters at present levels poses significant potential risks to the marine aquatic environment. These risks may be amplified through the food chain and ecological cycle. Therefore, further study, possibly further controls, and alternative chemicals should all be explored as approaches to the environmental management of these emerging contaminants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.05.120.

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Organic UV filter concentrations in marine mussels from French coastal regions

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ABSTRACT

The accumulation of EHMC, OCT and OD-PABA, three common UV filter compounds, was investigated in marine mussels. Wild *Mytilus edulis* and *Mytilus galloprovincialis* were sampled in ten sites along the French Atlantic and Mediterranean coasts from June to November. In mussel tissues, 100% of the samples had quantifiable EHMC concentrations ranging from 3 to 256 ng g^{-1} dry weight, while 55% of the samples had detectable OCT concentrations ranging from under 2 to 7 112 ng g^{-1} dry weight. These concentrations significantly increased with the rising air temperature in summer, the recreational pressure and the geomorphological structure of the sampling sites (its lack of openness to the wide). This is the first study to report bioaccumulation of UV filters in marine mussels, thus highlighting the need for further monitoring and assessment.

1. Introduction

UV filters are used in sunscreen and cosmetics to protect skin from UV radiation and thus to prevent harmful effects of solar radiation. These chemicals are also used as sunblocking agents for the protection of materials against UV. Some of these compounds, such as the 2-ethylhexyl-4-trimethoxycinnamate (EHMC also called OMC) and octocrylene (OCT) are "high production volume chemicals" (HPVC), corresponding to chemicals produced or imported at a rate of over 1000 t $year^{-1}$ in European Union. EHMC and OCT are among the most common organic UV filters present in cosmetic formulations (Rastogi, 2002). They are of global concern because of their uses and their potential endocrine disruption properties. Their potential for disrupting endocrine systems has been showed for mammals (Gomez et al., 2005; Schlumpf et al., 2008) and fish (Fent et al., 2008; Coronado et al., 2008; Christen et al., 2011). In fish, Zucchi et al. (2011) and Christen et al. (2011) showed that EHMC induced a decrease in spermatocytes in testes and a decrease in previtellogenic oocytes in ovaries. In addition, sun-blocking agents in mixtures showed synergistic interaction in vitro and additive to antagonistic activity in vivo (Kunz and Fent, 2009). This information is relevant as aquatic environments are exposed to mixtures of sun-blocking agents. Other observations on these substances showed that EHMC and benzophenone-3 (BP-3) were involved in coral bleaching by promoting viral infections (Danovaro et al., 2008).

Due to their wide use, UV filters enter the aquatic environment in two ways, either indirectly via wastewater treatment plants (WWTP) effluents or directly through swimming and other recreational activities. Previous studies on the prevalence of these compounds in the aquatic environment have revealed a series of organic UV filters found in WWTP sludge and effluents, at concentrations of up to 100 ng L^{-1} for EHMC, 270 ng L^{-1} for OCT and 7 ng L^{-1} for octyl dimethyl p-aminobenzoic acid (OD-PABA) (Balmer et al., 2005; Cuderman and Heath, 2007; Kupper et al., 2006; Li et al., 2007). UV filters have also been detected in receiving surface water such as rivers or lakes, with concentrations sometimes reaching 1040 ng L^{-1} for EHMC, 250 ng L^{-1} for OCT and 47 ng L^{-1} for OD-PABA (Balmer et al., 2005; Cuderman and Heath, 2007; Poiger et al., 2004; Rodil and Moeder, 2008; Kameda et al., 2011). Although these studies have concluded that WWTP effluents are the main source of UV filters in water, Balmer et al. (2005) have shown that in lakes UV filter concentrations are not correlated with WWTP effluents, thus suggesting a possible input from recreational activities. UV filters are also present in the marine environment. EHMC have been detected in surface microlayers in the Pacific Ocean (Goksoyr et al., 2009) and in the coastal zone, where the EHMC or OCT concentration in water was found to be dependent on recreational activities such as swimming (Langford and Thomas, 2008).

Due to the presence of UV filters in water, accumulation in freshwater animal species has been observed (Balmer et al., 2005; Buser et al., 2006; Fent et al., 2010; Zenker et al., 2008). OCT has been found in fish caught downstream from WWTP discharge in rivers, at

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concentrations of around 600 ng g^{-1} lipids (Buser et al., 2006). EHMC has been quantified in crustaceans (*Gammarus* sp.), in mollusks (*Dreissena polymorpha*) and in several fish species at levels reaching 133, 150 and 337 ng g^{-1} lipids, respectively (Fent et al., 2010). The swimming season was found to be another factor impacting the accumulation in species tissues. Indeed, Buser et al. (2006) showed that OCT concentrations recorded in fish in September were 3- to 5-times higher than those in May, before the swimming period. Similarly, Fent et al. (2010) showed that freshwater mussels collected in a lake where bathing was practiced had higher concentrations after summer than before.

Very few data are available on the presence of UV filters in seawater (Giokas et al., 2005; Goksoyr et al., 2009; Langford and Thomas, 2008), despite previous finding showing the impact of recreational activities in water and species contamination by UV filters. Almost no data is currently available on levels of UV filters in marine organisms.

In order to assess the prevalence of UV filters in marine organisms, this study focused on the presence and concentration of the most commonly used and most lipophilic UV filters (EHMC, OCT and OD-PABA) in tissues of wild marine mussels collected along the French coast. Two species were assessed: *Mytilus galloprovincialis* on the Mediterranean coast and *Mytilus edulis* on the Atlantic coast. Mussels are biomonitor organisms recognized for trace pollutants such as metals and PCB (Andral et al., 2011) and more recently for pharmaceuticals, perfluorinated compounds and pesticides (Wille et al., 2011). EHMC, OCT and ODPABA were thus selected for this work and their accumulation was studied in wild mussels. Ten sites were selected in the French coastal regions that were representative of different levels of spatial, recreational and tourist population pressure. Sampling was performed from June to November to account for temporal variations during the swimming period.

2. Material and methods

2.1. Reagents and standards

All chemicals were of high purity grade. 2-ethylhexyl-4-trimethoxycinnamate (EHMC) (E)-isomer, and octyl dimethyl p-aminobenzoic acid (OD-PABA) were from Merck (Darmstadt, Germany). Octocrylene (OCT) and the internal standard chrysene-d12 were from Sigma-Aldrich (Steinheim, Germany). The physicochemical properties of the selected UV filters are presented in Table 1. Stock solutions of individual standards and standard mixtures were prepared at 1000 mg L^{-1} in ethanol. Working standard solutions were obtained by further dilution of stock solutions with ethanol. All solutions were stored in the dark at −20 °C prior to use. Pesticide analysis-grade solvents (acetone, ethanol, ethyl acetate, n-heptane) and HPLC-grade solvents (acetonitrile, methanol MeOH) were from Carlo Erba (Val de Reuil, France). Ultrapure water was obtained with a Milli-Q system (Millipore). Prior to use, all glassware was cleaned twice with the solvent used in the different procedures, and gloves were worn throughout all sample processing steps in order to prevent background contamination, which is a common problem in UV filter determination at environmental levels. Blanks were carried out for all procedures in order to assess the absence of contamination.

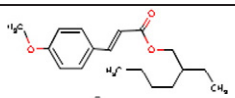
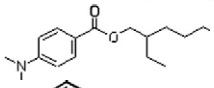
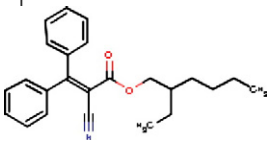
2.2. Investigated sites and sampling

Wild mussels were collected over a 6 month period during 3 to 5 sampling surveys (June to November 2008) from the 10 selected sites along the French coast, including a marina (site 8). The sites represented various anthropogenic pressures and different geomorphological configurations (Fig. 1). The influence of recreational activities was estimated with data on population and tourist accommodation from the French Institut National de la Statistique et des Etudes Economiques database (INSEE, 2007, 2008). Sampling sites positioned with GPS were also dependent on the presence and localization of wild mussels and characterized with distance from the beach, sampling depth (<2 m) and the openness to the wide. *M. galloprovincialis* (Mediterranean coast) and *M. edulis* (Atlantic coast) were manually collected with gloves and no sunscreen formulation was used by the personnel who performed the sampling. Weather conditions were recorded (MétéoFrance, 2008). Each sample contained 50 mussels, calibrated from 5 to 7 cm to ensure homogenous sampling. Mussels were kept in ice until treatment (within 4 h). Soft tissues were collected by dissection and stored in polyethylene bags at −20 °C, freeze dried (Heto Power dry LL 3000, Thermo) and ground into powder. The ratio of dry flesh weight to dry shell weight was used to determine a condition index (CI) for each sample.

2.2.1. Extraction and purification

3 ± 0.1 g of homogenated samples were extracted via microwave-assisted extraction in a Multiwave 3000 (Anton Paar) with 25 mL of an acetone/heptane mixture (1:1, v:v) after the addition of internal standard. The extraction temperature was increased to 110 °C within 15 min. After extraction, the liner was rinsed with three portions of 5 mL acetone/heptane mixture. All extracts were filtered (0.2 µm GF-C) through 10 g of anhydrous sodium sulfate, rotary evaporated to dryness and re-dissolved in 1.5 mL ethanol. The lipid content was determined gravimetrically. The purification was done on a RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 µm) from Waters (Ireland) with HPLC (Dionex-Ultimate 3000) adapted from Zenker et al. (2008). The elution started with MeOH and MilliQ water (70:30, v/v) for 4 min with an increase to 100% MeOH within 16 min, and kept there for 20 min. The flow rate was 1.5 mL min^{-1}. The fraction from 9 to 19.5 min was collected, evaporated to dryness and reconstituted in 1 mL heptane for analysis.

Table 1
Chemical structures and properties of the investigated compounds.

Substance	Structure	CAS number	M [g mol^{-1}]	Log K_{ow}	Water solubility 25 °C (mg L^{-1})
EHMC		5466-77-3	290.41	5.8	0.155
OCT		6197-30-4	361.49	6.88	0.00381
OD-PABA		21245-02-3	165.19	5.77	0.0053

Abbreviations: Kow, n-octanol–water partition coefficient; M, molar mass of the neutral species.

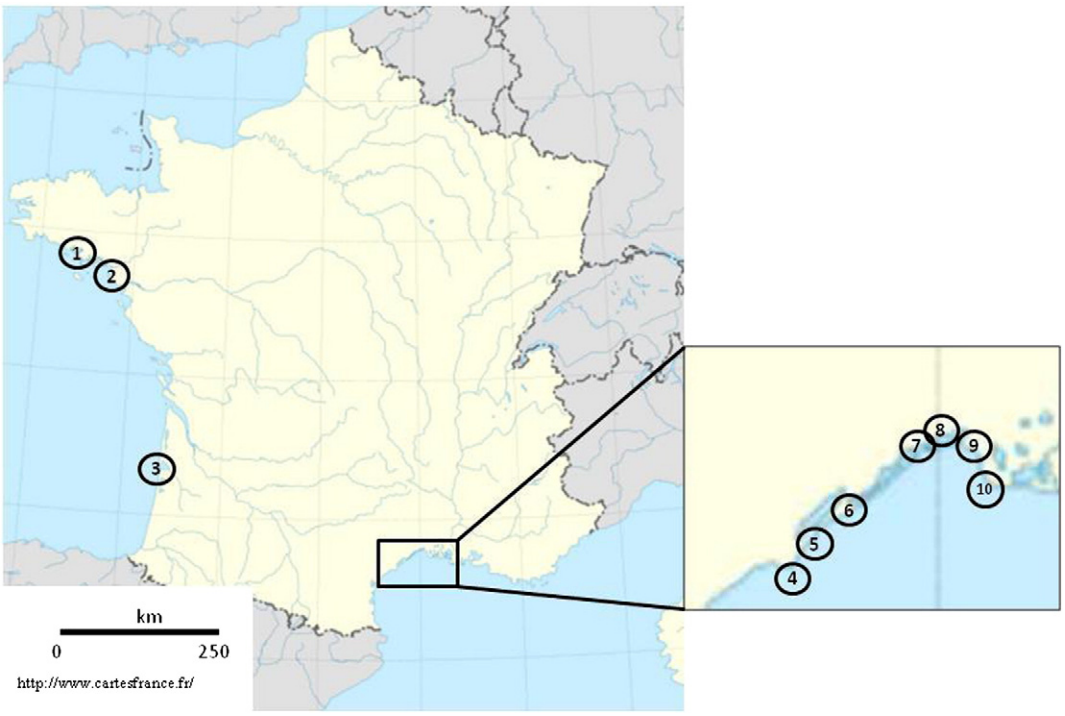


Fig. 1. The 10 selected sites along the French coast.

2.3. GC-MS^2 analysis

Analysis was carried out on a GC Ultra trace 3000 (Thermo) connected to an ion trap mass spectrometer (MSn) detector Polaris Q (Thermo). An SGE-BPX5 capillary column (30 m, 0.25 mm I/D, 0.25 μm film thickness) was used. 1 μl was injected with an autosampler (AI 3000) in splitless mode at 250 °C. Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The GC temperature program was as follows: 80 °C, 0.5 min isothermal, 20 °C min^{-1} to 150 °C, then at 15 °C min^{-1} to 200 °C, 2 min isothermal, 5 °C min^{-1} to 220 °C, finally at 20 °C min^{-1} to 300 °C followed by an isothermal hold of 10 min. The GC–MS interface temperature was 300 °C and the electron impact mode was set at 70 eV. Data were acquired in the selected ion monitoring (SIM) for standard chrysene-d12 and in the MS-MS mode for Z and E EHMC, OD-PABA and OCT. The collision energies, Qz and ion quantifications are presented in Table 2. Peak detection and integration were carried out using Xcalibur software (Thermo). Z EHMC was assumed to give the same response factor as E EHMC. The results are expressed as the sum of both E and Z EHMC.

2.4. Quantification and quality control

The procedural blanks never contained target compounds. Spiked samples were analyzed as described above together with a blank sample. The linearity (n = 7) of the analytical procedure was verified by spiking samples with the three UV filters over a concentration range of 5 to 300 ng g^{-1} dw (Table 3). Because no certified materials are available, recovery was determined in mussel tissues spiked with known amounts of the analytes (6 replicates of three concentrations 25, 50 and 100 ng g^{-1} dw). The mean extraction recoveries ranged from 89 to 116%, with a coefficient of variation of under 17% (Table 3). The accuracy, calculated as the percent deviation of the mean observed concentrations from the nominal concentration, was under 16%. The precision was determined by calculating the relative standard precision (RSD) of analysis performed on three different days. The limits of detection (LOD) and the limits of quantification (LOQ) were respectively 2 and 5 ng g^{-1} dw for EHMC, OCT and OD-PABA (Table 3).

Statistical analyses were performed with Statgraphics plus. The Spearman correlation was used to test the results obtained for Mediterranean beaches (with $p<0.05$).

Table 2
Analytical parameters used for quantification of EHMC, OCT and OD-PABA.

Compound	Parent ion	Excitation amplitude (V)	Qz	Quantification ions	Retention time (min)
EHMC	178	1.3	0.3	121 + 132 + 161	Z-19.77
					E-21.94
OD-PABA	165	1.3	0.45	91 + 118 + 148	21.40
OCT	248	1.45	0.3	220 + 219 + 176	24.15

3. Results

The site characteristics and the recreational pressure are presented in Table 4. In table 5, UV filter concentrations show that 100% of the samples had quantifiable EHMC ranging from 3 to 256 ng g^{-1} dw, while 55% of the samples had detectable OCT ranging

Table 3
Linear correlation coefficients (r^2) over a concentration range of 5 to 300 ng g^{-1} dw, recoveries, Accuracy, RSD (n = 6), limit of detection (LOD) and limit of quantification (LOQ).

Compound	r^2	Recoveries%	Accuracy (%)	RSD (%)	LOD	LOQ
		25 ng g^{-1} dw			ng g^{-1} dw	ng g^{-1} dw
		50 ng g^{-1} dw				
		100 ng g^{-1} dw				
EHMC	0.992	89 ± 10	−11	9	2	5
		99 ± 13	−1	12		
		95 ± 11	−5	11		
OD-PABA	0.977	111 ± 17	+11	13	2	5
		103 ± 9	+3	10		
		116 ± 12	+16	8		
OCT	0.987	100 ± 15	0	13	2	5
		89 ± 7	−11	9		
		101 ± 13	+1	10		

from under 2 to 7 112 ng g^{-1} dw. OD-PABA was never detected in mussels. No correlation was obtained between EHMC, OCT, condition index and mussel lipid content.

Two criteria were selected for categorizing studied sites, in order to illustrate the influence of the recreational activities and the influence of the geomorphological structure on the UV filter concentration in mussels. The first criteria concerned population size; the studied sites were representative of three population sizes, estimated by the sum of regular inhabitants and the capacity of tourist accommodation offered: class A with population over 50 000 inhabitants, class B and class C with population from 25 000 to 30 000 inhabitants and from 6000 to 12 000 inhabitants respectively. Following this criterion, two sites were classified as A (sites 4 and 5), five sites as B (sites 2, 3, 6, 9, 10) and three sites as C (sites 1, 7 and 8) (Table 4). The second criterion was geomorphological structure of the sampling sites. We considered the total beach length, the openness to the wide including manmade structures and the overall depth that allowed to determine three sites as closed to the wide (sites 5, 8, 9), two as partially closed (PC) (sites 4 and 6) and five as open (O) to the wide (sites 1,2,3,7 and 10). Fig. 2 represents the results in two closed sites for EHMC with different inhabitants' pressure (A and B) during the studied period. This figure shows a higher EHMC concentration for the higher inhabitants' pressure; the end of June and August had the highest concentrations in EHMC and a significant decrease is observed from the end of September, showing the recreational activity period influence. Fig. 3 illustrates the results on OCT for sites with different geomorphological structures (O and PC). The lowest OCT concentrations were observed in sites open to the wide and submitted to lower inhabitant pressure (7 O C) when compared to partially closed sites with higher inhabitants' pressure (4 PC A).

As expected, the highest UV filter concentrations were observed at the two sites with the highest potential recreational activity pressure (sites 9 and 5). The mussel OCT concentration reached more than 1000 and more than 7000 ng g^{-1} dw, respectively for sites 9 and 5. These high concentrations were significantly correlated ($p<0.05$) with the air temperature (21.8 to 24 °C), corresponding to the highest tourist activity and thus recreational pressure. OCT maxima for July and August were confirmed for all sites. The lowest EHMC concentrations were obtained in November, probably due to the lower recreational activity pressure—the concentration ranged from 3 to

Table 4
Recreational sampling site characteristics.

Site	Location	Species	Openness	Depth cm	Inhabitants*	Tourist accomodation*	Inhabitant pressure	Sampling date	Air t°C$^{s\ O}$
1	Atlantic:	ME	Open	Foreshore				20/07/08	17.0
	N 47°30,33						C	08/08/08	18.4
	O 03°07,10				2217	3785		03/09/08	16.9
								30/10/08	11.1
2	Atlantic:	ME	Open	Foreshore				10/07/08	17.2
	N 47°16,03						B	07/08/08	18.7
	O 02°24,61				16719	13648		17/09/08	14.7
3	Atlantic:	ME	Open	Foreshore				23/06/08	21.5
	N 44°34,61						B	17/07/08	20.3
	O 01°13,82				24616	6461		18/08/08	19.8
								17/09/08	17.7
								14/10/08	17.6
4	Mediterranean:	MG	Partially closed	0–150				03/07/08	22.5
	N 43°17,130						A	29/07/08	23.1
	E 03°31,53				21104	35112		27/08/08	21.8
								25/09/08	16.3
								26/11/08	8.3
5	Mediterranean:	MG	Closed	0–50				03/07/08	23.2
	N 43°23,566						A	29/07/08	24.0
	E 03°39,99				42972	7282		27/08/08	23.2
								25/09/08	17.4
								26/11/08	8.6
6	Mediterranean:	MG	Partially closed	0–150				03/07/08	23.2
	N 43°25,914						B	29/07/08	24.0
	E 03°46,70				23068	2664		27/08/08	23.2
								25/09/08	17.4
								26/11/08	8.6
7	Mediterranean:	MG	Open	0–200				02/07/08	23.1
	N 43°31,48						C	30/07/08	24.0
	E 03°55,80				6048	6587		26/08/08	22.7
								24/09/08	16.2
								24/11/08	7.7
8	Mediterranean:	MG	Closed	0–50				02/07/08	23.1
	N 43°31,48						C	30/07/08	24.0
	E 03°55,80				6048	6587		26/08/08	22.7
								24/09/08	16.2
								24/11/08	7.7
9	Mediterranean:	MG	Closed	0–50				02/07/08	23.3
	N 43°33,310						B	30/07/08	23.6
	E 04°05,31				8246	17118		26/08/08	22.5
								24/09/08	16.8
								24/11/08	8.2
10	Mediterranean:	MG	Open	0–200				02/07/08	23.1
	N 43°32,206						B	30/07/08	23.6
	E 04°08,02				8173	20010		26/08/08	22.1
								24/09/08	16.7
								24/11/08	8.2

ME M. *Edulis*, MG M. *Galloprovincialis*, *INSEE 2007–2008,° Meteo France.

Table 5
EHMC and OCT concentrations (ng g^{-1} dw) and conditions index (CI) in wild mussels, nd: not detected.

	Sampling date	CI	EMHC		OCT	
			Duplicates in ng g^{-1} dw		Duplicates in ng g^{-1} dw	
1	20/07/08	0.078	20	17	nd	nd
	08/08/08	0.102	15	15	17	11
	03/09/08	0.086	8	11	nd	nd
	30/10/08	0.077	7	11	nd	nd
2	10/07/08	0.110	5	8	nd	nd
	07/08/08	0.163	12	21	nd	nd
	17/09/08	0.123	6	7	nd	nd
3	23/06/08	0.137	12	10	nd	nd
	17/07/08	0.166	29	30	12	14
	18/08/08	0.110	45	42	23	23
	17/09/08	0.146	14	21	nd	nd
	14/10/08	0.115	32	24	9	10
4	03/07/08	0.085	15	22	36	43
	29/07/08	0.096	41	39	214	222
	27/08/08	0.107	22	24	168	176
	25/09/08	0.115	16	18	10	10
	25/11/08	0.100	9	6	nd	nd
5	03/07/08	0.153	25	26	248	252
	29/07/08	0.138	115	124	1286	1297
	27/08/08	0.126	224	256	7015	7112
	25/09/08	0.144	19	18	32	31
	25/11/08	0.130	9	9	nd	nd
6	03/07/08	0.129	25	24	36	30
	29/07/08	0.083	42	43	137	124
	27/08/08	0.092	30	27	95	75
	25/09/08	0.098	24	25	nd	nd
	25/1108	0.071	8	10	nd	nd
7	02/07/08	0.106	19	21	42	47
	30/07/08	0.112	35	26	119	132
	26/08/08	0.109	13	15	36	34
	24/09/08	0.098	40	39	nd	nd
	24/11/08	0.104	7	6	nd	nd
8	02/07/08	0.107	9	11	60	47
	30/07/08	0.091	21	24	65	61
	26/08/08	0.123	23	26	nd	nd
	24/09/08	0.119	8	13	nd	nd
	24/11/08	0.075	3	5	nd	nd
9	02/07/08	0.215	50	48	390	454
	30/07/08	0.199	62	53	1111	1141
	26/08/08	0.208	106	99	842	803
	24/09/08	0.150	26	30	22	29
	24/11/08	0.086	18	17	nd	nd
10	02/07/08	0.192	8	13	23	18
	30/07/08	0.214	11	16	98	94
	26/08/08	0.188	10	16	97	78
	24/09/08	0.182	16	20	nd	nd
	24/11/08	0.100	3	8	nd	nd

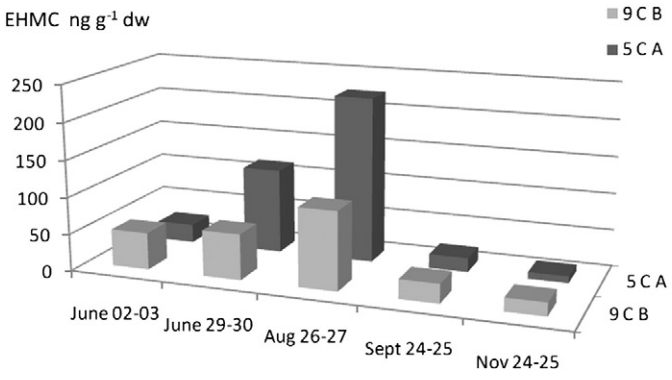


Fig. 2. EHMC ng g^{-1} dw in two closed sites (C): 5 with a population size A and 9 with a population size B.

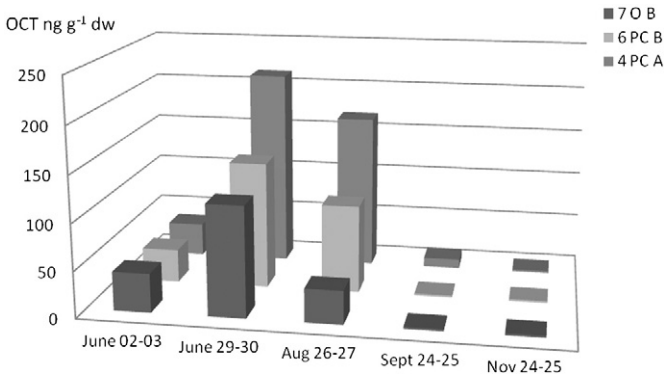


Fig. 3. OCT ng g^{-1} dw in an open (O) and two partially closed (PC) sites (4, 6 and 7) with a population size A or with a population size B.

18 ng g^{-1} dw. OCT was no longer detectable in mussels collected in November.

4. Discussion

Studies on environmental UV filter behavior are limited, especially compared to other pollutants. Their presence in the aquatic environment has been reported since the early 1980s, but mainly in freshwater systems. The highest concentrations were reported in surface water for sites receiving wastewater effluents and for sites with recreational activities (Rodil and Moeder, 2008; Kameda et al., 2011). A similar trend has been observed in aquatic organisms, with higher concentrations at sites affected by WWTP effluents or at recreational sites (Buser et al., 2006; Fent et al., 2010). As expected, in the present study on coastal sites subjected to recreational activities, UV filters such as EHMC and OCT were found in mussels collected during the warmest seasons. For EHMC, this study had equivalent or higher levels than those reported in freshwater mussels (*D. polymorpha*), ranging from 22 to 150 ng g^{-1} lipids (Fent et al., 2010) where samples were collected in a small river impacted by human activities. In freshwater fish, UV filters such as EHMC and OCT were also detected at concentrations ranging from 337 ng g^{-1} lipids for EHMC to 2 400 ng g^{-1} lipids for OCT (Buser et al., 2006; Fent et al., 2010). In the aquatic environment, when OD-PABA was assessed, concentrations in surface water were lower than those of EHMC or OCT, with a maximum 47 ng L^{-1} for OD-PABA (Balmer et al., 2005; Cuderman and Heath, 2007; Rodil and Moeder, 2008). To our knowledge, there is no data on OD-PABA in aquatic living resources. In this study, OD-PABA was never found in mussels collected along the French coast.

Previous studies have shown the relation between high recreational activity and UV filter concentrations in surface and coastal waters (Balmer et al., 2005; Langford and Thomas, 2008). In this study, we get in-depth description of the intensity of recreational activity and of the site geomorphological structure through two criteria. These criteria have their importance in understanding the behavior of UV filters and their accumulation in mussels. Concentrations detected in mussels from Mediterranean beaches were related to the attendance of recreational sites, with higher concentrations noted in July and August. UV filter concentrations were correlated with air temperature and not with the population size, thus highlighting the influence of recreational activities in coastal contamination. At closed sites, with a lower exchange of water and thus less dilution of contaminants, UV filters remain at the surface microlayer, therefore sampling mussel depth may well relate to the level of mussel contamination. UV filters such as EHMC have been detected in sea surface microlayers (Goksoyr et al., 2009), and as it has been shown, for PAHs (Baumard et al., 1998; Naes et al., 1995), the surface microlayer could be a fraction that is readily bioavailable to mussels. It is therefore possible to consider that higher levels in mussels collected near

the surface are due to the surface microlayer contamination by UV filters.

It is important to point out that OCT had a broad range of temporal variability, dropping as low as total absence, whereas EHMC remained present even after the recreational activity period. In the studied sites, the sources of EHMC and OCT should be considered to be similar and sources other than recreational activities assumed to have minor influence. So, EHMC may be hypothesized more persistent than OCT in mussels but this point needs further investigation of UV-filter bioaccumulation over time, at least in several sampling sites. This difference in behavior cannot be explained by their Kow values that are very similar (5.8 for EHMC and 6.88 for OCT). In a study performed with transplanted mussels, very little bioaccumulation was shown for the persistent and lipophilic perfluorinated compounds (PCFs) (Gomez et al., 2011). The increase of efflux transport activity of proteins belonging to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, which act as a first line of defense of the organism, was hypothesized to play a major role in the low accumulation observed. Similar mechanisms should be involved in the uptake and depuration of UV filters by mussels. There is no data on bioaccumulation and depuration rates of UV filters, nor on metabolism and excretion. There is a real need to address these processes in mussels and in aquatic living resources, mainly because adapted protocols should be developed, as highlighted by Liu et al. (2011) for PCFs.

A question that should be raised in regard to the obtained results concerns their toxicological relevance. The evaluation of risk to humans after consumption of marine species contaminated by UV filters cannot be assessed without considering direct exposure through dermal pathway. Indeed, accumulated UV filters could be toxic for wild mussels and other species in coastal environment. As UV filters showed different endocrine activities in fish, an interference with the endocrine system of mussels could be hypothesized. Current knowledge on the molluscan endocrine system and the role of endocrine disruptors (EDs) in invertebrates are discussed in the reviews of Janer and Porte (2007), Lafont and Mathieu (2007), and David et al. (2009, 2012). They noted that it is difficult to interpret the effects of EDs (like alkylphenols) on invertebrates because their endocrine system is not yet fully understood. Furthermore, vertebrate-type steroids, like 17β-estradiol (E2), have been reported in various invertebrate mollusc organs (Reis-Henriques et al., 1990; Stefano et al., 2003) but their function is still unclear and under debate. In addition, the fact that steroid compounds may bioaccumulate in mollusks (Labadie et al., 2007) could contribute to be a confounding factor. In this context, toxic effects of UV filters accumulated in mussels would be difficult to establish. Other possible effects of UV filter exposure could be investigated differently, for example, through the metabolic pathway consisting in esterification of estradiol when mussels were exposed to E2 or to alkylphenols (Janer et al., 2005; Lavado et al., 2006), through the modulation of CYP3A isoforms observed after exposure to E2 (Cubero-Leon et al., 2012), or by markers like cell shape changes, lysosomal destabilization and hydrolytic enzyme release that have been showed altered in *M. galloprovincialis* exposed to E2 (Canesi et al., 2004).

UV filters contaminate coastal marine environment and bioaccumulate in mussels collected along the French coast. Since these compounds are continuously released to the sea, the acute and long term effects require further study in regard to the metabolism and in the particular context of discontinuous exposure.

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Review

Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495

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ABSTRACT

Although there are no legal discharge limits for micropollutants into the environment, some regulations have been published in the last few years. Recently, a watch list of substances for European Union-wide monitoring was reported in the Decision 2015/495/EU of 20 March 2015. Besides the substances previously recommended to be included by the Directive 39/2013/EU, namely two pharmaceuticals (diclofenac and the synthetic hormone 17-alpha-ethinylestradiol (EE2)) and a natural hormone (17-beta-estradiol (E2)), the first watch list of 10 substances/groups of substances also refers three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), other natural hormone (estrone (E1)), some pesticides (methiocarb, oxadiazon, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid and triallate), a UV filter (2-ethylhexyl-4-methoxycinnamate) and an antioxidant (2,6-di-tert-butyl-4-methylphenol) commonly used as food additive. Since little is known about the removal of most of the substances included in the Decision 2015/495/EU, particularly regarding realistic concentrations in aqueous environmental samples, this review aims to: (i) overview the European policy in the water field; (ii) briefly describe the most commonly used conventional and advanced treatment processes to remove micropollutants; (iii) summarize the relevant data published in the last decade, regarding occurrence and removal in aqueous matrices of the 10 substances/groups of substances that were recently included in the first watch list for European Union monitoring (Decision 2015/495/EU); and (iv) highlight the lack of reports concerning some substances of the watch list, the study of un-spiked aquatic matrices and the assessment of transformation by-products.

Contents

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1. Introduction

Water is a valuable resource, crucial to all living organisms and for multiple human activities, such as domestic uses, agriculture and industry. However, several contaminants of emerging concern (CECs) end up in vital aquatic compartments, such as surface water, groundwater and even drinking water, at concentrations between few ng L^{-1} and several μg L^{-1} (Matamoros and Bayona, 2006), with negative impact on water quality. The occurrence of CECs in the environment is reported in thousands of publications during the last decades and reviewed by many authors (Bell et al., 2011; Bu et al., 2013; da Silva et al., 2012; da Silva et al., 2013; Li et al., 2015; Li, 2014; Liu and Wong, 2013; Sima et al., 2014; Zhang et al., 2014a), demonstrating an increasing concern about them. For instance, a series of periodic review articles focused on occurrence, fate, transport and treatment of CECs were published annually since 2007 (Wells et al., 2007) until 2011 (Bell et al., 2011), and then works on occurrence, fate and transport of CECs were reviewed separated from treatment since 2012 (da Silva et al., 2012) until 2015 (Li et al., 2015), due to the significant increase in the number of publications dealing with this particular topic.

CECs can be natural or anthropogenic substances such as pesticides, industrial compounds, pharmaceuticals, personal care products, steroid hormones, drugs of abuse and others (Ribeiro et al., 2015). Sources of CECs include: (i) industrial wastewater; (ii) runoff from agriculture, livestock and aquaculture; (iii) landfill leachates; and (iv) domestic and hospital effluents, from which micropollutants might follow many pathways (Mompelat et al., 2009), as represented in Fig. 1.

The management of industrial effluents resulting from the production of pharmaceuticals, personal care products, pesticides and other compounds, has been properly done in several countries where regulations are already implemented, but more strict regulations are still needed in other regions of the world. The runoff from agriculture and livestock areas is another important source of micropollutants, particularly in the case of pesticides used to improve productivity, as well as steroid hormones and antibiotics used for livestock (Birkett and Lester, 2002; Song et al., 2007). In addition, many contaminants and their intermediates can reach the fields when they are irrigated with treated wastewater and, as consequence, the receiving waters can also contain these substances (Pedersen et al., 2003). Other source of CECs is the leakage from landfills and sewage treatment facilities, industrial waste systems and septic tanks (Matthiessen et al., 2006). The release of effluents from municipal wastewater treatment plants (WWTPs) is other important route for the appearance of micropollutants in the aquatic environment (Tijani et al., 2013), the wastewater treated in these plants mainly resulting from domestic and/or industrial activities, as well as from hospitals.

In fact, most of the conventional WWTPs are not designed to completely eliminate organic compounds at low concentrations, making the treatment processes vulnerable to such problem of pollution (Tijani et al., 2013). In this context, the non-degradable or partially removed compounds in WWTPs are likely to be detected in surface water. In the cases of sewage sludge and soils, micropollutants can desorb and runoff to surface water or undergo direct leaching to groundwater aquifers with consequent contamination of drinking water (Feng et al., 2013).

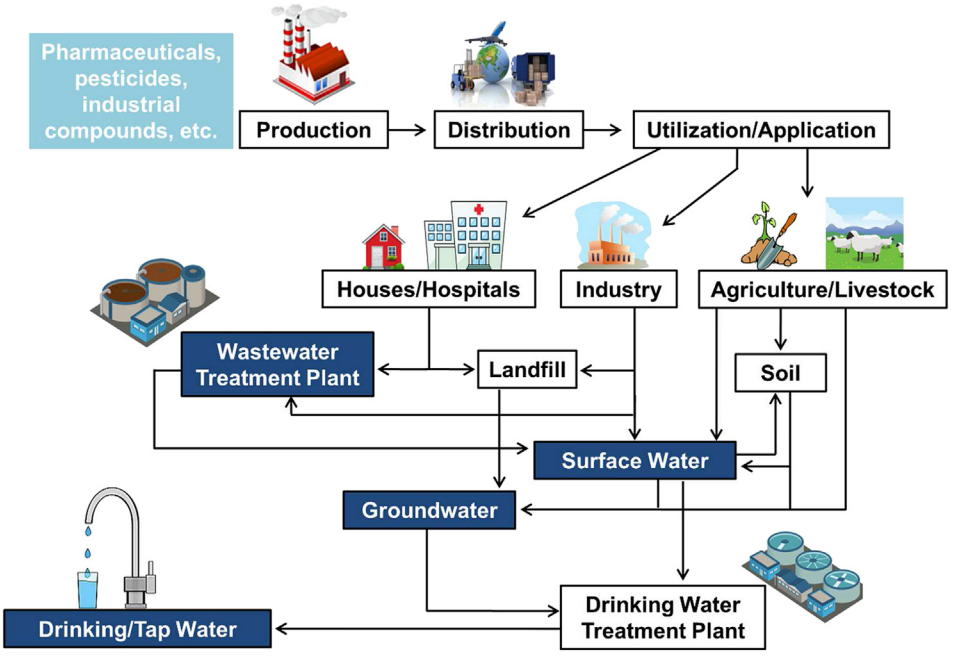


Fig. 1. Representative sources and routes of micropollutants in the environment.

Agricultural reuse of sewage sludge in particular as fertilizer, is a common practice to improve the soil structure and provide nutrients, but can represent a source of environmental contamination (Dichtl et al., 2007). Moreover, sewage sludge solids sourced by wastewater can be considered a sink of hazardous substances (e.g., such as pathogens, heavy metals and organic pollutants) that will accumulate in soils (Dichtl et al., 2007). Due to the increasing concern about human health impacts, land application gained interest to convert sludge into a safer material through the treatment by anaerobic digestion, composting or other biological processes (Zhang et al., 2014b). While composting is a controlled bio-oxidative process that converts sludge into stable and humic like materials, anaerobic digestion occurs in the absence of oxygen and has two main end products, a methane-rich biogas used as renewable energy source and the digested used as fertilizer (Zhang et al., 2014b). Removal of toxic organic contaminants by these processes was reported; however, their complete mineralization is difficult due to the adsorption mechanism and the formation of intermediates (Zhang et al., 2014b).

The fate and distribution of CECs will depend on the D_{ow}, which is a pH-dependent n-octanol–water distribution ratio that simultaneously considers hydrophobicity and ionogenicity (Wells, 2006, 2007). Although most regulators use octanol–water partitioning coefficient (K_{ow}) to evaluate the hydrophobic partitioning, the environmental fate and transport should be based in the parameter D_{ow}, which is more accurate for ionizable organic compounds.

The contamination of environmental compartments, such as surface water, groundwater and soils, which are continuously interrelated, may cause cumulative negative effects along multi-generational exposure in aquatic organisms and/or affect the human's health by drinking water contamination (Daughton, 2010). A great concern about the occurrence of micropollutants in the aquatic resources and the subsequent effects on humans and biota has been highlighted in the last few years. However, it is difficult to predict which environmental and public health implications may arise from the occurrence of CECs in freshwater ecosystems, since the individual concentrations usually found in the environment are lower than those able to cause direct negative effects (Quinn et al., 2009). For instance, concerning pharmaceuticals, toxicological studies have shown that they might have direct toxicity towards certain aquatic organisms (Crane et al., 2006). The main issues related to the frequent occurrence of recalcitrant compounds are their simultaneous presence as complex mixtures and the long term exposition that can lead to serious chronic effects, as reported by several studies (Kidd et al., 2007; Santos et al., 2010). Their constant but imperceptible effects can gradually accumulate, finally leading to irreversible changes on both wildlife and human beings (Daughton and Ternes, 1999; Jjemba, 2006).

Natural attenuation is a low-cost and simple process comprising physical, chemical and/or biological mechanisms to reduce contaminants concentrations (Khan et al., 2004; Kuppusamy et al., 2016). Volatilization, dispersion, dilution, sorption, photolysis, biodegradation/transformation are the main natural attenuation processes (Khan et al., 2004; Kuppusamy et al., 2016). While volatilization has a minor impact, dispersion and dilution can lead to a significant decrease on the concentrations of contaminants (Gurr and Reinhard, 2006). The dilution can decrease their concentration to levels for which no significant effects are verified for aquatic organisms. Sorption to sediments and suspended solids also reduce the concentration of CECs, but accumulation is enhanced. Indirect or direct photolysis can lead to removal of contaminants, but is highly dependent on the presence of suspended matter and solar radiation. CECs can also be degraded by biodegradation/transformation, by bacterial enzymes (Khan et al., 2004).

The upgrading of the treatment processes for effluents generated by conventional WWTPs might minimize the discharge of micropollutants into the receiving waters, and can even improve the overall quality status of effluents for possible reuse (Comninellis et al., 2008; De Luca et al., 2013). The design improvement of WWTPs to include advanced treatment technologies, aiming to transform CECs into less harmful compounds or even to mineralize them, is one of the promising strategies to achieve this aim, as recently implemented in Switzerland. Advanced water treatment processes include adsorption (e.g., granular activated carbon (GAC)), membrane and advanced chemical/oxidation technologies (Sudhakaran et al., 2013). Other option is the implementation of natural systems to depurate water, such as riverbank filtration (RBF), aquifer recharge and recovery (ARR) and constructed wetlands (CWs), which are reviewed in the literature (Li et al., 2014; Petrovic et al., 2009; Zhang et al., 2014a) and will not be discussed in this work.

1.1. European policy

Although there are no legal discharge limits for micropollutants, some regulations have been published. The Directive 2000/60/EC was the first mark in the European water policy, which set up a strategy to define high risk substances to be prioritized (Directive, 2000). A set of 33 priority substances/groups of substances (PSs) and the respective environmental quality standards (EQS) were ratified by the Directive 2008/105/EC (Directive, 2008). Two years ago, the European Union Directive 2013/39/EU recommended attention to the monitorization and treatment options for a group of 45 PSs (Directive, 2013), meeting the protection of the aquatic compartments and the human health. In that Directive, two pharmaceuticals (the non-steroid anti-inflammatory diclofenac and the synthetic hormone 17-alpha-ethinylestradiol – EE2) and a natural hormone (17-beta-estradiol – E2) were recommended to be included in a first watch list of 10 substances/groups of substances for European Union monitoring, to be launched within two years. In the first quarter of 2015, the watch list of substances for European Union-wide monitoring (as set out in Article 8b of Directive 2008/105/EC) was amended in the Decision 2015/495/EU of 20 March 2015. Besides the abovementioned substances (diclofenac, EE2 and E2), three macrolide antibiotics (azithromycin, clarithromycin and erythromycin) were included, together with other natural hormone (estrone – E1), some pesticides, a UV filter and an antioxidant commonly used as food additive, listed in Table 1. The frequent occurrence of CECs in the environment and the inefficiency of conventional WWTPs to remove such compounds, promoted the amendment of the framework to cover a larger set of hazardous compounds, as well as further recommendations for wastewater treatment steps or even new treatment scenarios. This actions should be implemented by the European Commission and regulated by the European country authorities.

This review aims to summarize some relevant data of occurrence and removal in aqueous matrices of the 10 substances/groups of substances (i.e., a total of 17 organic compounds) enlisted in the first watch list for European Union monitoring, defined in the Decision 2015/495/EU. Studies on the occurrence of the referred substances (3 estrogens, diclofenac, 2,6-di-tert-butyl-4-methylphenol, 2-ethylhexyl-4-methoxycinnamate, 3 macrolide antibiotics, methiocarb, 5 neonicotinoids, oxadiazon and triallate) are shown in Table 1, for different aquatic compartments, namely wastewater, surface water and groundwater. Reports dealing with the removal of these 17 substances, only in real matrices, are overviewed below. The search comprised publications since 2005 (last decade) in Scopus database, using as keywords each substance and the treatments herein reported. Most of the works refer to unspiked aqueous environmental samples treated at lab-, pilot- or

Table 1
List of 10 substances/groups of substances (total of 17 organic compounds) included in the watch list of EU Commission Decision 495/2015, and their occurrence in different aquatic compartments, namely effluents of wastewater (WW), surface water (SW), and groundwater (GW). *n.a. refers to not available data.

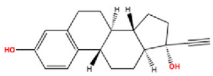
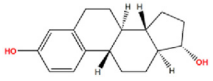
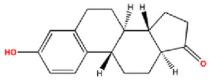
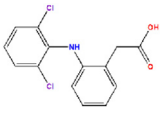
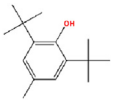
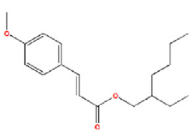
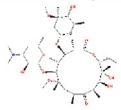
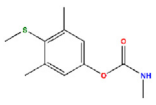
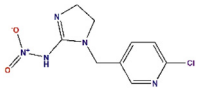
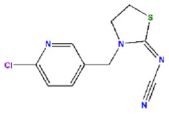
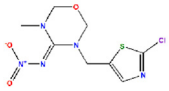
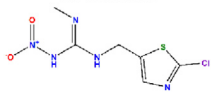
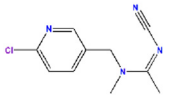
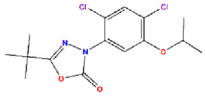
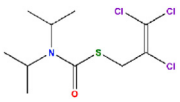
Name of substance/ group of substances	CAS number	(Substance) structure	Concentration (ng L^{-1}) Matrix Locations (number of samples)	Reference
17-Alpha-ethinylestradiol (EE2)	57-63-6		<1–8 WW Korea (n = 120), Germany (n.a.), South Africa (n = 12)	(Behera et al., 2011; Bolong et al., 2009; Manickum and John, 2014)
			0.2–1.9 SW China (n = 3), Korea (n = 120), Germany (n.a.), France (n = 73)	(Bolong et al., 2009; Luo et al., 2014; Vulliet and Cren-Olivé, 2011)
			0.5–230 GW France (n = 73), USA (n.a.)	(Luo et al., 2014; Vulliet et al., 2008; Vulliet and Cren-Olivé, 2011)
17-Beta-estradiol (E2) Estrone (E1)	50-28-2		<1–88 WW China (n = 3), Korea (n = 120), Sweden (n = 3), UK (n.a.), Germany (n.a.)	(Behera et al., 2011; Bolong et al., 2009; Nie et al., 2012; Zorita et al., 2009)
			0.2–10.1 SW China (n = 3), Korea (n = 120), Germany (n.a.), Japan (n = 517), France (n = 71)	(Bolong et al., 2009; Luo et al., 2014; Vulliet and Cren-Olivé, 2011)
			0.3–147 GW France (n = 73), USA (n.a.)	(Luo et al., 2014; Vulliet et al., 2008; Vulliet and Cren-Olivé, 2011)
	53-16-7		<1–220 WW China (n = 3), Korea (n = 120), Sweden (n = 3), UK (n.a.), Germany (n.a.)	(Behera et al., 2011; Bolong et al., 2009; Nie et al., 2012; Zorita et al., 2009)
			0.5–69.1 SW China (n = 3), Korea (n = 120), Germany (n.a.), France (n = 71)	(Bolong et al., 2009; Luo et al., 2014; Vulliet and Cren-Olivé, 2011)
			0.7–79 GW France (n = 73), USA (n.a.)	(Luo et al., 2014; Vulliet et al., 2008; Vulliet and Cren-Olivé, 2011)
Diclofenac	15307-86-5		14.9–4425 WW Spain (n.a.), Italy (n = 3), USA (n.a.), Portugal (n = 4)	(Al Aukidy et al., 2012; Lara-Martin et al., 2014; Pereira et al., 2015; Prieto-Rodriguez et al., 2012)
			0.8–1043 SW Spain (n.a.), Vietnam (n.a), Costa Rica (n = 86), Greece (n = 30)	(Li, 2014; Spongberg et al., 2011; Stasinakis et al., 2012)
			1.17–380 GW Spain (n = 30), France (n = 70)	(Li, 2014; Lopez-Serna et al., 2013; Luo et al., 2014)
2,6-di-tert-butyl-4-methylphenol	128-37-0		49–620 SW USA (n = 19), Sweden (n.a.)	(Bendz et al., 2005; Benotti et al., 2009)
2-ethylhexyl-4-methoxycinnamate	5466-77-3		4.7–505 WW China (n = 17), Norway (n = 5)	(Langford et al., 2015; Li et al., 2007; Tsui et al., 2014)
			12–1040 SW Japan (n = 23)	(Amine et al., 2012)
			770 GW Spain (n = 7)	(Díaz-Cruz et al., 2012)

Table 1 (*continued*)

Name of substance/ group of substances	CAS number	(Substance) structure	Concentration (ng L^{-1}) Matrix Locations (number of samples)	Reference
Macrolide antibiotics	83905-01-5	Azithromycin 	0.4–1220 WW Italy (n = 3), Slovakia (n = 3), USA (n.a.), Portugal (n = 4)	(Al Aukidy et al., 2012; Birosova et al., 2014; Gibs et al., 2013; Pereira et al., 2015)
			0.6–90.8 SW Vietnam (n = 2), China (n = 24)	(Hoa et al., 2011; Tong et al., 2014)
			0.6–1620 GW Spain (n.a), China (n = 69)	(Lopez-Serna et al., 2013; Ma et al., 2015; Tong et al., 2014)
	81103-11-9	Clarithromycin 	54–1890 WW Spain (n.a.), Italy (n = 3), Slovakia (n = 3), USA (n.a)	(Al Aukidy et al., 2012; Birosova et al., 2014; Lara-Martin et al., 2014; Prieto-Rodriguez et al., 2012)
			0.01–778 SW Vietnam (n = 2), Spain (n = 18), China (n = 24)	(Gracia-Lor et al., 2011; Hoa et al., 2011; Tong et al., 2014)
			0.2–20.5 GW Spain (n.a.), China (n = 15)	(Lopez-Serna et al., 2013; Tong et al., 2014)
	114-07-8	Erythromycin 	16–147.9 WW Spain (n.a.), Slovakia (n = 3), USA (n.a.), China (n = 3)	(Birosova et al., 2014; Gibs et al., 2013; Prieto-Rodriguez et al., 2012; Yan et al., 2014b)
			0.28–2246 SW Vietnam (n = 2), Spain (n = 18)	(Gracia-Lor et al., 2011; Hoa et al., 2011)
			4.8–154.3 GW Spain (n = 121), China (n = 54)	(Cabeza et al., 2012; Lopez-Serna et al., 2013; Ma et al., 2015)
Methiocarb	2032-65-7		4.73–14.92 WW Spain (n = 55)	(Campo et al., 2013; Masiá et al., 2013)
Neonicotinoids	105827-78-9 138261-41-3	Imidacloprid 	2–34.44 WW Spain (n = 55)	(Campo et al., 2013; Masiá et al., 2013)
			1.1–105 SW Spain (n = 24), USA (n = 35), Greece (n = 89), Portugal (n.a.), Australia (n = 13)	(Gonzalez-Rey et al., 2015; Masiá et al., 2013; Papadakis et al., 2015; Sánchez-Bayo and Hyne, 2014)
	111988-49-9	Thiacloprid 	20–400 SW Australia (n = 13)	(Sánchez-Bayo and Hyne, 2014)
	153719-23-4	Thiamethoxam 	40–1580 SW Brasil (n.a.), Vietnam (n = 11), Australia (n = 13)	(Chau et al., 2015; da Rocha et al., 2015; Sánchez-Bayo and Hyne, 2014)
	210880-92-5	Clothianidin 	20–420 SW Australia (n = 13)	(Sánchez-Bayo and Hyne, 2014)
	135410-20-7 160430-64-8	Acetamiprid 	20–380 SW Australia (n = 13)	(Sánchez-Bayo and Hyne, 2014)
Oxadiazon	19666-30-9		4–1440 SW Canada (n = 8)	(Furtula et al., 2006)

(*continued on next page*)

Table 1 (*continued*)

Name of substance/ group of substances	CAS number	(Substance) structure	Concentration (ng L^{-1}) Matrix Locations (number of samples)	Reference
Triallate	2303-17-5		n.a.	n.a.

full-scale, some describing the removal of these substances on spiked environmental matrices, and some including the comparison between the real matrix and ultrapure/deionized water. The first step of sample preparation is usually the filtration of the samples, and the works on occurrence take into account this step in the sample preparation protocol of the analytical method. Before such literature overview, the next sections (1.2, 1.3 and 1.4) present a brief description of the most commonly used conventional and advanced treatment processes.

1.2. Treatment by conventional processes

The efficiency of a conventional WWTP varies depending on the characteristics of the pollutant and on the treatment process employed. The main mechanisms for removal of micropollutants occurring during the secondary treatment at WWTPs are biological and/or chemical transformation and sorption (Radjenovic et al., 2009; Verlicchi et al., 2012). The most common employed processes are conventional activated sludge (CAS) and membrane biological reactors (MBRs).

The efficiency of a CAS system depends on the physicochemical characteristics of the substances and on the nature of the microbial community. The most important operational factors affecting the efficiency are the temperature, the hydraulic retention time (HRT) and the sludge retention time (SRT) (Oulton et al., 2010; Petrovic et al., 2009), a higher HRT favoring the removal of more refractory compounds and a higher SRT allowing a higher diversity of microorganisms (Petrovic et al., 2009). The usual SRT in the CAS systems is 7–20 days and the biomass concentration 3–5 kg m^{-3}, with a HRT typically ranging from 2 to 24 h (Verlicchi et al., 2012).

MBRs emerged as an alternative to CAS, integrating aerobic biodegradation and membrane separation, modestly more efficient than CAS in the extent of removal of several CECs (Oulton et al., 2010). MBR treatment differs mainly in the SRT that is normally longer (15–80 days) and the commonly higher biomass concentration (8–10 kg m^{-3}), HRT being often between 7 and 15 h (Verlicchi et al., 2012). Other important difference is the final stage using ultrafiltration (UF) or microfiltration (MF) membranes to separate the liquid from sludge. Therefore, MBR overcome the constraints of CAS treatment related to the sludge retention and settling characteristics, by applying these membranes to retain the biomass (Petrovic et al., 2009), decreasing the chemical oxygen demand while enhancing the removal of suspended solids and pathogens. Unlike the reports related to CAS, studies focusing on the performance of MBR processes to remove CECs are limited and difficult to compare due to the different operation conditions and target pollutants (Kim et al., 2014). Verlicchi et al. (2012) reviewed extensively the occurrence and removal of pharmaceutical compounds in municipal wastewater, comparing the effectiveness of the secondary treatment by CAS and MBR, with much more studies employing CAS and using generally 24 h composite water samples, avoiding diurnal variability and favoring the inter-studies comparison. Pharmaceuticals and hormones that are now included in the watch list of Decision 495/2015 were referred in that review, where it was concluded that average removals found in the literature were superior employing MBR than CAS, namely between 26 and 44% for CAS and higher than 60% for MBR, except for azithromycin (Verlicchi et al., 2012).

1.2.1. Intermediates formation

Overall, most studies on both CAS and MBR have been focusing on the parent compounds and little attention has been given to the produced intermediates. It is noteworthy that biological or chemical reactions occurring in the secondary clarifiers might lead to the accumulation of metabolites/by-products (Oulton et al., 2010). There are also some compounds (e.g., pharmaceuticals, hormones, drugs of abuse that are excreted by humans and/or animals) that can be found at higher concentrations in the WWTPs effluents than in the respective influents, due to their excretion as conjugates that are broken in the WWTPs. These conjugates are generally metabolized during biological treatment and the parent compound is released, often increasing the concentrations of the parent compounds at the outlet of the WWTPs. For example, E1 can be detected in the secondary effluent of a WWTP at a higher concentration than that found in the raw influent, due to the oxidation of E2 that enters into the WWTP. This fact explains the occasional negative removal efficiencies, sometimes at high extents, with the greatest contribution of the biological transformation (Verlicchi et al., 2012). There are other causes for negative removals occurring during the WWTP treatment. In most cases, the sampling protocol does not consider the HRT and/or SRT and as consequence effluent does not correspond to the same plug of influent (Campo et al., 2013). Sometimes the compounds can be released from particulate matter during treatment (e.g. macrolide antibiotics released from feces particles) (Kim et al., 2014). There are already some reports investigating the occurrence and removal of metabolites and/or intermediates; however, it is crucial to develop more studies on this matter, comprising the parent compounds, the possible by-products and the known metabolites in a broader and more comprehensive approach.

1.3. Separation by membrane technologies

Membrane filtration is mostly used for the removal of microorganisms and salts from water/wastewater. The most common membrane technologies include relatively low-pressure systems, such as MF and UF operating at pressures up to 5 and 10 bar, respectively, or high-pressure systems, namely nanofiltration (NF) operating at nearly 50 bar or reverse osmosis (RO) up to 70 bar (or 150 bar for high pressure RO systems) (Coday et al., 2014; Oulton et al., 2010; Peters, 2010). Among these types, the high pressure systems are more suitable for rejection of organic micropollutants, considering the size exclusion mechanism, but larger pores can be employed if electrostatic repulsion or adsorption are the main mechanisms involved in the process (Oulton et al., 2010). The parameters affecting the efficiency of the process include the molecular weight cut-off (MWCO), some membrane properties (e.g., hydrophobicity, surface roughness and charge) and

physicochemical characteristics of the compounds to be rejected (e.g., molecular weight, pKa,K_{ow} and polarity), among others (Oulton et al., 2010). Regarding the high pressure systems, the main characteristic of NF is the ion selectivity, where monovalent ions can pass through the membrane and multivalent anions are retained (Peters, 2010). The rejection rates are high for organic compounds with molecular weights above 100–200 g mol^{-1} (Hillis, 2000). This process is typically applied for dye/color removal, but recent studies focused on the removal of emerging micropollutants from drinking water and wastewater (Baker, 2012). In the case of RO, the organic and inorganic molecules are separated from the feed solution by their molecular weight (normally, less than 200 g mol^{-1}), size, charge and inability to permeate the active surface of the RO membrane (Lee et al., 2012). The applications range from the production of ultrapure water, to the desalination of seawater for drinking water production and the treatment of industrial wastewater (Peters, 2010). More recently, RO was also applied for the removal of micropollutants, the process depending on complex interactions (e.g., steric, electrostatic/repulsion and hydrophobic) between the contaminants, the solution and the membrane (Dolar et al., 2012). Among the membrane processes, RO was considered as the ultimate treatment step yielding highest pollutant rejection efficiencies (Theepharaksapan et al., 2011).

Forward osmosis (FO) and membrane distillation (MD) are some alternatives to the membrane processes exclusively based on hydraulic pressure. FO is an osmotically driven membrane process that consists on the osmotic pressure difference between the draw solution and the feed solution. Recently, FO has been more intensively investigated for water/wastewater treatment, as a single treatment or coupled to other membrane processes (Coday et al., 2014; Liu et al., 2015). MD (mainly developed for desalination) is based on a vapor pressure gradient across a porous hydrophobic membrane and can operate under different possible configurations (e.g., direct contact, vacuum, air gap and sweep gas MD) (Drioli et al., 2015; Silva et al., 2015; Wang and Chung, 2015). MD has also been studied to reject organic compounds in water treatment (Alkhudhiri et al., 2012) since a complete rejection of inorganic ions and non-volatile substances is theoretically expected.

One of the major disadvantages in this type of processes is the production of a concentrate containing all the retained compounds (Bagastyo et al., 2011; Justo et al., 2014). The disposal of the concentrate can be performed by sewer disposal, evaporation ponds and deep well injection (Umar et al., 2015), but direct discharge to water bodies (oceans, surface and groundwater) is common and constitute potentially serious threat to ecosystems (Justo et al., 2015; Pérez-González et al., 2012). Thus, careful environmental practices are recommended to handle such a concentrated waste before discharging into the aquatic environment (Westerhoff et al., 2009). Different approaches for the treatment of membrane concentrates have been investigated, mainly using AOPs, but also coagulation/flocculation and adsorption with activated carbon were reported (Bagastyo et al., 2011; Justo et al., 2013b, 2014). However, most of these emerging technologies have been developed at laboratory or pilot plant scale (Pérez-González et al., 2012). Good results have been achieved by AOPs for the removal of organic pollutants and persistent compounds, but the cost of these processes can limit their wide implementation at full-scale (Pérez-González et al., 2012; Westerhoff et al., 2009).

1.4. Degradation by advanced oxidation processes (AOPs)

Advanced oxidation processes (AOPs) are conceptually based on the production of highly reactive oxidizing species, such as hydroxyl radicals ($HO^{\bullet}$). AOPs are able to degrade unselectively organic pollutants (Hoigné, 1997) and can be used as pre- or post-treatment of a biological process. As pre-treatment, the aim of a single or a sequence of complementary AOPs is to obtain a more biodegradable effluent able to be treated by a conventional biological process. AOPs can be used as post-treatment to remove micropollutants and their by-products, ideally yielding as final products CO_2, H_2O and inorganic ions, if the aim is the direct discharge in natural water courses. One shortcoming often found in the application of AOPs for wastewater treatment is the frequent presence of radical scavengers in the wastewater, limiting the attack of the radicals to the organic pollutants. Commonly employed AOPs to investigate the treatment of micropollutants in real matrices, include the Fenton and photo-Fenton processes, (catalytic) wet peroxide/air oxidation, (catalytic) ozonation, heterogenous photocatalysis, electrochemical oxidation or combination of them. For the catalytic processes, different catalysts have been identified as the most active depending on the reaction system, including metal oxides (based on Ti, Cu, Zn, Mn, Fe, Co and Bi, among others), supported noble metals (e.g., Ru, Pt, Pd, Ir and Rh), or even metal-free carbon materials such as activated carbons, carbon xerogels, carbon nanotubes, carbon foams and fibers and graphite (Ribeiro et al., 2015).

Briefly, the Fenton process, based on the Fenton reagent (Fenton, 1894), employs H_2O_2 and a precursor of iron, generating $HO^{\bullet}$ at atmospheric pressure and room temperature. High efficiency, relatively cheap reagents, no need of energy to activate H_2O_2 and the consequent easy implementation and operation are the advantages of such treatment. Some disadvantages are the generation of a secondary waste (sludge) and the narrow range of optimal pH (2.5 − 3.0). The photo-assisted Fenton process can be more efficient than Fenton alone, mainly due to the faster regeneration of Fe^{2+} (Pastrana-Martínez et al., 2015). Other related options are electro-Fenton, where Fe^{2+} is produced from sacrificial cast iron anodes (Nidheesh and Gandhimathi, 2012), or even photo-electro-Fenton (Umar et al., 2010).

The concept of catalytic wet peroxide oxidation is similar to that of the Fenton process, but in this case any catalyst can be used (not only iron species) and slightly higher temperatures (50–70 °C) are typically employed (the operating pressure and temperature dramatically increasing in the case of wet air oxidation).

Regarding ozonation, this process involves the direct attack of ozone (quite selective for electron-rich organic molecules) mainly at low pH and/or indirect reactions through $HO^{\bullet}$ more prone at high pH (Ikehata and El-Din, 2004; Munter, 2001). The main handicap of ozonation is the typical low efficiency to mineralize the organic pollutants, while natural organic matter (NOM) and carbonate ions can have a significant interference with the ozone decomposition rate (Saquib et al., 2010). For this reason, different heterogeneous catalysts are under investigation to improve the process (Faria et al., 2008, 2009; Gonçalves et al., 2013; Gonçalves et al., 2010; Orge et al., 2012; Restivo et al., 2012).

Heterogeneous photocatalysis is other process that has been extensively investigated for water/wastewater treatment and is based on the use of wide band-gap semiconductors which generate electrons and holes (and subsequent chain reactions including $HO^{\bullet}$) when irradiated with photons of energy higher than the semiconductor band-gap (i.e., $h\nu \geq E_G$) (Frank and Bard, 1977; Fujishima and Honda, 1972). TiO_2 is the most widely used reference photocatalyst due to the outstanding activity, photochemical stability, good band gap energy, low cost and relatively low toxicity (Hoffmann et al., 1995; Kabra et al., 2004). The possible use of sunlight and the intrinsic anti-microbial ability of heterogeneous photocatalysis (Marin et al., 2011; McCullagh et al., 2007; Monteiro et al., 2015; Rincón and Pulgarin, 2004) are counterbalanced by its main shortcomings, such as the fast recombination of electron–hole pairs and the limited usage of solar light when bare TiO_2 is

Table 2
Some examples of studies dealing with the removal of E1, E2 and/or EE2. Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
E1	ng L^{-1} level	3 pilot WWTPs, one employing CAS; Average flow rate: 107 dm^3 d^{-1}; SRT 3 d; HRT 7 h; 24 h composite samples; Burlington Skyway municipal WWTP; Ontario, Canada.	Removal efficiency of CAS was greater than 65% for E1.	(Ogunlaja and Parker, 2015)
E2 EE2 E1	14.5 ± 4.5 ng L^{-1} n.d. 3.2 ± 4.1 ng L^{-1}	Municipal WWTP with biological and chemical treatment; Average flow rate: 20,000 m^3 d^{-1}; Kristianstad; South Sweden.	Removals of 78% and >47% were observed for E1 and E2, respectively.	(Zorita et al., 2009)
E2	10 μg L^{-1} (spiked wastewater)	Lab-scale MBR and CAS; Industrial-municipal mixed wastewater before secondary treatment.	E2 was almost completely removed (99%) applying both treatments.	(López-Fernández et al., 2013)
E1 E2 EE2	n.d./n.d.; up to 23.2/21.2 ng L^{-1}; up to 22.2/29.2 ng L^{-1}.	WWTPs: CAS or MBR coupled with UF or MF; 24 h composite samples composed by 4 h-aliquots collected by an automatic device; Granada, Spain.	The concentrations after the CAS and MBR treatments were respectively: up to 0.81 and 4.9 ng L^{-1} for E2 and up to 6.62 and 6.92 ng L^{-1} for EE2. MBR system was shown as good alternative to provide high-quality water for reuse. MBR with MF was more efficient for E2 removal.	(Camacho-Munoz et al., 2012)
EE2	140 ng L^{-1} (after primary clarifier)	WWTP with CAS-MF-GAC-ozonation; Average flow rate: 227,000 m^3 d^{-1}; Gwinnett County, GA, USA.	After CAS and MF, the concentration of EE2 decreased by more than 90%. Ozonation oxidized the remaining compounds by more than 60%.	(Yang et al., 2011)
EE2 E1	8.73 ng L^{-1} 20.69 ng L^{-1}	Pilot-scale combination of MBR and NF or RO; MBR permeate flux: 10.5 L m^{-2} h^{-1} (constant flux mode); 4-L samples of the influent and effluents of each MBR, NF and RO process.	Removal efficiencies higher than 70% (based on the detection limits) were verified for E1 and EE2 with each treatment process.	(Lee et al., 2008)
E1 E2 EE2	150 μg L^{-1} (spiked surface water)	Lab-scale UF prior to NF; NF experiments were conducted at 10 bar and 3.6 cm s^{-1} of cross-flow velocity; Surface water from Tagus river, Portugal.	High rejections (higher than 90%) were obtained for E1, E2 and EE2.	(Sanches et al., 2012)
E2 EE2	0.2 μg L^{-1} (spiked wastewater)	MF and RO or MF prior to a pilot-scale UV/H_2O_2; LP-UV lamp; H_2O_2: 3 mg L^{-1}.	Removal of 99% was achieved in both cases.	(James et al., 2014)
E2 EE2 E1	1 mg L^{-1} (spiked surface water)	Multi-barrier approach; Lab-scale NF followed by LP-UV (λ_{max} = 245 nm) or indirect (H_2O_2-assisted) LP-UV; H_2O_2: 0, 20, 40, 60, 80 or 100 mg L^{-1}; Surface water.	A rejection of 71% was verified using NF (for all the compounds). Direct photolysis led to high E1 removal, while a removal >74% was obtained by indirect (H_2O_2) photolysis. The multi-barrier approach led to higher overall removals (80, 90 and 95% for E2, EE2 and E1, respectively).	(Pereira et al., 2012)
E1 E2 EE2	1.65–3.59 μg L^{-1} (treated wastewater from the secondary clarifier)	Pilot plant O_3, O_3/UV, O_3/H_2O_2 and O_3/UV/H_2O_2; O_3: 3.15 g h^{-1}; 5% of ozone in gas mixture.	A removal higher than 99.7% was observed for the 3 estrogens.	(Pesoutova et al., 2014)
E1	3 μg L^{-1} – 5 mg L^{-1} (spiked wastewater)	Lab-scale O_3, UV, UV/H_2O_2, O_3/UV, O_3/H_2O_2 and O_3/UV/H_2O_2; Annular reactor (750 mL); LP-UV lamp (λ_{max} = 253.7 nm); O_3: 0.33–1.31 mg L^{-1}; H_2O_2: 20, 40 and 60 mg L^{-1}; Municipal wastewater (London, OR, Canada).	A complete removal after 30 min was achieved, employing all processes, except for UV (75 min). Ozonation achieved the higher removal rates of E1. Low TOC removal was observed for all the AOPs tested, with the degradation rate decreasing with higher TOC values.	(Sarkar et al., 2014)
E2 EE2 E1	0.035 mg g^{-1} (dw) 0.150 mg g^{-1} (dw) 0.125 mg g^{-1} (dw)	Lab-scale UV, H_2O_2 and UV/H_2O_2; Reactor with continuous recirculation (800 mL); 75 W LP Hg lamp (λ_{max} = 253.7 nm); H_2O_2: 0.5 mol L^{-1}; pH 3; Spiked waste activated sludge.	E2, EE2 and E1 were removed respectively by 92%, 95% and 97%, after 2 min. UV/H_2O_2 was more efficient than UV or H_2O_2 alone. The sludge matrix influenced the degradation rate.	(Zhang and Li, 2014)
EE2	10 mg L^{-1} (spiked wastewater)	Catalytic ozonation; O_3: 20 mg L^{-1}; Catalysts: 5 g of commercial γ-Al_2O_3 or synthesized Co_3O_4/Al_2O_3; Ultrapure water and secondary effluents pre-treated to remove its carbonate/bicarbonate content by stripping; Municipal wastewater from a WWTP; Badajoz, Spain.	EE2 was removed in less than 10 min, regardless the matrix or the presence of catalyst. Comparing with single ozonation, catalytic ozonation enhanced the COD and TOC removals, especially in the presence of the Co_3O_4/Al_2O_3 catalyst.	(Pocostales et al., 2011)

Table 2 (*continued*)

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
EE2 E1	<4.3–7.4 ng L^{-1} 1.6–2 ng L^{-1}	Pilot-scale ozonation plant; O_3: 86–153 g Nm^{-3}; O_3 consumption: 0.6 and 0.9 g O_3 g DOC_0^{-1}; Wastewater; Austria.	The application of 0.6 g O_3 g DOC^{-1} increased the removal of these compounds (to not detected).	(Schaar et al., 2010)
E2 EE2 E1	10–250 ng L^{-1} (spiked river water)	Ozonation; O_3: 3–4 mg L^{-1}; River water.	High removal (98–99%) after 10 min was achieved by ozonation process for all estrogens.	(Westerhoff et al., 2005).
EE2 E2 E1	391.4 ± 59.3 ng L^{-1} 110.4 ± 55.4 ng L^{-1} 20.2 ± 3.3 ng L^{-1}	Lab-scale photolytic ozonation, ozonation and photocatalysis; O_3 flow rate: 150 Ncm^3 min^{-1}; O_3: 50 g Nm^{-3}; MP Hg vapor lamp (UV/Vis λ > 300 nm); TiO_2 photocatalyst: 0.5 g L^{-1} load; Urban wastewater from the secondary treatment of a WWTP; North of Portugal.	Complete removal by photocatalytic ozonation was achieved for all estrogens, while EE2 was not completely removed using ozonation (77.2% only) and E1 was not completely removed using photocatalysis (61.8% only).	(Moreira et al., 2015)
EE2	2.0 μM (spiked surface water)	Quartz photolysis tubes (1.4 cm i.d. × 20 cm) at a 45° angle were used in photodegradation experiments; Lake water from Lake Quinsigamond.	EE2 showed very high resistance to microbial degradation while rapid photodegradation under sunlight irradiation occurred (half-life of 23 h).	(Zuo et al., 2013)

AOP, advanced oxidation process; CAS, conventional activated sludge; COD, chemical oxygen demand; DOC, dissolved organic carbon; dw, dry weight; GAC, granular activated carbon; HRT, hydraulic retention time; LP, low pressure; MBR, membrane biological reactor; MF, microfiltration; MP, medium pressure; n.a., not available; n.d., not detected; NF, nanofiltration; RO, reverse osmosis; SRT, sludge retention time; TOC, total organic carbon; UF, ultrafiltration; WWTP, wastewater treatment plant.

employed (i.e. only the UV fraction, near 3–5% of the overall spectrum) (Andreozzi et al., 1999). A recent approach is the hybridization of photocatalysis with membrane processes, with emphasis in the preparation of new filtration membranes with photocatalytic properties (Athanasekou et al., 2015; Pastrana-Martinez et al., 2015).

Sonolysis, supercritical water oxidation, γ-ray irradiation, microwaves and pulsed electron beam are less commonly applied AOPs (Ribeiro et al., 2015).

2. The watch list: occurrence and removal

This section aims to overview the substances and group of substances of the watch list for European Union monitoring, defined in the Decision 2015/495/EU, regarding their occurrence in aqueous matrices as well as their removal by using the above-mentioned treatments. Scopus database was used and the keywords were the name of each substance and the following treatments: CAS, MBR (conventional processes); RO, MF, UF, NF, FO or MD (membrane technologies); and UV- and peroxide-based, Fenton-based, heterogeneous photocatalysis or ozonation-based processes (AOPs). The studies selected for this review were performed using realistic matrices. Considering the huge amount of literature available for estrogens (EE2, E2 and E1) and for diclofenac, only some examples of studies related to treatment processes for these particular substances (Tables 1 and 2) are included in this review.

2.1. EE2, E2 and E1

Steroid hormones include highly active biological compounds able to induce the therapeutic effect at very low doses. Within this group, estrogens are the most usually found in the aquatic environment, existing either as natural or synthetic substances and acting as endocrine-disrupting compounds (EDCs) (Barreiros et al., 2016; Rocha et al., 2008). Estriol, E1 and E2 are natural estrogens mainly excreted from humans whereas EE2 is the most used oral contraceptive, also excreted by humans, causing injurious effects to the ecosystems such as feminization of male fishes, DNA and immunity alterations (Li, 2014). The effects of EDCs toward animals are well reported, for example, a 7-year experiment was developed (Kidd et al., 2007) and it was concluded that the chronic exposure of fathead minnow to 5–6 ng L^{-1} of EE2 led to feminization of male fish and altered oogenesis in females. Some studies suggested that the effect of EDCs exposure on human health includes a decrease in male sperm count, an increase in testicular, prostate, ovarian and breast cancers and reproductive malfunctions (Joffe, 2001). The major concern is related to fetuses and newborn babies, because of their higher vulnerability (Sharpe and Irvine, 2004). Recently, Kabir et al. (2015) reviewed extensively the mechanism of action and harmful effects of EDCs on human health; and Futran Fuhrman et al. (2015) highlighted the EDCs risk assessment, namely issues related to long-term and combined exposure, transgenerational and mixture effects. Due to the potential deleterious effects that can arise from their release into the environment, their occurrence is well described and reviewed by several authors (Khanal et al., 2006; Li, 2014; Teske and Arnold, 2008). Table 1 summarizes some studies on the occurrence of E1, E2 and EE2 (concentration, matrix and location), which are frequently found in water matrices, namely wastewater, surface and groundwater, at ng L^{-1} levels.

The removal of these hormones are reported in several studies (Fig. 2), varying depending on the processes (Table 2). Biological treatments coupled with membrane processes are reported as effective mean for elimination of these types of compounds (Camacho-Munoz et al., 2012). As example, more than 90% of EE2 was removed in an advanced wastewater reclamation plant employing a biological treatment and MF (Yang et al., 2011). Few studies were developed using other membrane technologies to remove E1, E2 and EE2 (Table 2), being highly removed by NF and/or RO (Cheng et al., 2010; Lee et al., 2008). AOPs are promising to remove this type of pollutants, with ozonation having the highest efficiency (Table 2). Data regarding these compounds can be consulted in article reviews that have been published in the last few years and that already encompass a significant amount of information dealing with their removal from water (Basile et al., 2011; Jung et al., 2015; Kaplan, 2013; Liu et al., 2009; Luo et al., 2014; Yu et al., 2013). Concerning the studies on the removal of the substances of the watch list, it can be concluded that E1, E2 and EE2 were the most studied in the last decade, employing all the types of processes herein referred (Fig. 2).

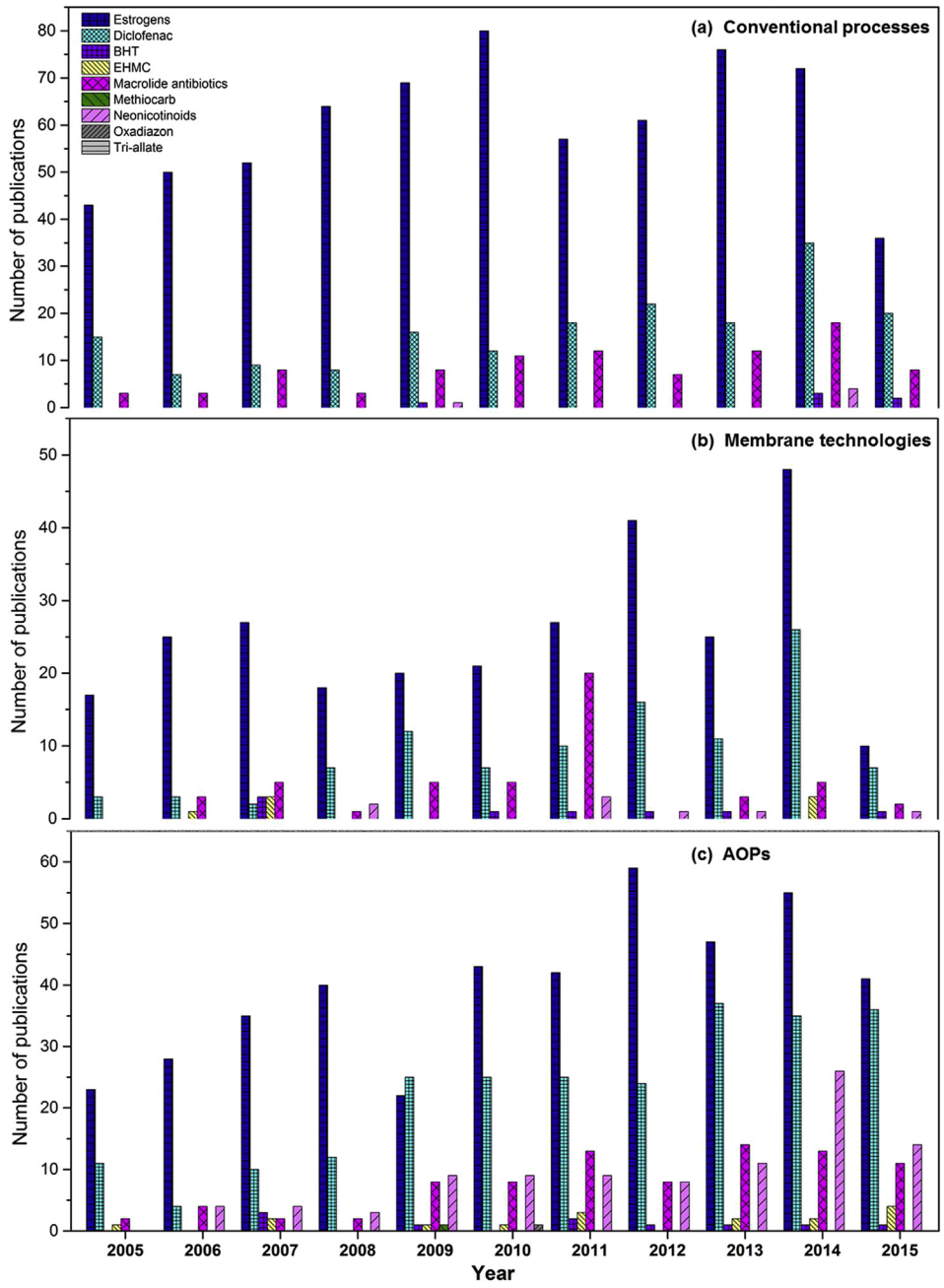


Fig. 2. Number of publications dealing with the removal of the 10 substances/groups of substances included in the first watch list for European Union monitoring (Decision 2015/495/EU). The search comprised publications since 2005 in Scopus database, using as keywords each substance and the treatments reported in the previous sections, namely (a) conventional processes (CAS or MBR); (b) membrane technologies (RO, MF, UF, NF, FO or MD); and (c) AOPs (UV-and peroxide based, Fenton based, heterogeneous photocatalysis or ozonation-based processes). In this particular search, any type of matrix (realistic and non-realistic) was considered.

2.2. Diclofenac

Regarding the non-steroidal anti-inflammatory drug (NSAID) diclofenac, it is considered harmful to several species at environmental concentrations, as indicated by Vieno and Sillanpää (2014), who overviewed its occurrence, fate and transformation processes during treatment in WWTPs. Diclofenac is often detected in WWTP influents and effluents, surface water and groundwater. Table 1 describes some studies on its occurrence in these aquatic compartments, with diclofenac found up to 4.4 μg L^{-1}. Information concerning the removal of diclofenac can be checked in article reviews that have been published in the last few years and which already included systematized data of its removal from water (Barra Caracciolo et al., 2015; Cherik et al., 2015; Fatta-Kassinos et al., 2011; Petrie et al., 2013; Ziylan and Ince, 2011). Diclofenac can be partially adsorbed on sludge and is usually poorly biodegradable, which means low removal rates during biological wastewater treatment (Table 3) (Vieno and Sillanpää, 2014; Zhang et al., 2008). Membrane technologies to remove diclofenac have been used, but more research is needed (Table 3). Concerning AOPs, some studies dealing with heterogeneous photocatalysis and/or photo-Fenton are described in Table 3, with a moderate diclofenac removal, most using a pilot compound parabolic collector (CPC) plant and a high reaction time. Ozonation as single process, or

Table 3
Some examples of studies dealing with removal of diclofenac. Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Initial diclofenac concentration	Treatment and sampling conditions	Concluding remarks	Reference
Up to 12.7 ng L^{-1} (CAS); up to 38 ng L^{-1} (MBR).	WWTP with CAS or MBR coupled to UF or MF; 24 h composite samples composed by 4 h aliquots collected by an automatic device; Granada, Spain.	Mean removal rates were between 54 and 71% for MBR and approximately 79% for CAS.	(Camacho-Munoz et al., 2012)
30 mg L^{-1} (spiked surface water)	Photocatalysis and solar photolysis; Advanced lab-scale reactor immersion-well (UV-RS-1) made of Pyrex glass (cut-off <290 nm); Solution volume: 400 mL; MP Hg vapor lamp: TQ 150 W Heraeus, Germany; TiO_2 load: 0.1 g L^{-1}; pH 6.2.	Water quality showed high influence in the treatment efficiency. For river water, solar photolysis showed higher removal compared to TiO_2 photocatalysis, with 66% and 82% diclofenac removal for photocatalysis and direct sunlight, respectively.	(Kanakaraju et al., 2014)
0.05 μg L^{-1} (spiked surface water)	Kagithane Drinking Water Treatment Plant; Flow rate: 700,000 m^3 d^{-1}; Lab-scale NF; MF: cross-flow rate of 3 L min^{-1} and water flux of 137 L m^{-2} h^{-1}; Raw water sources: Terkos Lake and Alibeyköy Dam.	Diclofenac overall rejection was approximately 61%.	(Vergili, 2013)
220 ng L^{-1} (after primary clarifier)	WWTP with CAS-MF-GAC-ozonation; Average flow rate: 227,000 m^3 d^{-1}; Gwinnett County, GA, USA.	Diclofenac was removed by CAS, between 51 and 80%, achieving the LOQ.	(Yang et al., 2011)
WWTP1: 507 ng L^{-1}; WWTP2: 1450 ng L^{-1}.	WWTP1: parallel CAS and MBR serving 28,000 inhabitants and treating 5544 m^3 d^{-1} by CAS and 7237 m^3 d^{-1} by MBR; WWTP2: CAS serving 100,000 inhabitants and treating 20,846 m^3 d^{-1}; 24 h composite samples; Lede, Belgium.	Removal was not observed in both treatments of WWTP1 employing parallel CAS and MBR. Almost no removal occurred in WWTP2, with 1391 ng L^{-1} in the effluent of CAS.	(Vergeynst et al., 2015)
Up to 2400 ng L^{-1}	15 WWTPs designed for 6850 to 756,000 population equivalents; Flow rates: 349–140,000 m^3 d^{-1}; Discharge points: main Portuguese rivers and Atlantic Ocean; 24 h composite influent and effluent samples; Portugal.	Concentration of diclofenac was quantified up to 670 ng L^{-1} in the effluent. The mean removal of this substance was 45.6%.	(Pereira et al., 2015)
Up to 0.49 μg L^{-1}	4 WWTPs impacted by effluents from mid-size hospitals, corresponding to a WWTP inflow between 1 and 30%; Flow rates: 1300–103,000 m^3 d^{-1}; WWTP 1/3 – SBR and UV-tertiary treatment; WWTP 2 – CAS; WWTP 4 – oxidation ditch activated sludge process; 24 h composite samples; New York, USA.	WWTP 2 and 4, employing CAS or oxidation ditch activated sludge, were more efficient than the others for the removal of diclofenac.	(Oliveira et al., 2015)
Up to 6.34 ng L^{-1}	4 WWTPs; Chongqing, China; 2 WWTPs: anaerobic/anoxic/oxic (A/A/O) activated sludge process; 1 WWTP: CAST; 1 WWTP: OD.	Although diclofenac was quantified up to 4.7 ng L^{-1}, the removal was not assessed due to its detection in some cases, below than LOQ.	(Yan et al., 2014a)
n.a.	MBR pilot plant in continuous operation (ca. 1% of diurnal hospital sewage); 2 h composite influent and effluent samples; Luxembourg.	Diclofenac was removed at an extent between 40 and 50%. UV was evaluated as post-treatment; degradation was improved by applying H_2O_2.	(Kohler et al., 2012)
6.01 ng L^{-1}	4th largest WWTP in China, serving 1,540,000 equivalent inhabitants; 600,000 m^3 d^{-1}. CAST followed by chlorination; Grab samples collected according to the residence time in each treatment; Southwest China.	The removal obtained after secondary treatment was 41.8% for diclofenac. Chlorination led to a reduction of 8.6%.	(Yan et al., 2014b)
361–911 ng L^{-1}	Pilot-scale MF followed by RO; MF: flow rate of 2 m^3 h^{-1} and flux of 323 L m^{-2} h^{-1}; Residence time 3 min; RO: flow rate of 1 m^3 h^{-1} and permeate flux of 34 L m^{-2} h^{-1}; Residence time 50 min; Treated effluent; Girona, Spain.	High removal of diclofenac was observed. RO reduced the concentration of diclofenac in the MF permeate to levels below the limit of detection.	(Rodriguez-Mozaz et al., 2015)
57–131 ng L^{-1}	Pilot-scale NF and RO; NF: Water flux between 12 and 62 L m^{-2} h^{-1}, depending on the type of membranes; RO: Water flux of 23.5 L m^{-2} h^{-1}; Treated effluent; Sydney, Australia.	RO was the most efficient treatment for the rejection of diclofenac, reaching concentrations lower than 5 ng L^{-1}.	(Shanmuganathan et al., 2015)
104.1 ng L^{-1}	Pilot-scale UF followed by a RO; UF permeate flux: 227 m^3 d^{-1}; RO permeate flux: 82 m^3 d^{-1}; Ansan, Gyeonggi-do, Korea.	Concentration of diclofenac considerably decreased by UF (permeate concentration: 69.7 ng L^{-1}). RO completely removed diclofenac.	(Chon et al., 2013)

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Table 3 (*continued*)

Initial diclofenac concentration	Treatment and sampling conditions	Concluding remarks	Reference
750 ng L^{-1}	Pilot-scale NF; Flux: 1–2 L m^{-2} h^{-1}; 24 h composite sample; Giessen, Germany.	Diclofenac decreased by at least 65%.	(Röhricht et al., 2010)
605 ng L^{-1}	2-L reactors at 25 °C; UV/H_2O_2: 3 LP Hg lamps (λ_{max} = 254 nm); H_2O_2 consumed ranged from 0.04 to 0.72 mg H_2O_2 mg TOC^{-1}; Ozonation: 10 g O_3 Nm^{-3}; RO concentrates from a municipal WWTP	UV/H_2O_2 exhibited higher performance than ozone in the removal of diclofenac, which had one of the lowest initial observed kinetic constants probably due to the matrix effects on the process.	(Justo et al., 2013a)
935 ng L^{-1}	2-L reactors at 25 °C; UV/H_2O_2: 3 LP Hg lamps (λ_{max} = 254 nm); H_2O_2 consumed ranged from 0.01 to 0.90 mg H_2O_2 mg TOC^{-1}; RO concentrates from a municipal WWTP in a coastal area of Catalonia, Spain.	Diclofenac was completely decomposed by UV, after the first minutes of treatment; it was also removed by UV/H_2O_2 and ozonation process.	(Justo et al., 2014)
283 ng L^{-1}	Biological activated carbon (BAC) process to treat municipal wastewater RO concentrate; Lab scale during 320 days of operation; BAC, combined UV/UV/H_2O_2–BAC and ozone–BAC.	54% of diclofenac was removed by the BAC filter. The integration of the UV/H_2O_2 or the ozonation processes was necessary to obtain a complete removal of diclofenac.	(Justo et al., 2015)
>750 ng L^{-1}	Photocatalysis in a pilot-scale CPC plant under natural solar irradiation; TiO_2 load: 20 mg L^{-1}; Effluents of the biological treatment of El Ejido WWTP; Almería, Spain.	Complete diclofenac removal was achieved after 480 min.	(Prieto-Rodriguez et al., 2012)
671–4941 ng L^{-1}	Photo-Fenton in a pilot-scale CPC plant; Fe^{2+}: 5 mg L^{-1}; pH: 3 and 10; H_2O_2: 50 mg L^{-1}; Complexing agents (humic acid and ethylenediamine-N,N′-disuccinic acid); Effluents of the secondary treatment in a municipal WWTP; Almería, Spain.	Diclofenac was removed by 97% in the photo-Fenton process (pH 3), after 50 min. Photo-Fenton with humic acids at neutral pH resulted in a longer treatment time required to reach a similar degradation.	(Klamerth et al., 2013)
≈70 ng L^{-1}.	Bench-scale UV and UV/H_2O_2 (λ_{max} = 254 nm); H_2O_2: 7.8 mg L^{-1}; Volume and HRT: 35 L and 5 min, respectively; Capacity: 10 m^2 d^{-1}; Municipal WWTP; Japan.	A complete removal of diclofenac was observed for both processes.	(Kim et al., 2009)
10 μg L^{-1} (spiked surface water)	UV/H_2O_2 in a pilot plant with three parallel reactors with MP, LP or dielectric barrier discharge UV lamps. Pre-treated surface water (by coagulation, flocculation and sedimentation in a natural reservoir, micro-straining and dual layer rapid sand filtration) from Meuse River (Netherlands), spiked with a mixture of 15 compounds.	The degradation of diclofenac was higher than 80%.	(Lekkerkerker-Teunissen et al., 2013)
n.a.	Sulfate radical based homogeneous photo-Fenton involving peroxymonosulfate as oxidant, ferrous iron (Fe(II)) as catalyst and simulated solar irradiation as light source; Biologically treated domestic wastewater effluents;	PMS/Fe(II)/UV–Vis advanced oxidation system using simulated solar irradiation has demonstrated better kinetic performances over TiO_2/UV–Vis system for diclofenac.	(Ahmed et al., 2014)
0.1 mg L^{-1} (spiked wastewater)	Heterogeneous photocatalysis and Photo-Fenton; Pilot-scale CPC solar plant at the Plataforma Solar de Almería (Spain); A: Photo-Fenton (pH 2; 5 mg L^{-1} of Fe^{2+}; 50 mg L^{-1} of H_2O_2; 5 mg L^{-1} of TiO_2); B: no pH adjustment; 50 mg L^{-1} of H_2O_2; 5 mg L^{-1} of Fe^{2+} (demineralized water); 5, 15 and 55 mg L^{-1} of Fe^{2+} (standard freshwater); 5 mg L^{-1} of Fe^{2+} (standard fresh water without $NaHCO_3$).	Solar TiO_2 photocatalysis showed complete diclofenac degradation. 20–50% of degradation in demineralised water was achieved in the dark (Fenton process) and photo-Fenton was the most effective treatment with a complete removal observed after 20 min. In standard fresh water, diclofenac was removed by Fenton process.	(Klamerth et al., 2009)
0.276 μg L^{-1}	Heterogeneous photocatalysis: Solardetox Acadus-2006 CPCs with 3.0 m^2 irradiated surface and 24 L of irradiated volume; TiO_2 load: 0.2 g L^{-1}; Effluent of a WWTP from the South East of Spain.	High diclofenac removal (≈88%) was observed after 3 h of treatment (bellow LOQ) applying solar TiO_2 photocatalysis.	(Bernabeu et al., 2011)
10 mg L^{-1} (spiked wastewater)	Catalytic ozonation; O_3: 20 mg L^{-1}; Catalysts: 5 g of commercial γ-Al_2O_3 or synthesized Co_3O_4/Al_2O_3; Ultrapure water and secondary effluents pre-treated to partially remove its carbonate/bicarbonate content by stripping; Wastewater from a municipal WWTP; Badajoz, Spain.	Diclofenac was removed in less than 10 min, regardless the matrix or the presence of catalyst. Comparing with single ozonation, catalytic ozonation enhanced the COD and TOC removals, in particular with a Co_3O_4/Al_2O_3 catalyst.	(Pocostales et al., 2011)

Table 3 (*continued*)

Initial diclofenac concentration	Treatment and sampling conditions	Concluding remarks	Reference
30–80 mg L^{-1} (spiked wastewater)	UVA, O_3, O_3/UVA, O_3/TiO_2, O_3/UVA/TiO_2; O_3: 5–30 g m^{-3}; HP Hg lamp; TiO_2 load: 0.5 and 2.5 g L^{-1}; Ultrapure water and urban wastewater from a municipal WWTP; Badajoz, Spain	Complete removal of diclofenac was verified by applying photocatalytic ozonation within 6 min (60–75% TOC reduction after 60 min, regardless the water matrix used). Photocatalytic ozonation showed the lowest ozone consumption compared to the other ozonation processes.	(Aguinaco et al., 2012)
30 mg L^{-1} (spiked surface water)	Single ozonation and catalytic ozonation; O_3: 10 g m^{-3}; pH = 7; Catalysts: 1 g L^{-1} of lab-prepared Mn–Ce–O or a commercial (N-150) catalyst; Synthetic effluent and river water collected from Mondego River; Portugal.	The catalysts had no significant effect on diclofenac removal when compared with single ozonation. However, both catalysts increased the COD removal per mg of ozone applied.	(Martins et al., 2015)
n.a.	Bench-scale photolysis; 150 W MP Hg lamp, which emits radiation between 200 and 450 nm; Municipal wastewater of secondary effluent of a biological WWTP; Portugal.	The degradation rate constants obtained for diclofenac in a filtered wastewater matrix were lower than in a pure water matrix.	(Salgado et al., 2013)
2.5 mg L^{-1} (spiked wastewater)	Lab-scale TiO_2 photocatalysis; 125 W black light fluorescent lamp (300–420 nm); Catalyst load: 0.2–0.8 g L^{-1}; Urban WWTP effluent.	TiO_2 photocatalysis showed a high removal of diclofenac (≈98%).	(Rizzo et al., 2009)
100 μg L^{-1} (spiked wastewater)	Solar photo-Fenton in a pilot-scale solar CPC reactor; H_2O_2 dose = 0–50 mg L^{-1}; Fe^{2+} = 5 mg L^{-1}; Municipal wastewater.	Diclofenac was completely removed (<LOQ) after 34 min.	(Klamerth et al., 2010)
464.8 ± 64.7 ng L^{-1}	Lab-scale photolytic ozonation, ozonation and photocatalysis; O_3: 50 g Nm^{-3}; O_3 flow rate: 150 Ncm^3 min^{-1}; MP Hg vapor lamp (UV/Vis λ > 300 nm); TiO_2 photocatalyst: 0.5 g L^{-1} load; Urban wastewater from the secondary treatment of a WWTP; North of Portugal.	For all processes, the complete removal was achieved.	(Moreira et al., 2015),
13.5–52.0 μg L^{-1} (spiked wastewater)	Lab-scale ozonation; O_3: 5.5–8.5 mg L^{-1}; O_3 flow rate: 0.39 Ndm^3 min^{-1}; Urban wastewater samples from the secondary clarifier of two WWTPs from West-Alcalá and Alcázar de San Juan; Spain.	High diclofenac removal (>90%) was observed.	(Rodriguez et al., 2012)
970–2300 ng L^{-1}	Pilot-scale ozonation plant; O_3: 86–153 g Nm^{-3}; O_3 consumption: 0.6 and 0.9 g O_3 g DOC_0^{-1}; Municipal wastewater; Austria.	The application of 0.6 g O_3 g DOC_0^{-1} increased the removal of diclofenac (to values < LOQ).	(Schaar et al., 2010)
5–20 mg L^{-1} (spiked wastewater)	UV-A/TiO_2 photocatalysis: 9 W lamp; Catalyst load: 50–1600 mg L^{-1}; H_2O_2 = 0.07–1.4 mM; Treated municipal effluent from Limassol; Cyprus.	UV-A/TiO_2 was efficient for the degradation and mineralization of diclofenac in treated municipal effluents.	(Achilleos et al., 2010)

BAC, Biological activated carbon; CAS, conventional activated sludge; CAST, cyclic activated sludge technology; COD, chemical oxygen demand; CPC, compound parabolic collector; DOC, dissolved organic carbon; GAC, granular activated carbon; HRT, hydraulic retention time; LOQ, limit of quantification; LP, low pressure; MP, medium pressure; MBR, membrane biological reactor; MF, microfiltration; n.a., not available; n.d., not detected; NF, nanofiltration; OD, oxidation ditch; RO, reverse osmosis; SBR, Sequential Batch Reactor; TOC, total organic carbon; UF, ultrafiltration, WWTP, wastewater treatment plant.

combined with photolysis and/or photocatalysis, has been widely investigated showing a high performance for diclofenac removal. Overall, diclofenac is the second most studied substance of the watch list in the last 10 years, employing all the types of processes (Fig. 2).

2.3. 2,6-di-tert-butyl-4-methylphenol

The anti-oxidant 2,6-di-tert-butyl-4-methylphenol (BHT) has been used as a common anti-oxidant to preserve and stabilize the freshness, nutritive value, flavor and color of food and animal feed products, since the 1950s (Fries and Püttmann, 2002; Tombesi and Freije, 2002). BHT can also improve the stability of pharmaceuticals and cosmetics and increase the durability of rubber and plastics. Approximately 40 countries allow the use of BHT as a direct or indirect food additive (Fries and Püttmann, 2002). The use of BHT as a food additive does not appear to pose a public health risk. However, in the natural environment, BHT is degraded biologically to 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO), reported by generating peroxides in mice and rats and inducing cellular DNA damage (Fries and Püttmann, 2004). The occurrence of the anti-oxidant BHT in the aquatic environment has been demonstrated (Table 1), with studies conducted in Sweden (Bendz et al., 2005) and USA (Benotti et al., 2009) reporting the presence of BHT in surface water up to 620 ng L^{-1} and 49 ng L^{-1}, respectively. In other studies, BHT was detected in wastewater (between 22 and 258 ng L^{-1}) (Fries and Püttmann, 2004), whereas higher values were quantified in surface water (up to 1560 ng L^{-1}) and groundwater (up to 2156 ng L^{-1}) in Greece and Germany (Fries and Püttmann, 2002, 2004; Papadopoulou-Mourkidou et al., 2001).

Table 4
Studies dealing with removal of 2-ethylhexyl-4-methoxycinnamate (EHMC). Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Initial EHMC concentration	Treatment and sampling conditions	Concluding remarks	Reference
n.a.	5 WWTPs equipped with different treatment levels: preliminary screening, primary sedimentation, secondary treatment; UV-disinfection, chlorination, sand filtration and RO; 24 h composite or grab samples, depending on the plant; Hong Kong.	Removal of EHMC varied depending on the season in the range of 30–50%.	(Tsui et al., 2014)
Up to 234 ng L^{-1}	Ozonation: nominal capacity of 3000 m^3 h^{-1}; 5 L glass reactor operating in semi-batch mode, at a temperature of 25 °C and pH 8.5; WWTP located in Madrid, which treats a mixture of domestic and industrial wastewater.	The UV filter EHMC was completely refractory to ozone.	(Rosal et al., 2010)
23.6 ± 8.1 ng L^{-1}	UV, visible light, photocatalysis (visible light), O_3; 15 W LP Hg vapor lamp (λ_{max} = 254 nm); Xe 150 Xe-arc lamp with spectral emission in the visible region; Photocatalyst: ceria-doped TiO_2 at 0.5 g L^{-1}; O_3: 22 g Nm^{-3}; Mixture of domestic and industrial wastewater from the secondary clarifier of a 3000 m^3 h^{-1} WWTP placed in Alcalá de Henares; Madrid, Spain	EHMC was removed up to 50% after 15 min of UV-photolysis, mainly during the first 2 min. Visible light Xe-lamp driven photolysis led to an EHMC removal near 20% after 15 min. Removal was not enhanced, applying visible light Ce/TiO_2 photocatalysis. EHMC was not significantly removed by ozone.	(Santiago-Morales et al., 2013)

n.a., not available; LP, low pressure; RO, reverse osmosis; WWTP, wastewater treatment plant.

Additional data are needed to support assessments of human health risks associated with the exposure to this compound in the aquatic environment and to establish possible pathways of removal in aquatic systems. Considering the lack of studies on its removal (Fig. 2), it is urgent to study its elimination from water matrices.

2.4. 2-ethylhexyl-4-methoxycinnamate

Organic UV filters are chemical filters used in many personal care products, alone or in formulations containing a physical filter like ZnO or TiO_2 nanoparticles (Kaiser et al., 2012a). Their occurrence in the environment has been described in several papers that have been given a great attention to the aqueous matrices. These CECs reach the environment by two pathways, wash off from skin or through wastewater or swimming pool water. Organic UV filters are likely to be present in sediments (Kaiser et al., 2012b), where they might induce toxicological effects. Their known estrogenic effects on biota and humans was recently reviewed by Ramos et al. (2015), who highlighted not only the recognized *in vivo* and *in vitro* estrogenic activity to fish and mammals, but also other non-estrogenic hormonal targets in such organisms. The UV filter 2-ethylhexyl-4-methoxycinnamate (EHMC), included in the watch list for Union-wide monitoring, is an EDC and was reported at concentrations levels of hundreds of μg kg^{-1} in diverse organisms including macroinvertebrates and fish (Kaiser et al., 2012a). Lake and rivers sediments are well characterized regarding this contaminant, which is usually present at μg kg^{-1} levels (Kaiser et al., 2012a, 2012b; Langford et al., 2015). This compound was also detected up to 260 ng L^{-1} in tap water from Barcelona (Spain), one of the most frequently found of a group of five UV filters included in that study (Díaz-Cruz et al., 2012). Little is known about the removal of EHMC in the aquatic environment (Table 4, Fig. 2), only three studies reporting its removal. The removal of EHMC varied (30–50%), depending on the respective treatment applied at the WWTP and season (Tsui et al., 2014). This UV filter was refractory to ozonation, without any degradation being observed after 15 min (Rosal et al., 2010) or after 22 min, but could be removed by UV treatment (Santiago-Morales et al., 2013).

2.5. Macrolide antibiotics

Among the different classes of pharmaceuticals present in the environment, particular importance has been given to antibiotics, which are the most often discussed pharmaceuticals due to their potential role in the development of resistant mechanisms by bacteria (Xekoukoulotakis et al., 2010). Macrolide antibiotics, such as clarithromycin, azithromycin and erythromycin are widely used in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing or treating serious infections induced by pneumococci, staphylococci and streptococci (Lange et al., 2006; Xekoukoulotakis et al., 2010). The conventional municipal WWTPs do not fully eliminate these drugs, which are found in WWTP effluents (Lange et al., 2006) and in other aquatic systems (Gracia-Lor et al., 2011; Hoa et al., 2011; Lopez-Serna et al., 2013; Tong et al., 2014). These antibiotics have been extensively detected in wastewater, surface and groundwater in several countries at ng L^{-1} levels, with some studies reporting antibiotics at several μg L^{-1} (Table 1). For instance, azithromycin, erythromycin and clarithromycin were found in effluents of a WWTP in Slovakia at ng L^{-1} levels (Birosova et al., 2014). Clarithromycin and erythromycin were reported in surface water in Spain and Vietnam (Gracia-Lor et al., 2011; Hoa et al., 2011). Lopez-Serna et al. (2013) also reported the occurrence of the three macrolide antibiotics in groundwater (Spain) in the range 1.6–1620 ng L^{-1}.

Elimination of this class of antibiotics in the environment has been reported in the last decade, for all the types of processes here discussed (Fig. 2). Biological treatments occurring at WWTPs are normally insufficient to remove such recalcitrant pharmaceuticals (Table 5). The combination of biological with advanced treatments can be fruitful, as example MBR and RO led to elimination rates above 99% (Dolar et al., 2012) for the macrolides included in the watch list. Hence, advanced methods should be applied to deal with this environmental concern. Membrane technologies alone are not enough for the complete removal of such micropollutants (Table 5). Studies reported in the literature employing AOPs for the removal of this type of antibiotics in environmental samples are focused only on photocatalysis (Bernabeu et al., 2011; Xekoukoulotakis et al., 2010), revealing a lack of knowledge regarding the efficiency of other AOPs to remove this compounds in real scenarios. In fact, some studies with other AOPs were already published considering these compounds, but not using real matrices and, thus, they are out of the scope of the present review; for instance, UV/TiO_2 and ozonation were studied for the removal of clarithromycin and erythromycin, ozonation apparently being more effective for the parent compounds (complete degradation), while catalytic ozonation improving the mineralization of erythromycin

Table 5
Studies dealing with removal of macrolides (azithromycin, clarithromycin and erythromycin). Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
Erythromycin	2600 ng L^{-1} (after primary clarifier)	WWTP with CAS-MF-GAC-ozonation; Average flow rate: 227,000 m^3 d^{-1}; 24 h composite samples; Gwinnett County, GA, USA.	Erythromycin was recalcitrant to the biological treatment, but it was removed at an extent of 74% by GAC adsorption. Ozonation oxidized the remaining compounds by more than 60%.	(Yang et al., 2011)
Azithromycin Clarithromycin Erythromycin	118 ng L^{-1} 2020 ng L^{-1} 49 ng L^{-1}	MBR coupled to RO; Coastal WWTP Castell-Platja d'Aro, Spain.	The combination of MBR and RO led to removal rates above 99% for the target pollutants, with RO showing removal rates always higher than 99%. MBR removed 75–85% of the antibiotics, and the remaining non-degraded macrolides were removed by RO.	(Dolar et al., 2012)
Azithromycin Clarithromycin Erythromycin	232.5–876.9 ng L^{-1} >0.1 μg L^{-1} 4.11–42.01 ng L^{-1}	WWTP1 with secondary treatment (anaerobic/anoxic/oxic (A/A/O) treatment + moving bed biofilm reactor (MBBR) + secondary clarifier) and tertiary treatment (rotary fiber disc filters (RFDFs)). WWTP2 with secondary treatment (C-Orbal OD process + secondary clarifier) and tertiary treatment (UV disinfection and RFDFs); 24 h composite wastewater samples at different sampling points; Wuxi City, Jiangsu Province, China.	Removal efficiencies were generally higher in the WWTP1 employing the A/A/O-MBBR process than those obtained by the conventional WWTP2 adopting the C-Orbal OD process, except for clarithromycin. The type of biodegradation process was the predominant factor in this study, the better performance being obtained with WWTP1.	(Yuan et al., 2015)
Erythromycin	0.2 μg L^{-1}	1 WWTP serving 500,000 population equivalent, with an industrial inlet lower than 10% of the total load; with biological treatment, final clarification and tertiary treatment by phosphorus precipitation; 2 h composite influent and effluent samples, during 24 h Nancy, France.	No elimination was reported for erythromycin in the liquid phase. This antibiotic was also not adsorbed on the particulate matter or the sludge.	(Pasquini et al., 2014)
Azithromycin, Clarithromycin Erythromycin	406–611 ng L^{-1} 785–941 ng L^{-1} 164–210 ng L^{-1}	1 WWTP equipped with MBR and UV treatment, serving 24,000 inhabitants; Membrane modules made of hollow-fiber membranes; Average flow rates: 8800 m^3 d^{-1}; 24 h composite influent and effluent samples; Canada.	The degraded fraction of azithromycin was approximately 49% and that of erythromycin was negligible. Clarithromycin was not removed during MBR treatment, being even formed during treatment.	(Kim et al., 2014)
Azithromycin	up to 719 ng L^{-1}	15 WWTPs, designed for 6850 to 756,000 population equivalents; Average flow rates: between 349 and 140,000 m^3 d^{-1}; Discharge points: Portuguese rivers and Atlantic Ocean; 24 h composite influent and effluent samples; Portugal.	The concentration of azithromycin in the effluent was up to 200 ng L^{-1}, with a mean removal of 94.6%.	(Pereira et al., 2015)
Clarithromycin Erythromycin	up to 0.33 μg L^{-1} up to 0.13 μg L^{-1}	4 WWTPs impacted by effluents from mid-size hospitals (250–600 beds) corresponding to a WWTP inflow ranging between 1 and 30%; Average flow rates: between 1300 and 103,000 m^3 d^{-1}; WWTP 1/3 – SBR and UV-tertiary treatment; WWTP 2 – CAS; WWTP 4 – OD activated sludge process; 24 h composite samples; New York, USA.	WWTP 2 and 4, employing CAS or OD activated sludge process were more efficient than the others for the removal of clarithromycin and erythromycin.	(Oliveira et al., 2015)
Azithromycin Erythromycin	up to 661.9 ng L^{-1} up to 338.2 ng L^{-1}	2 municipal WWTPs with anaerobic/anoxic/oxic (A/A/O) activated sludge process, one of them employing a cyclic activated sludge technology (CAST) whereas the other having an OD; Chongqing, China.	WWTP using the OD biological treatment process had the higher efficiency to remove the macrolide antibiotics.	(Yan et al., 2014a)
Clarithromycin Erythromycin	n.a.	MBR pilot plant in continuous operation ca. 1% of diurnal hospital sewage; 2 h composite influent and effluent samples; Luxembourg.	Erythromycin was almost totally removed by MBR, while clarithromycin was removed at extents between 40 and 50%. UV was evaluated as post-treatment, with improved degradation obtained by adding H_2O_2.	(Kohler et al., 2012)
Azithromycin Erythromycin	330.27–376.5 ng L^{-1} 238.6–275.4 ng L^{-1}	4th largest WWTP in China, serving 1,540,000 equivalent inhabitants and treating 600,000 m^3 d^{-1}. CAST (anaerobic/anoxic/aerobic (A/A/A) treatment secondary clarifier) followed by chlorination; Grab samples collected according to the residence time in each treatment; Southwest China.	The removal obtained after secondary treatment was 75.6% for azithromycin and 42.8% for erythromycin. Chlorination led to a reduction of 8.0% for azithromycin. Erythromycin was not removed during chlorination.	(Yan et al., 2014b)
Azithromycin Clarithromycin	160–279 ng L^{-1} 1129–1570 ng L^{-1}	Samples were collected in winter from four WWTP located in Kyoto and Shiga prefecture (Japan); WWTPs employed a wide variety of secondary treatment processes: CAS; anaerobic/anoxic/aerobic (A/A/A) and anoxic/aerobic (A/A).	Removal efficiency of the macrolide antibiotics were higher using CAS (39–83%) and A/A (34–86%) processes than using A/A/A (41–53%) process.	(Ghosh et al., 2009)

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Table 5 (*continued*)

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
Clarithromycin	up to 27.4 μg L^{-1}	MBR followed by NF and RO; Membrane surface of NF and RO modules: 2.5 m^2; Operation: cross flow membranes; NF/RO modules: maximum flux between 20 and 36 L m^{-2} h^{-1}; Hospital wastewater, Germany.	Clarithromycin was completely removed by RO and NF treatments (<LOQ).	(Beier et al., 2010)
Erythromycin Clarithromycin	337 ± 19.2 ng L^{-1} 377 ± 30.9 ng L^{-1}	Pilot-scale UF and RO treatments in sequence; UF flux range of 25–47 L m^{-2} h^{-1}; RO flux range of 22–31 L m^{-2} h^{-1}; Municipal WWTP; Tel-Aviv, Israel.	High removal rates were achieved after RO (99% for macrolides antibiotics).	(Sahar et al., 2011)
Azithromycin Erythromycin	187–367 ng L^{-1} 180–191 ng L^{-1}	Pilot-scale MF followed by RO; MF: flow rate of 2 m^3 h^{-1} and flux of 323 L m^{-2} h^{-1}; Residence time 3 min; RO: flow rate of 1 m^3 h^{-1} and permeate flux of 34 L m^{-2} h^{-1}; Residence time 50 min; Municipal treated effluent; Girona, Spain.	High removals were observed for these pharmaceuticals compounds. Even though the pharmaceuticals were present in the MF permeate at levels higher than 100 ng L^{-1}, RO filtration reduced their loads to the low ng L^{-1} range or to below the method LOQ.	(Rodriguez-Mozaz et al., 2015)
Clarithromycin	77 ng L^{-1}	2-L reactors at 25 °C; UV/H_2O_2: 3 LP Hg lamps (λ_{max} = 254 nm); H_2O_2 consumed ranged from 0.01 to 0.90 mg H_2O_2 mg TOC^{-1}; RO concentrates from a municipal WWTP in a coastal area of Catalonia, Spain.	Clarithromycin was completely removed by ozonation, but it was recalcitrant to UV (removal of 60%) and UV/H_2O_2 (removal of almost 80%).	(Justo et al., 2014)
Clarithromycin	46 ng L^{-1}	Biological activated carbon (BAC) process to treat municipal wastewater RO concentrate; Lab scale during 320 days of operation; BAC, combined UV/UV/H_2O_2–BAC and ozone–BAC.	70% of clarithromycin was removed by the BAC filter. Pretreatment of RO brine with UV/H_2O_2 or ozonation led to the removal of the pharmaceutical.	(Justo et al., 2015)
Clarithromycin Erythromycin	<750 ng L^{-1}	Pilot-scale photocatalysis: CPC plant under natural solar irradiation; TiO_2 load: 20 mg L^{-1}; Municipal effluents collected downstream of the secondary biological treatment of El Ejido WWTP; Almería, Spain.	Using a low TiO_2 load (29.2 mm photoreactor), the treatment was not effective due to the slow reaction rate; 85% of the pollutants were degraded after 480 min. Increasing the light-path of the reactor, the performance was enhanced (90% of the pollutants removed after 300 min).	(Prieto-Rodriguez et al., 2012)
Clarithromycin Erythromycin	≈0.0275 μg L^{-1} <0.05 μg L^{-1}	Pilot-scale photocatalysis: Solardetox Acadus-2006 CPCs; 3.0 m^2 irradiated surface; 24 L irradiated volume; TiO_2 load: 0.2 g L^{-1}; Wastewater.	Removal was high for all the compounds after 3 h of treatment (bellow LOQ).	(Bernabeu et al., 2011)
Azithromycin	1653.84 ng L^{-1}	O_3/H_2O_2; O_3: 24 g O_3 Nm^{-3}; Gas flow: 0.36 Nm^3 h^{-1}; H_2O_2: 0.15 mL of a 30% (w/v) solution; Wastewater from the secondary clarifier from a sewage treatment plant of Alcalá de Henares; Madrid, Spain.	The removal for azithromycin was 89.6% after 5 min.	(Rodríguez et al., 2011)
Clarithromycin	up to 0.1 μg L^{-1}	Bench-scale ozonation: at pH 8.5 (original) and at pH 7.0 (adjusted by adding H_2SO_4); O_3 doses (g O_3/g DOC): 0.25, 0.5, 1.0, and 1.5; H_2O_2/O_3 molar ratio = 0, 0.25, and 0.5; 24 h composite samples of hospital wastewater effluents from a pilot MBR; Baden, Switzerland.	The elimination of clarithromycin was efficient when the ratio of O_3/DOC was higher than 0.5. The LOQ was achieved and the removal was higher than 92% at both pH conditions. The average removal was 80% using a ratio of O_3/DOC of 0.25.	(Lee et al., 2014)
Azithromycin Clarithromycin Erythromycin	n.d. 228 ng L^{-1} 150 ng L^{-1}	Ozonation of secondary effluent; O_3: 3 mg L^{-1}; Samples were collected from a municipal sewage treatment plant; Tokyo.	The removal efficiencies of all the target macrolides antibiotics were up to 80%.	(Nakada et al., 2007)
Clarithromycin	363–469 ng L^{-1}	Lab-scale UV, UV/H_2O_2, solar irradiation, Fenton, solar photo-Fenton; UV-C irradiation (λ_{max} = 254 nm); H_2O_2: 25 mg L^{-1}; Fenton: 25 mg H_2O_2 L^{-1} and 5 mg Fe^{2+} L^{-1}; Photo-Fenton: 25 mg H_2O_2 L^{-1} and 5 mg Fe^{2+} L^{-1}; Municipal wastewater from Vidy WWTP; Lausanne, Switzerland.	From the five different treatments applied, only the UV-based processes were able to remove 80% of clarithromycin. After 30 min of treatment, the oxidation was significant, verified by COD and TOC removals. For the cases of solar light, Fenton and photo-Fenton processes, the degradation rates were lower.	(Giannakis et al., 2015)
Clarithromycin	469 ng L^{-1}	Solar Fenton treatment (natural solar driven oxidation) in a pilot-scale CPC plant; H_2O_2: 50 mg L^{-1}; Fe^{2+}: 5 mg L^{-1}; Municipal wastewater from the El Ejido municipal WWTP; Almería, Spain.	Clarithromycin was completely degraded, applying photolytic and solar Fenton experiments, with a removal of 77% at the end of the treatment time (250 min), when present at low concentrations and at low Fenton reagent dosages.	(Karaolia et al., 2014)
Erythromycin	170 ng L^{-1}	Pilot-scale ozonation plant; O_3: 86 –153 g Nm^{-3}; O_3 consumption: 0.6 and 0.9 g O_3 g DOC_0^{-1}; Wastewater; Austria.	The application of 0.6 g O_3 g DOC^{-1} increased the removal of erythromycin (to values < LOQ).	(Schaar et al., 2010)

Table 5 (*continued*)

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
Azithromycin Clarithromycin Erythromycin	139.9 ± 6.2 ng L^{-1} 116.4 ± 2.7 ng L^{-1} 27.0 ± 2.5 ng L^{-1}	Lab-scale photolytic ozonation, ozonation and photocatalysis; O_3: 50 g Nm^{-3}; O_3 flow rate: 150 Ncm^3 min^{-1}; MP mercury vapor lamp (UV/Vis λ > 300 nm); TiO_2 photocatalyst: 0.5 g L^{-1} load; Urban wastewater from the secondary treatment of a WWTP; North of Portugal.	It was verified a complete removal by photocatalytic ozonation for all macrolide antibiotics, while by ozonation only erythromycin was totally eliminated. Photocatalysis was the less efficient process in study.	(Moreira et al., 2015)
Erythromycin	0.7–0.9 μg L^{-1} (spiked wastewater)	Lab-scale ozonation; O_3: 5.5–8.5 mg L^{-1}; O_3 flow rate: 0.39 Ndm^3 min^{-1}; Urban wastewater samples (spiked) from the secondary clarifier of two treatment plants from West-Alcalá and Alcázar de San Juan; Spain.	High removal of erythromycin (>90%) was observed for both wastewaters studied.	(Rodriguez et al., 2012)

BAC, Biological activated carbon; CAS, conventional activated sludge; CAST, cyclic activated sludge technology; CPC, compound parabolic collector; DOC, dissolved organic carbon; GAC, granular activated carbon; LOQ, limit of quantification; MBR, membrane biological reactor; MF, Microfiltration; n.a., not available; n.d., not detected; OD, oxidation ditch; RFDFs, rotary fiber disc filters; RO, Reverse osmosis; SBR, sequential batch reactor; UF, ultrafiltration; WWTP, wastewater treatment plant.

(Derrouiche et al., 2013; Lange et al., 2006).

2.6. Methiocarb

Regarding pesticides, their use plays an important role in harvest quality and food protection, providing enormous benefits to increase production, as pests and diseases are usually responsible to damage up to one-third of crops (Herrero-Hernández et al., 2013). As consequence of massive global consumption, pesticides and their degradation products spread through the environment and can contaminate water resources. Surface and groundwater located in intensive agricultural areas are more susceptible to pesticide contamination, which is a major concern if the water is used for human consumption (Masia et al., 2013). The impact of these contaminants in the environment and to the wildlife is demonstrated by several injurious effects, including the enhancement of the incidence of cancer, birth defects, genetic mutations, or other problems such as damage in the liver or in the central nervous system (Dabrowski et al., 2014). The occurrence of pesticides in aquatic compartments and their possible effects to public health are a topic of considerable environmental interest.

Methiocarb (also known as mercaptodimethur, mesurol, 3,5-dimethyl-4-(methylthio)phenyl methylcarbamate) is one of the most commonly used carbamate pesticides worldwide. This pesticide has been applied since 1960s for a variety of invertebrate pests and also as a bird repellent on fruit crops (Altinok et al., 2006; Qiang et al., 2014). The detected concentrations of methiocarb in wastewater and groundwater are generally low (Table 1); however, it poses a serious health threat to aquatic life and humans considering its high toxicity (Qiang et al., 2014). A negative removal of methiocarb was reported in a Spanish sewage treatment plant (Table 6), probably due to the limitations on the sampling procedure, where both HRT (24–72 h) and SRT (7.5–25 days) were not taken into consideration, consequently higher concentrations were found in the effluents than in influents (Campo et al., 2013). Recent studies related to the removal of this compound by advanced treatment options were not found in the literature.

2.7. Neonicotinoids

In the last decade, the neonicotinoid group of insecticides has been one of the most broadly adopted conventional management tools to deal with insect pests of annual and perennial cropping systems. Benefits of the neonicotinoids include flexibility of application, a wide range of active ingredients and broad spectrum activity (Huseth and Groves, 2014; Morrissey et al., 2015). This group includes imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid, which are extremely toxic to all aquatic arthropods, except water fleas (Sánchez-Bayo and Hyne, 2014). However, as a result of structural differences in the polypeptide subunit containing the neonicotinoid-binding region of the vertebrates' nicotinic acetylcholine receptors, neonicotinoids pose a relatively low risk to fish and mammals (Sánchez-Bayo and Hyne, 2014). Neonicotinoids are systemic insecticides and are applied as seed dressings by sprays, owing to their solubility in water. Therefore, the main sources of this class of herbicides in the environment are the runoff from agriculture areas and leaching into groundwater, with the consequent subsurface discharge into wetlands and other surface water (Morrissey et al., 2015). As a result of their high water solubility and persistence in soil, neonicotinoids cause a threat for water contamination, mainly after storm events that produce runoff pulses (Sánchez-Bayo and Hyne, 2014). Other sources of these compounds are soluble or insoluble fractions transported via snowmelt, decay of treated plants in water bodies, and deposition of treated seeds or soil into water bodies (Morrissey et al., 2015). Recent studies from Spain, Portugal, USA, Australia and other countries (Table 1) have confirmed the occurrence of this group of pesticides in the aquatic ecosystems (Campo et al., 2013; Chau et al., 2015; da Rocha et al., 2015; Gonzalez-Rey et al., 2015; Masiá et al., 2013; Papadakis et al., 2015; Sánchez-Bayo and Hyne, 2014).

There is a lack of literature concerning the removal of this class of pesticides in the environment (Fig. 2). The majority of the reports refers to the performance of AOPs, dealing with their degradation at laboratory or pilot-scale conditions and mostly using spiked water or spiked simulated water (Pena et al., 2011). Photolysis,

Table 6
Studies dealing with removal of methiocarb. Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Initial methiocarb concentration	Treatment and sampling conditions	Concluding remarks	Reference
3.77–5.74 ng L^{-1} (2010); 1.26 –105.31 ng L^{-1} (2011).	Sewage treatment plants monitored in the four River Basins of Ebro River; Spain.	The removal of methiocarb was negative. The higher concentrations in effluents than in influents were attributed to the sampling limitations: influent and effluent samples were collected at the same day, without considering the HRT (24–72 h) and SRT (7.5–25 days).	(Campo et al., 2013)

HRT, hydraulic retention time; SRT, sludge retention time.

Table 7
Studies dealing with removal of neonicotinoids (imidacloprid, thiacloprid, clothianidin and acetamiprid). Pollutants included in these studies that are out of the scope of 495/15/EU Decision are not discussed.

Compound	Initial concentration	Treatment and sampling conditions	Concluding remarks	Reference
Acetamiprid	<0.05 μg L^{-1}	Pilot-scale photocatalysis: Solardetox Acadus-2006 CPCs 3.0 m^2 irradiated surface; 24 L of irradiated volume; TiO_2 load: 0.2 g L^{-1}; Water taken from the outlet of a WWTP from the South East of Spain.	High removal was obtained for all the emerging contaminants after 3 h of treatment (bellow LOQ).	(Bernabeu et al., 2011)
Thiacloprid	0.05 −0.38 mM (spiked spring water)	Photocatalysis: Lab-scale reactor operated in a circular 'closed-loop' mode; Six 18 W UV lamps (λ_{max} = 366 nm); ZnO load: 0.5–3.0 g L^{-1}; Thermal water collected from the spring of Kistelek, Hungary.	Very low degradation was verified by direct photolysis. A removal of 86.6% was observed for thiacloprid, with a ZnO load of 2 g L^{-1} and pH 6.8. The efficiency of the thiacloprid removal in filtered and un-filtered thermal water was about two times lower than from the distilled water, indicating that the removal was due to the dissolved substances.	(Abramović et al., 2013)
Thiacloprid	0.32 mM (spiked river water)	Lab-scale UV and UV/H_2O_2; 125 W HP Hg lamp (emission bands λ = 304, 314, 335, 366 nm) (λ_{max} = 366 nm); H_2O_2 concentration: 0–162 mM; pH: 2.8–9; Spiked water from Begej river at Itebej, Serbia.	The removal rate of thiacloprid was influenced by the presence of HCO_3^-. Very low degradation rates were observed for single UV and H_2O_2. High removal of thiacloprid was achieved after 120 min of UV/H_2O_2. The removal rate for natural water was lower compared with distilled water (45 mM H_2O_2) at pH 8.2. However, the removal in natural water adjusted at pH 2.8 was higher than in distilled water due to the naturally occurring photosensitizers, i.e. dissolved organic matter.	(Abramovic et al., 2010)
Clothianidin	n.a.	Sulfate radical based homogeneous photo-Fenton involving peroxymonosulfate as an oxidant, ferrous iron (Fe(II)) as a catalyst and simulated solar irradiation as a light source; Biologically treated domestic wastewater effluents.	PMS/Fe(II)/UV–Vis advanced oxidation system using simulated solar irradiation has demonstrated better kinetic performances over TiO_2/UV–Vis system for clothianidin.	(Ahmed et al., 2014)
Acetamiprid	100 μg L^{-1} (spiked wastewater)	Pilot-scale photocatalysis: CPC; Wastewater of Almería, Spain.	The removal of acetamiprid was poor in the wastewater matrix.	(Jiménez et al., 2015)
Imidacloprid	60 mg L^{-1}	Pilot-scale CPC plant (60 L); Fe(III)-EDDS as complexing agent; Spiked tap water from the groundwater well of Plataforma Solar de Almería, Spain.	Photolysis of the complexing agent generated radical species able to act independently of carbonate scavengers that are present in natural water.	(Papoutsakis et al., 2015)

CPC, compound parabolic collector; EDDS, ethylenediamine-N,N'-dissucinic acid; HP, high pressure; LOQ, limit of quantification; n.a., not available; WWTP, wastewater treatment plant.

photocatalysis and photo-Fenton were applied to study the removal of these compounds from water, photocatalysis being the most applied (Table 7). Studies dealing with real water and other treatment processes should be performed to bring a more realistic overview of the elimination of this group of pesticides. Some other studies with these substances were already published, but not using real matrices and, therefore, they are out of the scope of the present review; for instance the degradation of imidacloprid (Peng et al., 2015; Tang et al., 2011; Wang et al., 2014; Zabar et al., 2012; Zarora et al., 2010), thiamethoxam (Mir et al., 2013; Šojić et al., 2012; Zabar et al., 2012), clothianidin (Zabar et al., 2012) and acetamiprid (Mitsika et al., 2013) were studied with photo-assisted and ozonation processes.

2.8. Oxadiazon

The oxadiazole herbicide oxadiazon [5-tert-butyl-3-(2,4-dichloro-5-propan-2-yloxyphenyl)-1,3,4-oxadiazol-2-one] has been habitually used to combat weeds in various agricultural crops such as rice, cotton, soybean, potato, peanut and onion. Oxadiazon is an organic contaminant causing a great environmental concern due to its relatively long half-life (Rahman, 2010). Previous studies on the leaching of oxadiazon in soils indicated that, the strong adsorption of the herbicide to soils reduces the displacement towards the sub-surface layers (Pinilla et al., 2008). However, oxadiazon was found in surface water in Canada (Table 1) at ng L^{-1} levels (Furtula et al., 2006). In contrast, the removal of oxadiazon in aquatic matrices is still unknown.

2.9. Triallate

Triallate (S-2,3,3-trichloroallyl di-isopropyl thiocarbamate) is a carbamothioate herbicide widely used to control annual and perennial grasses in wheat, barley, legumes and a number of other crops (D'Orazio et al., 1999; Volpe et al., 2004). This pesticide is often used in mixture with other chemicals (chloridazon, isoproturon, metoxuron) and its use, in the last decades, has exceeded 500 tons per year in some European countries (Volpe et al., 2004). Triallate has a high hydrophobic partitioning (Hornsby et al., 1996), therefore it adsorbs to loam and clay soils and is not readily dissolved in water (Bernal et al., 1996). This information indicates that this herbicide is not likely to move through the soil, even though it has a long soil half-life (82 days). Nevertheless, if there is significant moisture and/or low levels of organic matter in the soil, triallate may become desorbed from soil particles (D'Orazio et al., 1999). Leaching and consequent groundwater contamination would be possible in such situations, but Environmental Protection Agency (EPA) suggests that triallate leaching does not cause a threat to the environment, since it is usually used where the water table is relatively low (Kamrin, 1997). A lack of knowledge exists about its occurrence and removal in the aquatic environment due to its chemical nature.

3. Conclusions

Despite the considerable amount of studies reported on the occurrence and removal of E1, E2, EE2, diclofenac and macrolide antibiotics (azithromycin, clarithromycin and erythromycin), a lack

of knowledge exists concerning the pesticides (methiocarb, neonicotinoids, oxadiazon and triallate), the UV filter (EHMC) and the antioxidant (2,6-di-tert-butyl-4-methylphenol), which are included in the watch list of Decision 2015/495/EU for European Union monitoring. Thus, more investigation is needed regarding the occurrence and removal of neonicotinoids, EHMC and 2,6-di-tert-butyl-4-methylphenol and the performance of different treatments to remove the substances included in the watch list under realistic conditions. These compounds are usually present at residual concentrations, as mixtures in the different environmental compartments (e.g., municipal wastewater, surface water, groundwater, solid matrices) and comprehensive works considering it are scarce. As shown by different studies, the efficiency of the treatment processes can decrease considerably when realistic water matrices are used instead of simulated ones. For example, the presence of carbonates and bicarbonates can decrease the efficiency of AOPs, principally due to competition by $HO^{\bullet}$. Since multiple factors can affect the efficiency of the treatments, experiments should be performed as close as possible to the real conditions. Additionally, the formation of intermediates should be attempted in this type of studies, considering that the produced by-products might be more toxic and/or persistent than the parent compounds. Toxicological studies are needed to determine the deleterious effects on the ecosystems and human health of parent compounds and by-products formed in real matrices. Considering the scale up of the treatment option, these processes can be expensive both in the implementation and maintenance, therefore it is of major importance to perform cost effectiveness analysis for each of them under a common base of comparison.

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Cosmetics as a potential source of environmental contamination in the UK

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Chemicals of emerging concern (CECs) are frequently used in cosmetic formulations and can potentially reach the environment at concentrations that may cause harm. A methodology was developed to assess over 120 chemicals assembled from product ingredient listings to identify and validate potential CECs in cosmetics, based on Annex XIII of REACH legislation. Ten potential CECs were identified: polydimethylsiloxane, butylated hydroxylanisole (BHA), butylated hydroxytoluene, triclosan, nano titanium dioxide, nano zinc oxide, butylparaben, diethyl phthalate, octinoxate methoxycinnamate and benzophenone. These chemicals were quantified based on their consumption and concentrations in cosmetics and percentage market penetration. The initial predicted environmental concentrations ($PEC_{initial}$) were estimated to determine their exposure to the environment. With the exception of BHA, the $PEC_{initial}$ highlighted levels of exposure to the environment that triggered the need for further investigation of the chemicals. These chemicals were linked to cosmetics to highlight products with the potential to cause environmental harm. Skin care products had the highest quantities of CECs, with titanium dioxide and zinc oxide nanomaterials being dominant potential contaminants. Further research is required to assess the exposure pathways and fate of these chemicals to determine environmental risks associated with their use and disposal.

Keywords: cosmetics; personal care products; chemicals of emerging concern; predicted environmental concentrations

Introduction

Cosmetics referred to as 'personal care products' (PCPs) under the European Union Cosmetics Directive (76/768/EEC), as amended, which governs their manufacture, are defined as any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition. Hence, it is not surprising that these products cover everything from soap, shower gel, shampoo, shaving cream and toothpaste to make-up, skin creams and perfume. This directive is implemented in each member state via national legislation, which in the UK is the Cosmetic Products Safety Regulations 2004. The European cosmetics and toiletries market is estimated to have increased by 3.1% over the past four years [1], with the UK having the third fastest growth rate among its European counterparts. In 2008, annual sales figures for cosmetics and toiletries in the UK were estimated at approximately £7.3 billion with over 2.6 billion units of PCPs sold [1–3]. The frequency of demand and use for these products suggest that PCPs have become an essential part of our daily lives, and hence the chemicals present in these products should be assessed and adequately managed to avoid environmental contamination.

Cosmetics are an extraordinarily diverse group of products containing multiple chemical ingredients [4]. Several of the chemicals are used intensively in cosmetics and can be persistent, bioactive and exhibit accumulation potential as well as cause endocrine disruptions [4,5]. Although the acute toxicity of these chemicals is supposed to be low, some of these chemicals still show signs of being environmental concerns. For example, parabens can act as weak endocrine-disrupting substances [6,7], and triclosan can be converted, under certain conditions, into more toxic and persistent compounds, such as chlorophenols, dioxins or methyl triclosan [8]. Methylbenzylidene camphor (MBC), a sunscreen agent, was also detected in aquatic fauna in a German lake. The bioaccumulation factor was calculated as a quotient of the MBC concentration in the whole fish (21 pg/kg) versus that in the water (0.004 pg/L) and exceeded 5200, indicating high lipophilicity [4].

Despite this, the environmental fate and effects of cosmetics have been given limited attention in scientific work, and have frequently been assessed under the umbrella of pharmaceuticals and PCPs, despite the use of these chemicals as active ingredients or preservatives in PCPs and the continuous use of cosmetics as compared with drugs. Although the development and use of sophisticated tests

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for monitoring environmental contamination has revealed the presence of these types of potential contaminants at lower levels, down to nanograms per litre, limited or insufficient physicochemical and toxicological data have made it difficult to predict the levels and/or combination of chemicals that may cause environmental harm [5,9]. Some of the chemicals are therefore considered chemicals of emerging concern (CECs) since many of these substances have not been newly introduced to the environment, but have recently gained attention with the revelation of new aspects of their environmental occurrence, often because of improved analytical chemistry detection levels.

Legislation such as the European Union (EU) Cosmetics Directive (76/768/EEC), as amended, has been the principal driver promoting the safe use of cosmetics, by issuing precautionary bans on the use of certain chemicals in products. Its primary purpose is to protect human safety by controlling what may or may not be put in cosmetics. However, legislation governing the possible environmental consequences arising from the use of these products has been disregarded until recently. The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) initiative addresses both the safety and long-term environmental impacts of the ingredients used in cosmetics, by controlling the use of chemicals so exposure to every chemical can be demonstrated as safe for the environment [10,11]. However, the guidelines under REACH for assessing these CECs are extremely complex and entail a lengthy process.

Additionally, relaxed legislation governing the proper labelling of cosmetics (Contaminants (Hazard Information and Packaging for Supply) Regulations 2009), as well as marketing strategies of manufacturers to avoid listing the dangers/hazards of chemicals used and passing this information to their customers, has resulted in, and can further result in, numerous products containing chemicals of concern being placed on the market for use and ultimate disposal [12]. For example, current EU law does not require full disclosure of cosmetic ingredients; hence chemicals of concern such as diethyl phthalate (DEP) are currently masked under the name 'parfum' or are not included in ingredient lists on labels [13]. Research illustrated that, of the 34 products tested positive for phthalates, none listed phthalates as an ingredient on the label; this was even true for some products containing more than 10 g DEP/kg [13].

This paper describes aspects of a novel study to develop a methodology to identify, quantify and assess CECs in cosmetics. This is an essential initial step in considering the exposure of the environment to these chemicals, in order to determine whether consumption of cosmetics could be a possible means for CECs to reach the environment and hence to identify the need for further assessment. This paper also highlights gaps in the data, to facilitate an understanding of why some of these chemicals are emerging and can be potential contaminants.

Methodology

Cosmetics comprise a huge range of products, and to facilitate their study these products were grouped using the European Cosmetic, Toiletry and Perfumery (COPLIA) market's product categorization. The five product categories used in this study were chosen based on their use on, or application to the body; they included decorative cosmetics, skin care products, hair care products, toiletries and fragrances. These were subdivided into product groups using an illustrative list of cosmetic products outlined in the EU Cosmetics Directive (76/768/EEC) and its amendments (Table 1).

A list of chemicals used in cosmetics was compiled from Euromonitor's International Passport database on cosmetics and toiletries [3]. These chemicals were screened and sorted using the methodology developed below (Figure 1) to identify CECs with the potential to affect the environment. The methodology was developed based on REACH Annex XIII of EC Regulation No. 1907/2006 [10]. Annex XIII sets out the criteria for the identification of persistent, bioaccumulative and toxic (PBT) substances based on a technical guidance document on *Information Requirements and Chemical Safety Assessment* under Regulation (EC) No 793/93 and Directive 67/548/EEC, and on Regulation 850/2004 on persistent organic pollutants.

The screening process eliminated chemicals present in $<1\%$ of the products sampled (500 products) from the study because of insufficient use of these chemicals in PCPs. Also, if there was either a 0% or 100% gap of data on their physicochemical properties they were removed from the data set. A 0% data gap meant that all information with respect to environmental concerns for the chemical is known and the chemical can be assessed effectively in terms of safety. A chemical with a 100% data gap meant that data on this chemical were unknown, and hence it is impossible to adequately analyse this chemical in terms of potential environmental concerns. All other chemicals were assessed on the assumption that an existing data gap meant that the chemical is still in its emerging phase.

The chemicals were then sorted using PBT in full criteria outlined in Annex XIII of the REACH Regulation, 2006 (Table 2). Data were obtained from published journal articles, the Toxicology Data Network (TOXNET) database, approved under REACH and the United States Environmental Protection Agency (US EPA), as well as the Danish quantitative structure-activity relationship (QSAR) database using Syracuse Research EPIWIN software. Data were collected for over 120 chemicals found to be present in cosmetics to characterize these chemicals based on their PBT properties and to determine potential environmental concerns.

Table 1. Cosmetic classification based on the Cosmetics Directive 76/768/EEC and COLIPA product groups.

Product category	Product group	Product examples
Decorative cosmetics (products used for making up the face, eyes and lips)	Facial make-up products	Foundation/concealer, bronzer/blusher/highlighter & powder
	Lip products	Lip moisturizer, lip gloss, lipstick & lip pencil
	Nail products	Nail polish, nail treatment/strengtheners & nail polish remover
	Eye products	Mascara, eye shadow & eye liner
	Face care products	Day creams, night creams, masks, exfoliation, anti-wrinkle & anti-ageing creams for men and women, treatment series & anti-blackhead creams
Skin care (creams, lotions, gels and oils for the skin – hands, face, body & feet)	Face cleansers & toners	Liquid/cream/gel/bar cleansers & toners
	Body creams and lotions	General purpose body creams including hand lotion; baby care cream, lotion & milk; & firming/anti-cellulite body care
	Sun care products	Sun blocks, before or after sun care sticks/lotions, sun tanning & baby sun care products
	Shampoos and conditioners	Shampoos, conditioners, two-in-one (shampoo/conditioner), body shampoos & baby shampoos
Hair care (products used for cleansing, treating, conditioning and styling of hair)	Hair styling agents	Hair sprays, setting lotions & setting mousses, hair creams, brilliantine & hair gels
	Hair treatment agents	Colouring shampoos, bleaches & hair dyes; perms & relaxants
	Toilet soaps	Liquid hand wash lotions, bar soaps, dermatological soaps & baby soaps
	Oral care products	Toothpastes, mouth washes (before and after brushing) & other dental products which are in contact with the mouth (sprays etc.)
	Shaving products	Foams, creams, gels, soaps for pre- and post-shaving
Toiletries	Talcum powders	After-bath powders & baby powders
	Bath and shower products	Bath foams, salts, bath oils, bath & shower gels
	Deodorants and antiperspirants	Roll-ons, sprays, creams & sticks
	Depilatories	Creams & waxes for hair removal
Fragrance	Perfume, toilet waters and eau de cologne	Extracts, perfumes, eau de parfum, eau de toilette, eau de cologne, eau de lavande, parfum de toilette

All chemicals meeting the EU's PBT criteria for at least one property were considered to be CECs. Each chemical was quantified based on its consumption (per annum) in 53 different cosmetic products, using Equation (1):

$$TCcec\left(\frac{mg}{yr}\right) = \sum_{1}^{53} Ccec = \sum_{1}^{53}(P_{cec} \times Conc_{cec}) \quad (1)$$

where:

- TCecc (mg/yr) = total consumption of chemical of emerging concern in cosmetics,
- Cecc (mL) = consumption of chemical of emerging concern in cosmetics,
- P_{ecc} (no. of products sold containing the chemical of emerging concern) = per cent of products sampled containing chemical of emerging concern × no. of products sold,
- $Conc_{ecc}$ (concentration of chemical of emerging concern per unit of product) (μg/L) = chemical of emerging concern concentration in product × volume of product per unit.

Initial exposures of the environment to these chemicals were then estimated by calculating the initial predicted environmental concentrations ($PEC_{initial}$) for the CECs in surface water (Equation (2)).

$$PEC_{initial}(mg/L) = (DOSEai \times Fpen)/(WASTEWinhab \times DILUTION) \quad (2)$$

where:

- DOSEai (mg/d/inh) = maximum amount of chemical of emerging concern consumed daily per inhabitant

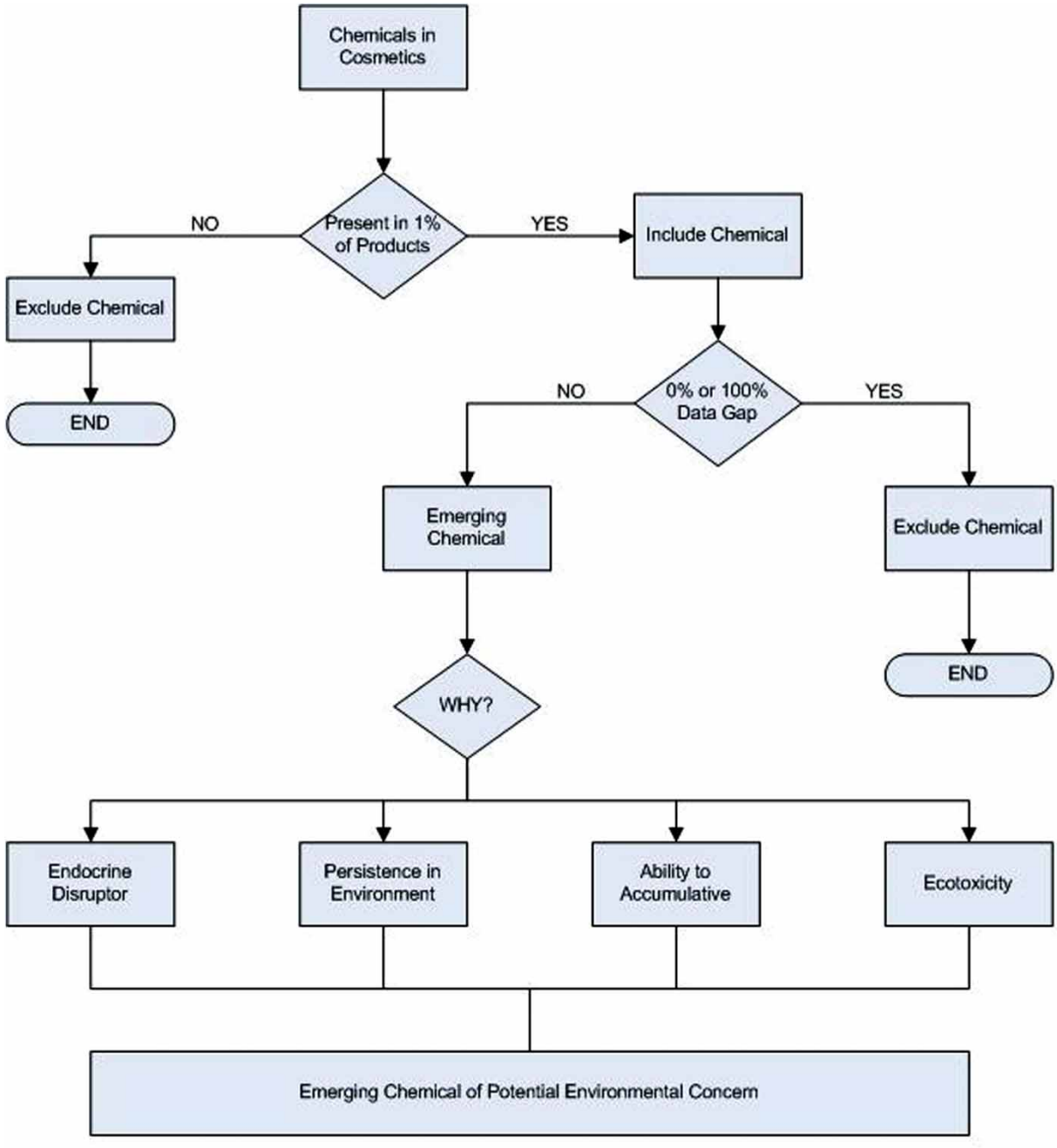


Figure 1. Screening and sorting of chemicals in cosmetics.

— Fpen (%) = percentage of market penetration
— WASTEWinhab (L/d/inh) = amount of wastewater per inhabitant per day = 200 [14]
— DILUTION = dilution factor for consumer products = 10 [14].

DOSEai and Fpen were estimated using Equations (3) and (4), respectively. DOSEai (maximum amount of chemical of emerging concern consumed daily per inhabitant) was calculated based on raw data collected from Euromonitor International on ingredients in cosmetics and quantities sold in the UK, and data collected from ingredient listings of products and material safety data sheets to determine concentrations of chemical used in PCPs. Maximum percentage concentrations of the emerging chemicals were used to predict a worst case scenario of environmental exposure to these potential contaminants. Fpen is defined as a percentage of market penetration and was calculated based on the

Table 2. PBT criteria according to Annex XIII of REACH Legislation.

Property	Description	PBT criteria
Persistence	Resistant to biological/chemical breakdown in the environment	Half-life (T1/2) > 40 days in fresh- or estuarine water, or T1/2 > 120 days in fresh or estuarine sediment, or T1/2 > 120 days in soil OR Not readily or inherently biodegradable OR Predicted biodegradability in a time frame of weeks to months
Bioaccumulation	Bioconcentrate in food chain, wildlife and humans	BCF[1] > 2000 L/kg OR Log K_{ow}^{2} > 4.5
Toxicity	Toxicity of flora, fauna and ecosystems, toxicity of humans and/or interference with the body's natural hormonal system	Acute L(E)C < 0.1 mg/L OR Chronic NOEC[3] < 0.01 mg/L OR Substance is CMR: carcinogenic (category 1 or 2), mutagenic (category 1 or 2), or toxic for reproduction (category 1, 2 or 3) OR Endocrine-disrupting effects

[1]BCF: bioconcentration factor; [2]K_{ow}: octanol–water partition coefficient; [3]NOEC: no observed effect concentration.

proportion of the population using a specific CEC on a daily basis.

$$\mathrm{DOSEai(mg/d/inh)} = \mathrm{TCecc}/(365\mathrm{Pop}) \quad (3)$$

$$\mathrm{Fpen}(\%) = [(\mathrm{Pop_{ecc}}/\mathrm{Pop}) \times 100]/365 \rightarrow \mathrm{Pop_{ecc}} = (\mathrm{Pop}/\mathrm{P_t}) \times \mathrm{Pecc} \equiv 100\mathrm{P_{ecc}}/365\mathrm{P_t} \quad (4)$$

where:

- $\mathrm{Pop_{ecc}}$ = population using PCPs containing chemical of emerging concern,
- Pop = total population in the UK for 2008 (60,943,912),
- $\mathrm{P_t}$ = total number of PCPs sold in the UK (2.6 billion units).

These $\mathrm{PEC_{initial}}$ values represented an initial assessment to estimate the potential of these CECs to affect the environment. If the $\mathrm{PEC_{initial}}$ value was below an action limit (0.01 μg/L), and no other environmental concerns were apparent, it was assumed that the CEC is unlikely to represent a risk for the environment following its usage in PCPs. If the $\mathrm{PEC_{initial}}$ value was equal to or above the action limit, it was assumed that the chemical could pose a potential risk to the environment and further analysis (environmental fate and effects) would be required. The $\mathrm{PEC_{initial}}$ was calculated for each chemical with the potential to cause environmental harm, irrespective of its route of exposure and environmental fate. This initial calculation was restricted to the aquatic compartment and assumed the following:

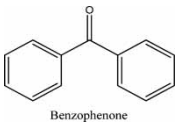
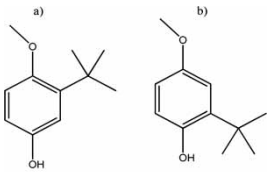
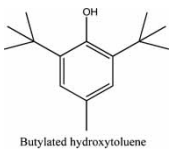
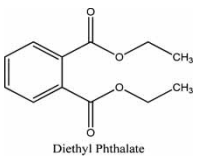
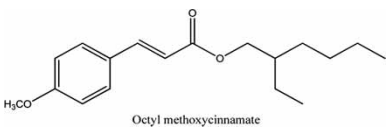
- the predicted amount of PCPs used per year is evenly distributed over the year and throughout the geographic area (UK)
- the sewerage system is the main route of entry of the chemicals from PCPs into the surface water
- there is no biodegradation or retention of the contaminant in the sewage treatment plant.
- dermal exposure to the contaminant is not taken into account.

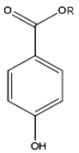
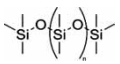
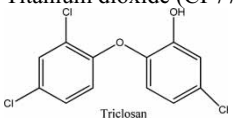
The CECs with potential for environmental concern were linked back to the products to determine the distribution and quantities of these emerging chemicals within the products, to assess whether cosmetics could be a potential source of environmental contamination. All data were analysed using Excel spread sheets.

Results and discussion

Over 2.6 billion cosmetic products are sold in the UK per annum, and the potential for environmental persistence, accumulation, toxicity and endocrine disruption of these chemicals means that there is a possibility of environmental contamination. The screening and sorting process identified two classes of CECs, namely preservatives and sunscreen agents, and 10 candidates from these with the potential to cause environmental concerns based on

Table 3. CECs commonly used in cosmetics based on the EU's PBT criteria.

CECs	Structure and CASRN[1]	Function/use	Persistence (T1/2 – freshwater)	BCF[2] (l/kg)	Toxicity: Acute L(E)C_{50} *Daphnia magna* (mg/L)	Toxicity: ED	Toxicity: CMR	Reference
BP3	 Benzophenone CAS: 119-61-9	Preservative	100	12	0.28	Yes	Yes	[15–20]
BHA	 The two isomers : Butylated Hydroxyanisole a) 2-tert-butyl-4-hydroxyanisole b) 3-tert-butyl-4-hydroxyanisole CAS: 25013-16-5	Preservative	28	269	7.0	Yes	Yes	[15,19,21–23]
BHT	 Butylated hydroxytoluene CAS: 128-37-0	Preservative	7	2500	3.06	ND[4]	Yes	[15,19,24]
DEP	 Diethyl Phthalate CAS: 84-66-2	Fragrance	28	117	52	Yes	Yes	[15,19,25,26]
OMC	 Octyl methoxycinnamate CAS: 5466-77-3	Sunscreen agent	21	5900	0.13	Yes	Yes	[15,19,27]

BP	 CAS: 94-13-3; 94-26-8; 99-76-3;	Preservative	43	110	4.0	Yes	Yes	[15,19,28–33]
PDMS	 CAS: 9016-00-6	Emollient	70	2000	0.14	Yes	Yes	[19,34–40]
Nano TiO_2	Titanium dioxide (CI 77891)	Sunscreen agent	ND	56563	5.5	ND	Yes	[39,41–43]
Triclosan	 CAS: 3380-34-5	Anti-microbial	730	5000	0.42	Yes	Yes	[19,44–47]
Nano ZnO	Zinc oxide (CI 77947)	Sunscreen agent	ND	ND	0.62	ND	Yes	[48]
EU limit			T1/2 > 40	BCF > 2000	$L(E)C_{50}$ < 0.1	Yes	Yes	

[1]CASRN is a single unique identifier number assigned to a material by the Chemical Abstracts Service (CAS) of the American Chemical Society (ACS).
[2]BCF is a value of the bioconcentration factor for CEC in animal tissue.
[3]$L(E)C_{50}$ represents the concentration of the chemical of emerging concern to which the organisms were exposed that causes mortality (LC_{50}) or some other defined effect (EC_{50}).
[4]ND means no data available for CEC.

their physicochemical characteristics and behaviour in the environment (PBT characteristics). The 10 CECS were benzophenone (BP3), butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), diethyl phthalate (DEP), octyl methoxycinnamate (OMC), butylparaben (BP), polydimethylsiloxane (PDMS), nano titanium dioxide (nano TiO_2), triclosan and nano zinc oxide (nano ZnO) (Table 3). Although the acute toxicity of these CECs did not meet the EU criterion of L(E)C50 < 0.1 mg/L, the toxicity levels were still moderate, ranging between 0.1 and 1.0 mg/L for most chemicals. The CECs were all linked to carcinogenic and/or mutagenic properties, and/or reproductive disorders in aquatic and terrestrial organisms. With the exception of nano TiO_2, nano ZnO and BHT, all other chemicals showed endocrine-disrupting properties. Studies assessing the endocrine-disrupting properties of the nanomaterials and BHT were either non-existent or non-conclusive, hence restricting the assessment of these potential contaminants.

Based on the calculated quantities of CECs present in cosmetics, the estimated per capita use of these chemicals ranged from a minimum of 0.081 mg/d/inh for BHA to a maximum for nanomaterials (81.85 mg/d/inh for nano ZnO and 134.28 mg/d/inh for nano TiO_2) (Table 4). Although by weight the average per capita use of nanomaterials in cosmetics was the greatest from among the CECs investigated, only about 42% of the products surveyed contained these potential contaminants. By contrast, over 70% of cosmetics contained DEP and BP, but estimated weights used of these products on a daily basis were much lower (2.80 mg and 10.30 mg, respectively) (Table 4). Diethyl phthalate is a main ingredient in synthetic fragrance, which is used in numerous products for odour enhancement, whereas UV filters (nano ZnO and TiO_2) are used only in selected products for the specific function of UV protection. The market penetration for products further emphasized this, as DEP showed market penetration at 0.23%, greater than the products containing nano TiO_2 (0.11%) and nano ZnO (0.05%) (Table 4).

The initial exposure of the environment to CECs used in cosmetics was analysed by comparing the amounts of products consumed, the concentrations of CECs in each product and the frequency of use. This estimation was based on an amended model derived by the Committee for Medicinal Products for Human Use (EMEA/CHMP) for environmental risk assessment of medicinal products for human use [49], since no methodologies exist for assessing the environmental exposure of chemicals/contaminants in cosmetics. The percentage market penetration (F_{pen} value) as determined by the EMEA/CHMP represents the proportion of a population being treated daily with a specific drug substance. It is usually a default value (0.01) based on the defined daily dose (DDD) values for drug substances derived by the World Health Organization. This DDD value cannot be derived for some of the CECs, as not all of the chemicals used in cosmetics have therapeutic uses; hence Fpen was corrected and calculated based on the proportion of the population using a specific CEC on a daily basis. Similar use and disposal patterns for medicinal products and cosmetics validate the use of this model for estimating the exposure of the environment to CECs in PCPs. As in the case of pharmaceuticals, surface water receives the largest amount of PCPs, via sewage and treated wastewater after use or disposal, hence the initial assessment of environmental exposure to these chemicals was directed to the aquatic environment [4,50,51].

The findings demonstrated that $PEC_{initial}$ exceeded the action limit (0.01 μg/L) for 9 of the 10 CECs identified in this study. Nano TiO_2 (7.621 μg/L), PDMS (4.285 μg/L) and nano ZnO (1.95 μg/L) were significantly greater than the action limit, whereas BHA was the only chemical selected whose $PEC_{initial}$ was below the action limit (Table 4). Cosmetics can therefore represent a potential source for environmental contamination based on the quantities of the CECs used in cosmetic formulations and the frequency of use and disposal of these products. However, detailed environmental risk assessments are needed to assess whether these CECs are reaching the environment at sufficient concentrations to pose potential risks to aquatic and terrestrial organisms. This is dependent on refining the $PEC_{initial}$ values and comparing them to

Table 4. $PEC_{initial}$ for CECs found in cosmetics in the UK.

CECs	Quantity consume in cosmetics (tonnes/yr)	Consumption per capita (DOSEai) (mg/d/inh)	Products sampled containing emerging chemical (%)	Market penetration (Fpen) (%)	$PEC_{initial}$ (μg/L)
BP3	55	2.471	18.9	0.02	0.024
BHA	1.8	0.081	18.9	0.02	0.001
BHT	39.5	1.776	43.4	0.15	0.135
DEP	62.4	2.807	73.6	0.23	0.320
OMC	133.6	6.007	30.2	0.04	0.124
BP	229.2	10.303	71.7	0.17	0.871
PDMS	1364.9	61.357	62.3	0.14	4.285
Nano TiO_2	2986.9	134.275	45.3	0.11	7.621
Triclosan	51.4	2.312	49.1	0.19	0.217
Nano ZnO	1820.8	81.852	37.7	0.05	1.951

Table 5. Consumption of CECs per product in the UK.

Products	CECs consumed (tonnes/d)	No. of products sold (m.u./d)[1]	CEC per product (mg)	Average size per product (mL)	% CECs per product
Face cleansers/toners	5.55	68.5	81.0	200	40.5
Sun care and tanning	3.41	41.8	81.5	200	40.8
Facial make-up	2.21	78.5	28.2	30	94.0
Deodorants & antiperspirants	2.08	417.5	5.0	200	2.5
Body creams and lotions	1.81	110.7	16.3	200	8.2
Face care	1.07	76.3	14.1	50	28.1
Shampoos & conditioners	0.72	247.2	2.9	250	1.2
Shaving products	0.30	37.6	7.9	200	3.9
Bath & shower products	0.24	329.5	0.7	500	0.1
Eye products	0.22	60.8	3.6	10	36.1
Depilatories	0.19	11.8	16.5	100	16.5
Hair styling & treatment	0.19	158.9	1.2	150	0.8
Nail products	0.17	23.7	7.3	20	36.3
Toilet soaps	0.15	522.3	0.3	250	0.1
Lip products	0.08	84.3	1.0	6	16.1
Perfumes	0.05	43.9	1.1	50	2.1
Oral care products	0.04	271.7	0.1	100	0.1
Talcum powder	0.0009	15	0.1	500	0.01

[1]m.u.: million units.

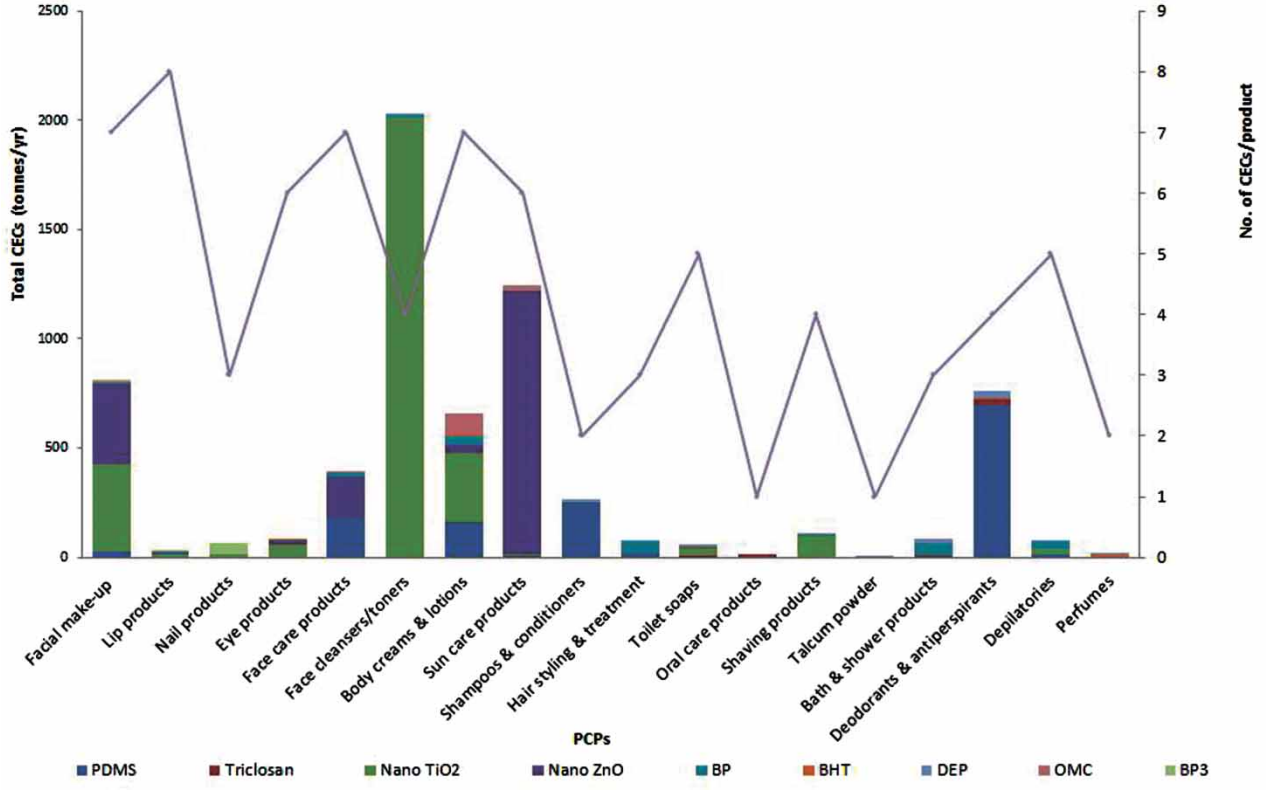


Figure 2. Distribution of CECs in cosmetics in the UK.

predicted no-effect concentrations based on toxicity assessments. Physicochemical data for these CECs, especially nanomaterials, are scarce, posing a challenge for further evaluation of these chemicals. There is also a lack of information regarding the potential impacts associated with the CECs' occurrence, fate and ecotoxicological effects in the environment, since few of the chemicals investigated are inventoried or regulated worldwide, and no legal requirements exist to assess the impact of long-term exposure to low concentrations of these CECs [5].

The CECs with the potential to cause concerns in the environment were traced back to products. Whilst products

such as talcum powder, toilet soaps, oral care products, bath and shower care products, and products used for hair styling and treatment contained only less than 1% of these CECs per product, other products such as cleansers/toners and sun care products contained over 40%, and facial make-up had over 90% of these CECs per product (Table 5). However the distribution of these potential contaminants in products and the quantities used within each product varied greatly (Figure 2). The findings indicated that lip products contained eight different CECs but in lesser quantities, whereas products such as face cleansers/toners contained only four CECs, with one chemical (nano TiO_2) being dominant and used in larger quantities (Figure 2). For example, approximately 2011.76 tonnes of nano TiO_2 are used in face cleansers/toners per annum in the UK, as compared with 12.33 tonnes of BP and 1.50 tonnes of triclosan. The same was evident for sun care products, which primarily contained nano ZnO (1204.50 tonnes used per annum), and deodorants and antiperspirants, which had higher levels of PDMS (698.13 tonnes) (Figure 2). It can therefore be suggested that nanoparticles are dominant ingredients used in cosmetics with the potential for environmental contamination. Further assessment of these chemicals, to fully understand the environmental implications of their use and disposal, and management of products containing these CECs are needed. Although other CECs were present in products, their consumption and distribution within the products were low, indicating a reduced chance for environmental contamination.

Limited data availability made it necessary to estimate some values for the quantities of CECs in different PCPs. Exact concentrations of chemicals in products were not always readily available from ingredient listings or material safety data sheets. Attempts to contact international personal-care manufacturing companies to collect this data were futile owing to company non-disclosure agreements. Estimates were used based on the existing concentration range for the specific CECs from other products. Where upper limits exist, in the Cosmetics Directive, for the use of certain chemicals in PCPs, these values were utilized for the study.

Conclusion

Cosmetics in the UK contain CECs. Some of these emerging chemicals, in particular nanomaterials, have the potential to cause concerns on exposure to the environment. The $PEC_{initial}$ calculated for the CECs identified in this study illustrated the need for further research to assess whether these chemicals pose a significant risk to the environment upon exposure, since these chemicals are used continuously. Products such as facial make-up, face cleansers/toners and sunscreen/tanning products contained high levels of emerging chemicals, with nanomaterials being dominant. Hence, the use of cosmetics containing CECs has the potential to contribute to some of these chemicals reaching the environment in concentrations that might cause concern. This research highlights the need for further work to assess the risks associated with the use and disposal of CECs and to assess the need for management of products containing CECs.

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European Commission

JRC TECHNICAL REPORT

Development of the first Watch List under the Environmental Quality Standards Directive

Directive 2008/105/EC, as amended by Directive 2013/39/EU, in the field of water policy

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Abstract

According to Directive 2008/105/EC (the Environmental Quality Standards Directive, EQSD), a new mechanism is needed to provide high-quality monitoring information on the concentrations of polluting substances in the aquatic environment across the EU. The aim of this mechanism is to support the identification of priority substances for regulation under the Water Framework Directive. A restricted number of substances (up to 10) are to be included in a dynamic Watch List, remaining there for limited time. Three compounds, i.e. diclofenac, 17-beta-estradiol (E2), and 17-alpha-ethinylestradiol (EE2) have already been identified for inclusion in the first Watch List, for the specific purpose of better informing the determination of suitable risk reduction measures. Therefore, up to seven additional substances should be identified for inclusion.
This report describes the procedure to identify a short-list of substances, based on the suspected risk to or via the aquatic environment, as well as on the unavailability of sufficient monitoring data or data of sufficient quality to identify the risk posed by those substances, and to prioritise them at EU level. From the short-list, seven additional substances are proposed for inclusion in the first Watch List.

Development of the first Watch List under the Environmental Quality Standards Directive

Table of Contents

Abbreviations

ADI	Acceptable Daily Intake
BCF	Bioaccumulation Factor
BMF	Biomagnification Factor
CIS	Common Implementation Strategy
dw	Drinking Water
EMA	European Medicines Agency
EqP	Equilibrium Partitioning method
fw `	Freshwater
hh	Human Health
Koc	Organic carbon adsorption coefficient
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MS	Member States
NOAEL	No-Observed Adverse Effect Level
NOEC	No-Observed Effect Concentration
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
PPP	Plant Protection Products
PS	Priority Substance
RBSP	River Basin Specific Pollutants
RQ	Risk Quotient (PEC/PNEC)
sec pois	Secondary poisoning
sed	Sediment
TDI	Tolerable Daily Intake
TG n. 27	Reference no. 18
WFD	Water Framework Directive

1. Introduction

According to the Environmental Quality Standards Directive 2008/105/EC as amended by Directive 2013/39/EU (EQSD) [1], a new mechanism is needed to provide high-quality monitoring information on the concentrations of potentially polluting substances in the aquatic environment to support future prioritisation exercises in line with Article 16(2) of Directive 2000/60/EC (Water Framework Directive, WFD) [2], and thereby to improve the protection of the aquatic environment and of human health via the environment. The mechanism is aimed at emerging pollutants and other substances for which the available monitoring data are either insufficient or of insufficient quality for the purpose of identifying the risk posed across the EU. It involves creating a Watch List with a limited number of such substances and monitoring them EU-wide for up to 4 years. A maximum number of 10 substances or groups of substances shall be included in the first Watch list, increasing by one at each update, up to a maximum of 14 substances or groups of substances [1]. Frequent reviews of the list will ensure that substances are not monitored longer than necessary, and that substances for which a significant risk at EU level is confirmed are identified as candidate priority substances with as little delay as possible.

However, three compounds, i.e. diclofenac (CAS n. 15307-79-6), 17-beta-estradiol (E2) (CAS n. 50-28-2), and 17-alpha-ethinylestradiol (EE2) (CAS n. 57-63-3), have already been selected for inclusion in the first list in order to collect sufficient monitoring data for the determination of risk reduction measures. The Joint Research Centre (JRC) has been tasked with proposing 7 substances as candidates for the completion of the first Watch List and identifying analytical methods for their monitoring.

This report describes the procedure and criteria used to identify a short-list of substances, proposed for inclusion in the Watch list.

2. Initial List of Substances

The main criteria for inclusion in the initial list of candidate substances were that i) the substance is suspected of posing a significant risk to, or via, the aquatic environment, meaning there is reliable evidence of hazard and of a possible exposure to aquatic organisms and mammals, but ii) there is not enough information to assess the EU-wide exposure for the substance, i.e. insufficient monitoring data or data of insufficient quality, nor sufficient modelled exposure data to decide whether to prioritise the substance.

Article 8b of the EQSD [1] sets out a comprehensive list of information sources to be considered when establishing the Watch List. These were further elaborated in the Document on the Development of the 1st Watch List under the EQS Directive, produced by DG ENV and presented at the 2nd Meeting of the WFD CIS Working Group Chemicals in March 2014 [3], and include:

(a) the results of the most recent regular review of Annex X to Directive 2000/60/EC provided for in Article 16(4) of that Directive (in particular substances ranked highly but not prioritised because of a paucity of monitoring data);

(b) research projects (even though these are likely to be the same research projects assessed in the priority substances review, the results would be considered more frequently for the watch list updates; their reliability should be considered);

(c) recommendations from the stakeholders referred to in Article 16(5) of Directive 2000/60/EC (these may include recommendations from the SCHER, MS, the EP, EEA, research programmes, international organisations, European business organisations inc SMEs, and environmental organisations);

(d) Member States' characterisation of river basin districts and the results of monitoring programmes, under Articles 5 and 8 of Directive 2000/60/EC respectively (consideration of river basin specific pollutants (RBSPs) if there is not already enough evidence from enough MS);

(e) Information on production volumes, use patterns, intrinsic properties (including, where relevant, particle size), concentrations in the environment and effects, including information gathered in accordance with Directives 98/8/EC, 2001/82/EC and 2001/83/EC, and with Regulations (EC) No 1907/2006 and (EC) No 1107/2009.

The above information sources have been considered for the compilation of the initial list of candidate substances (Table 1). Firstly, substances short-listed during the last review of priority substances, but not finally proposed for prioritisation [5] have been considered for the Watch List. Secondly, substances highlighted in some pieces of literature regarding research projects were considered, in particular those identifying emerging substances of concern. Thirdly, a few MS and Stakeholders have suggested substances for inclusion in the Watch List.

The document on the Development of the 1st Watch List included, in addition to the main (primary) criteria for identifying substances, the following secondary criteria: i) the need for a sufficiently sensitive analytical method to be available by the time monitoring has to begin (e.g. LoD less than or equal to PNEC or 2xPNEC, depending upon likely concentrations and nature of substance); and ii) no immediate ban on the production or use of the substance in the EU to be foreseen (unless, in the event of a ban, emissions from secondary sources such as imported products might be expected, and/or the substance is PBT or vPvB). As regards the first point, the JRC has done a preliminary assessment, and the second point has been taken into account in the final recommendation, having in mind that changes in the authorisation conditions for some substances could lead to future changes in concentrations of substances beyond the Watch List monitoring period.

The above document also identified some further criteria for prioritising substances, suggesting that account be taken of the particular hazardous properties of each substance, the relevance of the substance to drinking water quality, the irreversibility or severity of potential effects on ecosystems, the extent and nature of use; the possibility that monitoring data might very soon become available from other sources, and the ease of monitoring substances together, e.g. in same sample matrix (therefore for similar periods), at similar locations, as groups of substances. Some of the comments received from WG Chemicals members queried the value or precise meaning of some of these points, other comments were largely supportive. In line with the overall message, the approach presented in this document has focussed mainly on the risk quotient, on resolving information gaps identified during the last priority substances review, and on trying to give some attention to "emerging" pollutants.

Table 1. Initial list of substances

CAS n.	Substance name	Source [a]
294-62-2	Cyclododecane	[5]
60207-90-1	Propiconazole	[5]
731-27-1	Tolylfluanid	[5]
1066-51-9	Amino-methyl phosphonic acid (AMPA)	[5]
25057-89-0	Bentazone	[5]
80-05-7	Bisphenol A	[5]
298-46-4	Carbamazepine	[5]
1897-45-6	Chlorothalonil	[5]
1333-82-0	Chromium trioxide	[5]
81103-11-9	Clarithromycin	[5]
1085-98-9	Dichlofluanid	[5]
60-00-4	Edetic Acid (EDTA)	[5]
1071-83-6	Glyphosate	[5]
15687-27-1	Ibuprofen	[5]
93-65-2; 7085-19-0	Mecoprop (MCPP)	[5]
1113-02-6	Omethoate	[5]
2303-17-5	Tri-allate	[5]
52-68-6	Trichlorfon	[5]
7440-66-6	Zinc and its compounds	[5]
57-12-5	Cyanide – free (HCN and CN-)	[5]
723-46-6	sulfamethoxazole	[5]
53-16-7	Estrone	[6-8]
128-37-0	2,6-di-tert-butyl-4-methylphenol	MS
50-00-0	Formaldehyde	MS
85-01-8; 90640-80-5	Phenanthrene	MS
52645-53-1	Permethrin	EEB
121-75-5	Malathion	EEB
61-82-5	Aminotriazole	NORMAN
83905-01-5	Azithromycin	NORMAN
5466-77-3	2-ethylhexyl 4-methoxycinnamate	NORMAN
83164-33-4	Diflufenican	NORMAN
82419-36-1	Ofloxacin	NORMAN
114-07-8	Erythromycin	NORMAN
115-86-6	Triphenyl phosphate	NORMAN
85721-33-1	Ciprofloxacin	NORMAN
87674-68-8/163515-14-8	Dimethenamid/ dimethenamid-P	NORMAN
2032-65-7	Methiocarb	NORMAN
19666-30-9	Oxadiazon	NORMAN
105827-78-9; 138261-41-3	Imidacloprid	[9-11]

153719-23-4	Thiametoxam	[9-11]
210880-92-5	Clothianidin	[9-11]
111988-49-9	Thiacloprid	[11]
135410-20-7/160430-64-8	Acetamiprid	[11]

[a] Sources indicated as reference [5] are those listed in Table 4.1 of that document.

2.1 Substances from the last review of the PS list

During the last review of the priority substances list in accordance with Article 16(2) of the WFD [2], 12 priority substances (PS) were added to the initial list of 33 PS in Annex X to that Directive. Both modelling- and monitoring-based exercises, starting from initial lists of 2014 and 316 substances, respectively, were performed during the prioritisation process [4], resulting in a short-list of substances. However, not all of those substances were ultimately prioritised, in several cases because hazard information was lacking or monitoring data were available for too few Member States.

Therefore, the substances short-listed during the last review of the PS but not finally proposed for prioritisation, for which a detailed dossier had been produced and sometimes EQS had been derived, were included in the Initial List of substances as candidates for the Watch List. These substances have been identified in Table 4.1 of the Prioritisation scoping report [5], and are also listed above in Table 1. An exception is the substance musk xylene (CAS n. 81-15-2), which despite having been short-listed in the last prioritisation exercise, was not included in the current list of candidate substances because a ban has been imposed on its use in Europe. Firstly, the International Fragrance Association decided in their 44th amendment a voluntary ban on the use of musk xylene in fragrance products. Secondly, the European Commission has issued a ban on musk xylene with a sunset date of 21/07/2014.

2.2 Substances proposed by MS and other stakeholders and/or flagged in the literature

Member States representatives and other stakeholders which are part of the WFD Common Implementation Strategy (CIS) Working Group Chemicals were invited to propose substances for the Watch List based on the experience gained in the implementation of monitoring programs under the WFD, and previous prioritisation schemes followed in Europe. The proposed substances are listed in Table 1, as well as some substances flagged in the literature as being of possible concern.

The estrogenic hormone estrone (E1) is a product of E2 oxidation and although it has lower estrogen receptor binding/transactivation potency than E2 *in vitro* [6,7], it is usually found at higher concentrations (by a factor of about 10) in WWTP effluents and surface waters [6-8]. Because of its chemical similarity, E1 is usually analysed together with E2 and EE2.

Other substances proposed for inclusion in the initial candidate list were 2,6-di-tert-butyl-4-methylphenol (UV stabilizer and fuel antioxidant), formaldehyde, phenanthrene, the insecticides permethrin and malathion, and five neonicotinoid pesticides. The use of three of these neonicotinoids (imidacloprid, thiamethoxam, clothianidin) was recently restricted for two years (Regulation (EU) No 485/2013, Art. 2) by prohibition of seed treatment, soil treatment and foliar application before flowering for specific crops, to address concern that they pose a risk to bees [9]. Risks to other organisms have also been identified, and laboratory studies suggest that some of these substances have a half-life in soil that

can reach three years [10], while the field DT_{50soil} values in the EU Review Report 2005 were up to 305 d for clothianidin [11]. Use of the two other neonicotinoids, thiacloprid and acetamiprid, has not been restricted because they show lower toxicity to bees [12].

The initial list also includes top-ranked substances in "Category 2" [1] of the NORMAN Prioritisation scheme, including the plant protection products aminotriazole, diflufenican, dimethenamid, methiocarb and oxadiazon, the flame retardant triphenyl phosphate, the sun screen ingredient 2-ethylhexyl 4-methoxycinnamate and the antibiotics azithromycin, ofloxacin, erythromycin and ciprofloxacin. Moreover, antibiotics as a group and mixtures of unknown composition were also suggested by a stakeholder for inclusion in the initial list. Information on exposure to antibiotics in the environment is needed not only because of their potential direct toxic effects, but also because of the increasing concern regarding antimicrobial resistance (AMR), although the latter is not the issue in the present risk assessment [13-15].

2.3 Criteria for De-selection of Substances

From the initial list of 43 candidate substances for the Watch List, a criterion was defined to de-select substances with sufficient monitoring data available to conclude on a European-wide risk, i.e. with monitoring data from at least four MS. This is because a threshold for availability of monitoring data relating to at least four MS has been proposed as a criterion for including substances in the monitoring-based ranking of the next prioritisation exercise [16]. Therefore, such substances are considered to have sufficient monitoring data for a possible prioritisation and were therefore excluded from the Watch List. To identify the number of MS for which monitoring data are available, the period 2006-2014 was considered, and three databases were searched, i) WATERBASE, hosted by the European Environment Agency (EEA) and containing official monitoring data, aggregated by year, gathered under the State of the Environment (SoE) reports by MS, ii) IPCheM, with regard to the monitoring data compiled during the previous prioritisation exercise and iii) NORMAN database containing monitoring data from official sources, projects and literature.

Even though several substances on the initial list have been identified as River Basin Specific Pollutants (RBSP) in some MS [17], few additional data on those substances, apart from those available in the above databases, were forthcoming.

The selection criteria for consideration of monitoring data were the following:

1. Clear indication of the sampling site (site name, code, etc)
2. Clear identification of the analysed substance (determinand)
3. Clear identification of the measurement unit
4. Samples collected from 2006 on

[1] The substances have been selected on the basis of the occurrence data available in the NORMAN EMPODAT database and they fulfil the following criteria: a) hazard assessment is based on experimental data (AF maximum 50 for the derivation of the Lowest PNEC, mostly based on existing Assessment Reports) AND b) there is at least 1 site with exceedance of the Lowest PNEC (evidence of a potential risk) AND c) further monitoring data are needed for better assessment of exposure and risk at the European scale.

5. Either LOD or LOQ (at least one) clearly reported
6. Identification of the analysed fraction (only for NORMAN, such information was not available for WATERBASE)
7. LOD or LOQ ≤ substance-specific limits[2] when the value is reported to be below LOD or LOQ[3]
8. LOD or LOQ < PNEC when the value is reported to be below LOD or LOQ[3]

2.4 Dataset Filtered

After the application of the above criteria, 16 substances were de-selected based on availability of sufficient monitoring data. These substances are estrone, propiconazole, AMPA, bentazone, bisphenol A, carbamazepine, chlorothalonil, glyphosate, ibuprofen, mecoprop, omethoate, zinc, sulfamethoxazole, phenanthrene, permethrin and malathion. Monitoring data were available for dimethenamid from four MS, and only dimethenamid-P was considered for further assessment in this exercise. Therefore, a final number of 27 substances were taken forward for the purpose of ranking according to the risk they pose to the environment.

3. Methodology for Ranking of Substances

3.1 Overall Methodology

A risk assessment of all the substances in the filtered dataset was done by combining the substance-specific hazard data and information on exposure to the substance in or via the aquatic environment. According to the substance's physico-chemical properties, the receptors and compartments at risk were identified and an assessment done for each route of exposure, including the estimation of specific PEC and PNEC values, as summarized in Figure 1. In general, the criteria to identify the required assessments followed those specified in the Technical Guidance No. 27 of the Common Implementation Strategy (CIS) of the WFD [19].

The risk for direct toxicity to pelagic organisms from the presence of substances in the water column was always assessed, considering both a PEC_{fw} and a $PNEC_{fw}$ for surface water.

Depending on the sorption potential of a substance, a risk assessment for the sediment compartment was performed, i.e. whenever the organic carbon adsorption coefficient trigger value (Log K_{oc} or Log K_{ow}) ≥ 3, by estimating a PEC_{sed} and the $PNEC_{sed}$.

For the protection of organisms from secondary poisoning, an assessment was made for those substances with a potential to bioaccumulate, using as trigger value a bioconcentration factors (BCF) ≥

[2] Two substance-specific limits were calculated as the 99th percentile of all LOD and LOQ values for a certain substance (for NORMAN database separate limits were calculated for the dissolved fraction and for the "whole water" fraction).

[3] In WATERBASE data are aggregated by year, but information is available on the number of samples collected and the number of samples resulting below the LOQ. Hence these criteria were only applied if all samples were reported to be below LOQ.

100 or if no valid measured BCF was available a Log $K_{ow} \geq 3$ and the substance being not readily biodegradable [20]. In this case, a PEC_{biota} was considered and a $PNEC_{biota, sec\ pois}$ was estimated for top predators, as well as a $PNEC_{biota, hh}$ to assess the risk for human health arising from the consumption of fishery products.

Finally, an effect assessment was conducted for all substances regarding the protection of human health from consumption of drinking water, by estimating a $PNEC_{dw, hh}$ to be compared to the PEC_{fw}.

After estimating all the above PEC and PNEC values, risk quotients (PEC/PNEC) were calculated for the different compartment and receptor scenarios. The highest risk quotient calculated for a substance was used in the final ranking of substances (from highest to lowest risk).

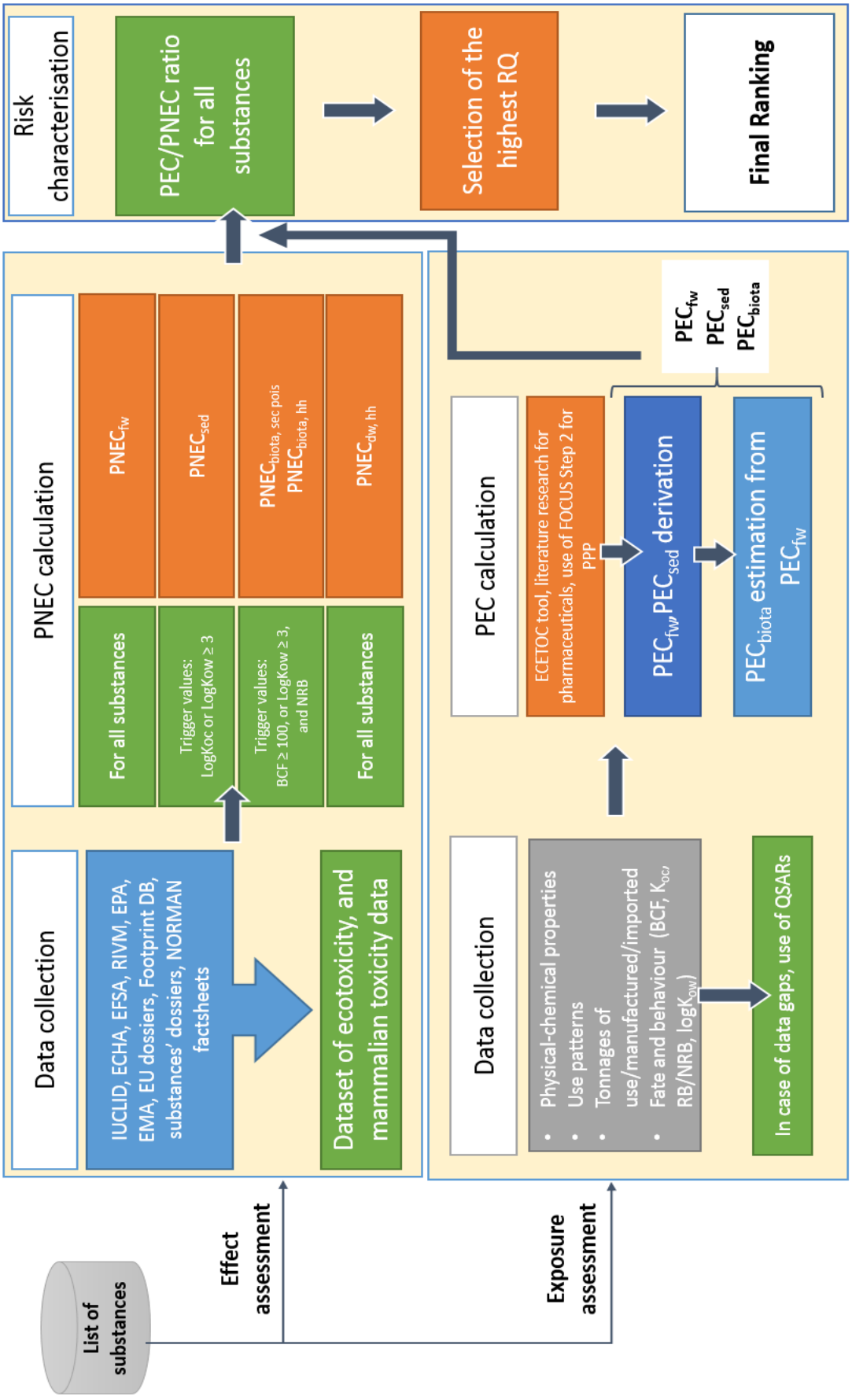

Figure 1. Summary of the overall methodology for the risk assessment of candidate substances for the Watch List.

3.2 Data Collection

For the collection of data, information was retrieved from several databases and reports, including IUCLID, ECHA, EFSA, RIVM, EMA, EU-Review reports, EU-RAR, EU Pesticides DB, Footprint Pesticides Properties DB, EPA, substances dossiers prepared during the last prioritisation exercise and substances factsheets provided by NORMAN. The relevance and reliability of the data retrieved from the above sources was deemed acceptable for the purpose of this exercise, since it was considered that the data had been reviewed by a competent authority, and no further review of the original study reports was conducted.

Data collected for the exposure assessment and PEC calculation comprised physical and chemical properties (molecular weight, water solubility, vapour pressure, biodegradability, sorption potential and bioaccumulation potential), tonnage (of use, manufacture and import) and Environmental Release Category (ERC) codes.

The collection of hazard data for the aquatic and sediment compartments included acute and chronic ecotoxicity (typically the most sensitive LC/EC50 or NOEC/EC10 endpoints). Regarding mammalian or human toxicity effects from oral exposure, data were collected for repeated dose toxicity, carcinogenicity, mutagenicity and effects on reproduction, focusing on typical endpoints such as NOAL, DNEL, ADI and TDI values. When new literature was considered that had not been retrieved from the sources listed above, reliability assessment of the ecotoxicological data was done by using a literature evaluation tool (LET), based on the Criteria for Reporting and Evaluating ecotoxicity Data (CRED) check list (Kase et al., personal communication).

3.3 Hazard assessment – estimation of PNEC values

3.3.1 PNEC for direct toxicity to freshwater organisms

Chemical risk assessment in the water compartment is relevant for the protection of organisms inhabiting the water column. Therefore, the protection threshold concentrations $PNEC_{fw}$ have been estimated for all substances.

For the estimation of the $PNEC_{fw}$, a deterministic approach has been used by selecting the most stringent valuable endpoint from the available aquatic toxicity data, and applying an assessment factor (AF), which was chosen based on the guidelines for the derivation of the $QS_{fw,\,eco}$ retrieved from the TG n. 27 - CIS WFD [18]. However, for some substances for which the PNEC was retrieved directly from other sources, a probabilistic method was used.

3.3.2 PNEC for the toxicity to benthic species

The threshold safety value for the protection of benthic organisms $PNEC_{sed}$ has been derived for those substances with a potential for sorption into the sediment compartment using as trigger values: logKoc or LogKow ≥ 3. The $PNEC_{sed}$ has been calculated following the TG n. 27- CIS WFD [18] and the ECHA Guidance (2012) [21], using equation A.

$$PNEC_{sed} = \frac{K_{\text{sed-water}}}{RHO_{sed}} \times PNEC_{fw} \times 1000 \qquad (A)$$

RHO_{sed} is the bulk density of wet sediment, $K_{sed\text{-}water}$ is the partition coefficient between sediment and water and 1000 is the conversion factor from m^3 to litre.

Since the final $PNEC_{sed}$ was calculated in terms of dry weight, a conversion step was required, by using the following equations B and C.

$$CONVsed = \frac{RHO_{sed}}{Fsolid_{Sed} \times RHOsolid} \qquad (B)$$

$$PNEC_{Sed} = CONVsed \times PNEC_{sed-ww} \qquad (C)$$

For the calculation of $K_{Sed\text{-}water}$, the following equation D was used.

$$K_{sed-water} = Fair_{sed} \times K_{air-water} + Fwater_{sed} + Fsolid_{sed} \times \frac{Kp_{Sed}}{1000} \times RHO_{solid} \qquad (D)$$

Default values for $Fair_{sed}$, RHO_{solid}, $Fwater_{sed}$, $Fsolid_{sed}$ and Foc_{sed} were taken from TG n. 27 - CIS WFD [18]. Since the given default value for $Fair_{sed}$ was *zero* [18], the first part of the equation ($Fair_{sed} \times K_{air\text{-}water}$) is not reported in the description of the calculations in the substances' factsheets.

3.3.3 PNEC for the toxicity to top predators from secondary poisoning

The WFD provides for the protection of top predators such as birds and mammals from risks of secondary poisoning arising from the consumption of aquatic organisms from lower trophic levels contaminated with toxic substances.

A $PNEC_{biota,\ sec\ pois}$ has been derived for all substances with a potential to bioaccumulate, as indicated under section 3.1.

The derivation of a $PNEC_{biota,\ sec\ pois}$ started from toxicological endpoints reporting on dietary and oral exposure such as the no-observed adverse effect level (NOAEL) or no observed effect concentration for ingestion ($NOEC_{oral}$). Since the $PNEC_{biota,\ sec\ pois}$ is expressed as concentration in food, conversion factors from NOAEL to NOEC have been used, for bird and mammalian toxicity studies, following the TG n. 27 - CIS WFD [18].

3.3.4 PNEC for hazard to human health via consumption of fishery products

Regarding the protection of human health from the consumption of contaminated fishery products, a $PNEC_{biota,\ hh}$ has been derived in a similar manner as the $QS_{biota,\ hh\ food}$ in the TG n. 27 - CIS WFD [18], by using endpoints such as the acceptable daily intake (ADI), tolerable daily intake (TDI) or $NOAEL_{oral}$ (divided by an AF).

$$PNEC_{biota,\,hh} = \frac{0.1 \times TL \times 70}{0.115} \qquad \text{(E)}$$

The $PNEC_{biota,\,hh}$ is expressed in μg·kg^{-1}, and uses a default value of human body weight of 70 kg, and a daily consumption of fishery products of 0.115 kg. In addition, it is assumed that fishery products make up no more than 10% of the threshold level value (0.1 × TL) [18].

3.3.5 PNEC for hazard to human health via drinking water consumption

Drinking water is a possible route of human exposure to substances in water, and protection threshold concentrations $PNEC_{dw,\,hh}$ have been derived for all substances, based on human toxicity data.
If available, WHO [21] or EU [22] drinking water standards have been used as the $PNEC_{dw,\,hh}$ values for that substance.
When a WHO drinking water standard was not available, the $PNEC_{dw,\,hh}$ was calculated according to the following equation F, retrieved from the TG n. 27 - CIS WFD [19].

$$PNEC_{\text{dw, hh}} = \frac{0.1 \times TL_{hh} \times bw}{uptake_{dw}} \qquad \text{(F)}$$

A human body weight (bw) of 70 kg and a daily uptake of drinking water ($uptake_{dw}$) of 2 litres were used. A fraction of 0.1 of the human toxicological standard (TL_{hh}, usually the acceptable daily intake (ADI) or the tolerable daily intake (TDI)) is allocated to intake of the substance via drinking water.

3.4 Exposure assessment – estimation of PEC values

Regarding the exposure assessment, information on tonnage and use pattern for all substances was searched for. To facilitate the ranking of substances based on risk, it was attempted to use a similar method for the PEC calculation for all substances, the ECETOC PEC calculation tool based on EUSES [23] This tool requires tonnage information, as well as usage information as input values. Unfortunately, the required input information was not available for all substances, and additional models were required, as detailed below. When more than one PEC value was calculated by different methods, the worst case value was generally used for the ranking of substances.

3.4.1 PEC for freshwater

Tonnages and usage information were retrieved from IUCLID for six substances, also for an additional seven substances from the last prioritisation exercise. Thus, for these substances, with the exception of PPPs, the ECETOC tool was used for the PEC calculation with the respective ERC codes (when more than one use was reported, the ERC corresponding to the worst-case scenario was selected for the calculation).

Unfortunately, the required input information for the remaining substances was not sufficient to run the model, and additional methods were sought for the PEC calculation depending on the availability of input data.

For one substance the PEC was retrieved from European Union Risk Assessment Reports (EU-RAR), while for eleven pesticides, the PEC was calculated with the model FOCUS Step 2 [24]. Finally, for four

antibiotics, given the unavailability of information on production or sales at European level, PEC values were calculated by using the following equation G, retrieved from the publication of Besse et al, 2008 [25]

$$PEC_{fw} = (consumption \times F_{excreta}) / (WWinhab \times hab \times dilution \times 365) \qquad (G)$$

where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants, $F_{excreta}$ is the excretion factor of the active substance (retrieved from the same publication), *dilution* is the dilution factor (default value of 10), consumption is the quantity (mg/year) of active ingredient consumed by the population during 1 year.

Data on human consumption of antibiotics in the list were available for six MS (France, Greece, Portugal, Latvia, Germany and Denmark), retrieved respectively from Besse et al, 2008 [25], Iatrou et al, 2014 [26], UBA report [27], and directly provided to the JRC for PT, LV and DK.

The worst case PEC value among those estimated for each antibiotic was selected for the risk quotient (RQ) calculation. However, human consumption is not the only use for the considered pharmaceuticals, and veterinary uses and intermediate uses (for erythromycin and azithromycin), are not accounted for in the above mentioned calculated PEC. Moreover, data on human consumption at European scale are still lacking. For this reason, the upper end values from the available monitoring data were used to calculate the risk, which is shown for comparison in the final ranking.

A summary of the sources of information and the models used for the PEC calculation are given in Table 2.

Table 2. Data sources and methods used for PEC calculation.

CAS n.	Substance name	Tonnage source	ERC code used	PEC calculation method
128-37-0	2,6-di-tert-butyl-4-methylphenol	IUCLID	ERC8d [a]	ECETOC [b]
135410-20-7/ 160430-64-8	Acetamiprid	-	-	FOCUS Step 2
61-82-5	Aminotriazole	(Last prioritisation exercise)[c]	(ERC8d)[c]	FOCUS Step 2
83905-01-5	Azithromycin	-	-	Eq. G
1333-82-0	Chromium trioxide	-	-	EU-RAR
85721-33-1	Ciprofloxacin	-	-	Eq. G
81103-11-9	Clarithromycin	-	-	Eq. G
210880-92-5	Clothianidin	-	-	FOCUS Step 2
57-12-5	Cyanide- free (HCN and CN-	-	-	-
294-62-2	Cyclododecane	IUCLID	ERC6a	ECETOC [b]
1085-98-9	Dichlofluanid	Last prioritisation exercise	ERC8b	ECETOC [b]
83164-33-4	Diflufenican	-	-	FOCUS Step 2 [d]

163515-14-8	Dimethenamid-P	-	-	FOCUS Step 2
60-00-4	Edetic Acid (EDTA)	IUCLID	ERC10b [a]	ECETOC [b]
114-07-8	Erythromycin	IUCLID	ERC8a	ECETOC [b] and Eq. G
5466-77-3	2-ethylhexyl 4-methoxycinnamate	Last prioritisation exercise	ERC8a	ECETOC [b]
50-00-0	Formaldehyde	IUCLID	ERC8d [a]	ECETOC [b]
105827-78-9/ 138261-41-3	Imidacloprid	-	-	FOCUS Step 2
2032-65-7	Methiocarb	(Last prioritisation exercise)[c]	(ERC8d)[c]	FOCUS Step 2
82419-36-1	Ofloxacin	-	-	Eq. G
19666-30-9	Oxadiazon	-	-	FOCUS Step 2 [d]
111988-49-9	Thiacloprid	-	-	FOCUS Step 2
153719-23-4	Thiamethoxam	-	-	FOCUS Step 2
731-27-1	Tolylfluanid	Last prioritisation exercise	ERC8b	ECETOC [b]
2303-17-5	Tri-allate	(Last prioritisation exercise)[c]	(ERC8d)[c]	FOCUS Step 2
52-68-6	Trichlorfon	Last prioritisation exercise	ERC8a	ECETOC [b]
115-86-6	Triphenyl phosphate	IUCLID	ERC8a [a]	ECETOC [b]

[a] Worst-case use scenario was selected among many others

[b] Koc value were used, in addition to default input values

[c] Calculations with ECETOC were made for comparison with FOCUS Step 2 results. However, the latter was used for the risk assessment.

[d] Output from FOCUS Step 2 retrieved from EFSA conclusion report

3.4.2 PEC for sediment

For the calculation of the PEC_{sed} ECETOC results were used whenever available, and for pesticides the results were retrieved from Focus Step 2. Similar to the PEC_{fw}, PEC_{sed} values for chromium trioxide were retrieved from the EU-RAR. For those pharmaceutical substances passing the trigger value Log Koc and Log Kow ≥3, the sediment equilibrium partition method (EqP) was used considering the PEC_{fw}. Similar to what was done for the PNEC sediment in Section 3.3.2 (equations B-D), the $PEC_{sed\text{-}ww}$ in terms of wet weight (ww) was calculated using equation G:

$$PEC_{sed-ww} = \frac{K_{sed\text{-}water}}{RHO_{sed}} \times \mathrm{PEC_{fw}} \times 1000 \qquad (H)$$

Since the final PEC_{sed} was calculated in terms of dry weight, a conversion step was required, by using the following equations I and J.

$$CONVsed = \frac{RHO_{sed}}{Fsolid_{Sed} \times RHOsolid} \qquad (I)$$

$$PEC_{Sed} = CONVsed \times PEC_{sed-ww} \qquad (J)$$

For the calculation of $K_{Sed\text{-}water}$, the following equation K was used.

$$K_{sed-water} = Fair_{sed} \times K_{air-water} + Fwater_{sed} + Fsolid_{sed} \times \frac{Kp_{Sed}}{1000} \times RHO_{solid} \qquad (K)$$

Default values for $Fair_{sed}$, RHO_{solid}, $Fwater_{sed}$, $Fsolid_{sed}$ and Foc_{sed} were taken from TG n. 27 - CIS WFD [18]. Since the given default value for $Fair_{sed}$ was *zero* [18], the first part of the equation ($Fair_{sed} \times K_{air\text{-}water}$) is not reported in the description of the calculations in the substances' factsheets.

3.4.3 PEC for biota

For the calculation of the PEC_{biota} the following equation L was used [28].

$$PEC_{biota} = PEC_{fw} \times BCF \times BMF \qquad (L)$$

BCF values were retrieved when available or, for 5 substances, calculated from QSARS.
Default BMF values were retrieved from TG n. 27 - CIS WFD [18].

3.5 Final Ranking

Risk quotients (RQ) were estimated for all the relevant receptors at risk, i.e. RQ_{fw}, RQ_{sed}, $RQ_{biota,\ sec\ pois}$, $RQ_{biota,\ hh}$, $RQ_{dw,\ hh}$, and are available in the substances fact sheets, in Annex I. The highest RQ for the different compartments and/or receptors was selected for the final ranking of substances (Table 3).

4. Results

4.1 Overview of the exposure, hazard and risk assessment

The current exercise has attempted to quantify the risk associated with the substances in the candidate list, for which a lack of EU-wide monitoring data has been identified that makes it difficult to decide whether to propose them as priority substances in the EU.
Details of the considered sources of information on hazard and exposure, as well as the calculations of PEC and PNEC done for each individual substance for the different receptors and compartments, can be found in the factsheets in the Annex.

First results for the estimated PEC and PNEC values, as well as the different RQ, were presented and discussed at the Working Group Chemicals meeting (16-17 October 2014), following which Member States and Stakeholders provided additional comments and information in writing. New information on exposure or hazard has been considered and used to update the substance assessments. The final results are summarised in Table 3. Substances are ranked from the highest to the lowest RQ. The provision of additional monitoring data for several substances means that there are now sufficient data to evaluate some of them in the monitoring-based exercise of the ongoing review of PS. These substances have been deselected from the candidate list (Table 4). However, a detailed factsheet is still presented in the Annex for these substances, including information on the measured environmental concentrations. For the two substances with the lowest ranking, ofloxacin and EDTA, the risk quotient was below 1 in the present assessment and they have also been excluded from the candidate list.

In addition to the ranking based on the risk quotient, the uncertainty of the PEC and PNEC calculations was also taken into account for the final recommendation. Additionally, existing or imminent non-authorisation of use was taken into account to avoid proposing substances for inclusion in the Watch List for which measures are already in place that are expected to reduce the risk to the environment. Finally, it was considered whether methods are available to allow the monitoring of each proposed substance at concentrations close to its PNEC.

A number of specific issues were identified during the risk assessment of the candidate substances, or brought to our attention by Member States and Stakeholders during or following the WG Chemicals Meeting (16-17 October 2014). In several cases, these issues relate to uncertainties in the PEC and PNEC calculation due to unavailability of data that could result in over or underestimation of the risk. These issues are discussed in more detail below by substance.

Table 3. Results from the risk-based ranking of substances using the highest risk quotient calculated for each substance

ID Rank	Substance	$PNEC_{fw}$ [a] (mg/L)	$PNEC_{sed}$ (mg/kg dw)	$PNEC_{biota, sec\ pois}$ (mg/kg food)	$PNEC_{biota, hh}$ (mg/kg food)	$PNEC_{dw, hh}$ (mg/L)	PEC_{fw} [b] (mg/L)	SOURCE of PEC_{fw}	PEC_{Sed} [b] (mg/kg)	SOURCE of PEC_{sed}	PEC_{biota} ($mg/kg_{wet\ fish}$)	Highest RQ for ranking		Comment
1	Trichlorfon	9.6E-07	NR	N.R.	N.R.	0.158	0.034	ECETOC	0.120	ECETOC	0.09	35312	RQ_{fw}	High uncertainty regarding its spatial use, and AF 1000 for $PNEC_{fw}$
2	Cyclododecane	NA	NA	5	38.043	2.188	0.468	ECETOC	306.45	ECETOC	64074.9	12815	$RQ_{biota, sec\ pois}$	No effects on aquatic organisms found at concentrations below water solubility. Doubts regarding analytical methods
3	Imidacloprid	9E-06	N.R.	N.R.	N.R.	0.21	0.008	FOCUS Step 2	0.018	FOCUS Step 2	0.005	889	RQ_{fw}	Sufficient monitoring data only pre-restriction
4	Diflufenican	1E-05	0.02	16.7	12.174	0.7	0.006	FOCUS Step 2	0.112	FOCUS Step 2	9.18	575	RQ_{fw}	Sufficient monitoring data
5	Oxadiazon	8.8E-05	0.05	0.24	0.219	0.0126	0.039	FOCUS Step 2	0.496	FOCUS Step 2	9.48	443	RQ_{fw}	Evaluation process of Confirmatory data is on-going.
6	Methiocarb	1E-05	0.0005	0.591	0.791	0.0455	0.004	FOCUS Step 2	0.026	FOCUS Step 2	0.30	395	RQ_{fw}	Sufficient monitoring data

													only pre banning as molluscicide
7	2,6-ditert-butyl-4-methylphenol	3.16E-03	1.290	16.7	15.217	0.875	0.423	ECETOC	367.640	ECETOC	2115	283	RQ_{sed}	
8	Thiacloprid	5E-05	N.R.	N.R.	N.R.	0.035	0.011	FOCUS Step 2	0.042	FOCUS Step 2	0.03	218	RQ_{fw}	
9	Tri-allate	6.7E-04	0.145	1.67	1.522	0.0875	0.118	FOCUS Step 2	2.560	FOCUS Step 2	165.20	176.12	RQ_{fw}	
10	Aminotriazole	3.20E-02	N.R.	N.R.	N.R.	0.004	0.501	FOCUS Step 2	0.459	FOCUS Step 2	1.19	143	$RQ_{dw,hh}$	Commission vote is expected in Dec 2014, regarding the renewal of the authorisation.
11	Chromium trioxide	3.4E-03	3.4	17	0.055	0.003	0.350	EU-RAR	0.152	EU-RAR	0.98	111	$RQ_{dw,hh}$	Last application date in 2016, sunset date 2017.
12	Thiamethoxam	1.4E-04	N.R.	N.R.	N.R.	0.091	0.011	FOCUS Step 2	0.007	FOCUS Step 2	0.03	78.6	RQ_{fw}	
13	Clothianidin	1.3E-04	N.R.	N.R.	N.R.	0.340	0.008	FOCUS Step 2	0.014	FOCUS Step 2	0.03	61.54	RQ_{fw}	
14	Erythromycin	0.0002	0.006	No info	0.043	0.002	0.005 [c]	ECETOC	0.3185	ECETOC	0.255	52.9	RQ_{sed}	
							0.0002 [d]	Eq. G	0.006	EqP	0.01	1.00	$RQ_{fw/sed}$	Human consumption
							0.0006	MEC	0.0185	EqP	0.03	3.07	$RQ_{fw/sed}$	Monitoring
15	2-ethylhexyl 4-methoxycinnamate	NA	0.2	N.R.	N.R.	7.875	0.006	ECETOC	8.390	ECETOC	2.73	41.9	RQ_{sed}	
16	Dichlofluanid	2.65E-04	0.018	3.33	21.30	1.225	0.005	ECETOC	0.732	ECETOC	0.38	40.2	RQ_{sed}	
17	Formaldehyde	4.7E-01	2.44	N.R.	N.R.	0.525	13.53	ECETOC	70.200	ECETOC	13.53	28.8	RQ_{fw}	
18	Dimethenamid-p	2.70E-03	0.005	N.R.	N.R.	0.070	0.066	FOCUS Step 2	0.109	FOCUS Step 2	3.81	24.3	RQ_{fw}	Sufficient monitoring data

19	Triphenyl phosphate	3.70E-03	0.240	N.R.	N.R.	0.140	0.015	ECETOC	5.490	ECETOC	2.16	22.9	RQ_{sed}	
20	Acetamiprid	5E-04	N.R.	N.R.	N.R.	0.245	0.005	FOCUS Step 2	0.005	FOCUS Step 2	0.02	10.0	RQ_{fw}	
21	Ciprofloxacin	8.9E-05	0.272	N.R.	N.R.	0.006	5.4E-04 [d]	Eq. G	1.642	EqP	0.0017	6.04	$RQ_{fw/sed}$	Human consumption
		8.9E-05	0.272	N.R.	N.R.	0.006	0.0012	MEC	3.78	EqP	0.004	13.93	$RQ_{fw/sed}$	Monitoring
22	Tolylfluanid	2.65E-04	0.058	8	6.087	0.350	9.7E-04	ECETOC	0.217	ECETOC	0.07	3.66	RQ_{fw}	Sufficient monitoring data
23	Clarithromycin	1.3E-04	0.0012	No info	0.012	0.001	4.38E-04 [d]	Eq. G	0.004	EqP	0.025	3.37	$RQ_{fw/sed}$	Human consumption
							6E-04	MEC	0.006	EqP	0.036	4.96	$RQ_{fw/sed}$	Monitoring
24	Azithromycin [c]	9E-05	0.014	No info	0.103	0.006	1.3E-04 [d]	Eq. G	0.020	EqP	0.03	1.42	$RQ_{fw/sed}$	Human consumption
		9E-05	0.014	No info	0.103	0.006	5.83E-04	MEC	0.09	EqP	0.117	6.48	$RQ_{fw/sed}$	Monitoring
25	Ofloxacin	1.3E-04	N.R.	N.R.	N.R.	No info	9.4E-05 [d]	Eq. G	0.0002	EqP	0.0003	0.72	RQ_{fw}	RQ < 1
26	Edetic Acid (EDTA)	2.2	118.58	N.R.	N.R.	0.6	0.2486	ECETOC	26.93	ECETOC	0.447	0.41	$RQ_{dw,hh}$	RQ < 1
27	Cyanide-free	0.00026	-	-	-	-	-	-	-	-	-	-	-	Many MS are monitoring CN, a few are already monitoring free CN. Improvements in analytical capabilities are likely to soon generate sufficient data.

[a] PNEC values were updated according to the comments received. New monitoring data from Sweden and Italy were considered as well, and therefore 5 substances may be moved to the priority substances review because there are monitoring data from at least 4 MS.

[b] $PEC_{fw,sed}$ values for plant protection products were calculated using FOCUS Step2.

[c] Erythromycin and azithromycin are registered in ECHA as having an intermediate use.

[d] PEC_{fw} values for antibiotics were re-calculated by using the following formula: PEC_{fw}= *(consumption X* $F_{excreta}$*) / (WWinhab X hab X dilution X 365)* from Besse et al. (2008)[25] where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants, $F_{excreta}$ is the excretion factor of the active substance, *consumption* is the quantity (mg/year) of active ingredient consumed by the population during 1 year.

Besse et al (2008)[25] made calculations based on data of consumption from France. Consumption data were taken from Iatrou et al, 2014[26] for EL, from UBA report for DE[27], and directly provided to JRC for PT, DK and LV by the respective MS. A similar calculation was done for these MS. The worst case PEC_{fw} value, calculated with the consumption data from single MS, was selected for the risk assessment.

The above calculations of PEC values for antibiotics do not consider any veterinary use. In fact, the MEC, based on monitoring data from just a few MS, show that the calculated PEC are likely an underestimation of the real environmental concentrations.

5. Discussion and Recommendations

- **Trichlorfon**: this substance has been banned as a PPP since 2007 but is currently still used as a veterinary pharmaceutical. For the PEC calculation, only a pre-banning tonnage was available (year 2000), and no PEC value was available from the literature related to its current use. For this reason, the available tonnage value was used for the PEC calculation, even though it is likely an overestimation, and an ERC code applicable to veterinary pharmaceuticals was selected (ERC8a). Additionally, as suggested at the WG Chemicals meeting 16-17 Oct 2014, a simulation was done to calculate the risk considering a reduction in the tonnage. From this simulation, it could be seen that even reducing the tonnage by 80%, the substance would still rank in the 2nd position in this exercise. It should be noted that six Member States (MS) have derived an EQS for trichlorfon, but it is unclear whether monitoring data have been collected by at least four MS for the time period 2006-2014, in which case the substance could automatically be considered under the monitoring-based exercise of the ongoing PS review. There is also significant uncertainty in the derivation of the PNEC, with the use of an AF of 1000. Regarding the available analytical methods, there are some difficulties reported with the extraction and analysis and in reaching the low PNEC of the substance [29]. It is considered that additional data are required on tonnage and hazard to better identify the risk from trichlorfon, before recommending its inclusion into the Watch List, and for this reason, the substance has been deselected from the candidate list.
- **Cyclododecane**: No effects on aquatic organisms found at concentrations below water solubility and no reliable QSARs predictions were found for the substance. Therefore, it was not possible to calculate $PNEC_{fw}$. The highest risk was calculated for biota. Even though cyclododecane has a high BCF and is expected to accumulate in biota, the derived PEC_{biota}, seems like an overestimation. The PEC_{biota} was calculated from the PEC_{fw} using ECETOC. However, the PEC_{fw} (also calculated using ECETOC) is about 30 times higher than the water solubility. In addition, there is no information on how widespread the use of cyclododecane is in Europe and no analytical methods could be retrieved from the literature [29]. For the reasons stated above, it is recommended to deselect cyclododecane from the candidate list for the WL.
- **Diflufenican**: The exposure of diflufenican was assessed using different FOCUS Step models and a risk was identified even using the high-tier FOCUS Step 3, considering the derived PNEC. The number of MS with monitoring data for the water compartment in the period 2006-2014 has been reassessed, and now reaches four MS, which is considered sufficient to assess the risk posed by the substance based on measured environmental concentrations in this compartment in the monitoring-based exercise of the ongoing review of the PS list. Even though the substance is likely to partition into the sediment (high Koc and DT50 sed values, see factsheet), a higher risk has been derived for the water compartment. However, PNEC exceedances were found in the two compartments (water and sediment).
- **Oxadiazon**: The substance is a RBSP in one MS, and monitoring data are available for only two MS, while there is information that the substance is used in nine MS. The exposure of oxadiazon was assessed using FOCUS models and a risk was identified even using the high-tier FOCUS Step 3, considering the derived PNEC. The available monitoring data, particularly those retrieved from IPChem,

also indicate a risk from this substance. Furthermore, a risk was determined for drinking water (RQ_{dw} 3.1). There are analytical methods capable of analysing the substance at low concentrations [29]. Although, an evaluation process of Confirmatory data on the substance is currently on-going, oxadiazon is still recommended for inclusion in the Watch List because of the apparently very high RQ, which could remain high even if the conditions of approval are changed.

- **Tri-allate**: The substance was evaluated during the last review of the PS list. The highest RQ calculated in the present report is for surface water (RQ_{fw}). The substance seems to be in use in eight MS, while monitoring data are available from only two MS (for water), with no PNEC exceedance. By contrast, the PEC calculation with FOCUS Step models indicates an exceedance of the PNEC even using FOCUS Step 3. There are analytical methods capable of analysing the substance at low concentrations in both water and soil [29].
- **Methiocarb**: Following the WG Chemicals meeting (16-17 October 2014), new monitoring data have become available and the total number of MS with monitoring data for methiocarb is now five. A PEC_{fw} has been calculated using both ECETOC and FOCUS Step 1 and Step 2 models. The results from the first two models were quite in agreement with the measured concentrations (MEC_{95}), except for SE, where all the measurements were below LOQ. Thus, both predicted and most measured concentrations are consistent with a risk for the water compartment in several MS. However, recently, the Commission Implementing Regulation (EU) No 187/2014 has restricted the uses of this active substance by withdrawing the authorisation for its use as a molluscicide. It is likely that this restriction will reduce the use of this substance in Europe and given the fast degradation of Methiocarb in the environment, no further aquatic exposure is expected from former use as a molluscicide. It is recommended that methiocarb is still considered for the Watch List to gather information on the post-banning environmental concentration (which at the moment is not available for any MS), in order to assess whether the banning as a molluscicide has been effective in eliminating the risk from this substance. In this respect, there are analytical methods capable of analysing the substance at low concentrations [29].
- **Imidacloprid**: This neonicotinoid has a widespread use in Europe as PPP and biocide, and some of its uses are currently restricted [30]. The PEC was derived using FOCUS Step models, where the PEC calculation was based on crop applications that accounted for the restriction in the uses of the substance. The calculated PEC shows an exceedance of the PNEC, even using FOCUS Step 3. Monitoring data are available for 5 MS (pre-restriction), and even though the MEC is lower than the predicted concentration, it still shows an exceedance of the PNEC. The PNEC has been derived using a probabilistic method and an AF of 3. Although there are monitoring data from more than 4 MS, the risk assessment of this substance in the prioritisation process may need to take account of the effects of the restriction, if it is extended, therefore monitoring data under those conditions should be obtained. There are analytical methods capable of analysing the substance at low concentrations [29].
- **2,6-ditert-butyl-4-methylphenol**: The substance is classified as having industrial uses with applications in a broad range of products. However, no monitoring data are available for the surveyed period (2006-2014), except from SE, for sludge and treatment plant effluents.

A recent tonnage value is available in IUCLID and this was used to calculate the PEC using an ECETOC model. Both $PNEC_{fw}$ and $PNEC_{sed}$ were retrieved from the ECHA file, with an AF of 100. The risk was higher for the sediment fraction. Given the high risk calculated for this substance, its widespread use and the fact that it is not readily biodegradable, and has a high BCF value, its inclusion into the Watch List is recommended. The literature indicates that methods are available for analysing the substance at the low ng/L level [29].

- **Thiacloprid**: The substance has a widespread use in Europe as PPP and biocide. Since no tonnage was available for its use as biocide, the PEC calculation was done with the application rate considering its use as PPP only using FOCUS Step models. Even using the higher-tier Step 3, the calculated PEC exceeded the PNEC. Monitoring data are available from four MS, in two of which there was exceedance of the PNEC. Hazard data for PNEC calculation were retrieved from the Biocide Assessment report for the substance and the lowest endpoint from the aquatic species tested corresponded to the midge *Chironomus riparius* to which an AF of 10 was applied to derive the PNEC. Although having sufficient monitoring data to be included in the monitoring-based exercise, it is recommended to keep thiacloprid on the candidate list, since the predicted risk is already reinforced by exceedances of the PNEC in 50% of the four MS with monitoring data. There are analytical methods capable of analysing the substance at low concentrations [29].
- **Aminotriazole**: A significant risk was calculated for this substance, even considering a high-tier FOCUS step 3 for the calculation of the PEC. However, monitoring data are only available from one MS, where the MEC_{95} is slightly below the PNEC. A high risk was determined for drinking water (RQ_{dw} 143). It was brought to our attention that a Commission vote is expected in December 2014, regarding the renewal of the authorisation of aminotriazole. For this reason, it is recommended to postpone the decision on an eventual proposal of aminotriazole for the WL until it is known whether a restriction on its use will be issued. The aminotriazole factsheet could then be reassessed on the occasion of the first revision of the WL. Furthermore, there seem to be some issues with the extraction and analysis of aminotriazole [29].
- **Clothianidin**: The substance has a widespread use in Europe (20 MS, according to the EU pesticides database) as PPP and biocide. A restriction on the use of clothianidin was imposed in 2013 [30]. The PEC has been calculated considering the application rate as PPP using the FOCUS Step models; the crop used for the PEC calculation was not listed in the restricted uses. Even using the higher-tier Step 3, the calculated PEC exceeded the PNEC. Monitoring data are available from only one MS, where all values were below the LOQ (which was below the PNEC). As for other neonicotinoid substances, the midge *Chironomus riparius* was the most sensitive species from all the hazard data in the Bioacide assessment report of the substance, and the PNEC was calculated using the deterministic approach with an AF of 10. Due to the high risk calculated for the aquatic environment, and insufficient information on environmental concentrations, it is considered that clothianidin is a good candidate for the WL, despite some of its uses currently being restricted. There are analytical methods capable of analysing the substance at low concentrations [29].

- **Chromium trioxide**: Chromium trioxide was short-listed during the last review of the PS list. A ban on the use of chromium trioxide and other hexavalent ions has been recently introduced, with the last application date in 2016, and a sunset date of 2017. Since there are already measures in place that are expected to decrease the environmental concentrations for this substance, it is recommended to deselect chromium trioxide as candidate for the Watch List and from the review of the PS list.
- **Thiamethoxam**: This neonicotinoid has a widespread use in Europe as PPP and biocide, and some of its uses are currently restricted [30]. The PEC was derived using FOCUS Step models, where the PEC calculation was based on crop applications that accounted for the restriction in the uses of the substance. The calculated PEC shows an exceedance of the PNEC, even using a PEC calculated from Step 3. Monitoring data are available for four MS, in one of which the PNEC has been exceeded. The highest MEC_{95} is lower than the predicted concentration, and also lower than the PNEC. The PNEC was derived using the deterministic approach with an AF of 100, following the Biocide Assessment report. Given its widespread use, and significant calculated RQ, the risk assessment of this substance could benefit from more extensive monitoring across Europe, to resolve the discrepancy between the PEC and the MEC, therefore it is recommended to consider thiamethoxam as a candidate for the Watch list. There are analytical methods capable of analysing the substance at low concentrations [29].
- **2-Ethylhexyl 4-methoxycinnamate**: This substance and UV filters in general have a widespread use worldwide. The tonnage value used for the PEC calculation was relative to year 2000, and considering the increase in the use of UV-filters in recent years, the tonnage could be even higher currently. The highest risk has been calculated for the sediment fraction from the PEC estimated with ECETOC and estimated $PNEC_{sed}$ and no toxicity was found for pelagic organisms below the water solubility of the substance. There are monitoring data for only two MS, where the MEC_{95} was lower than the PNEC for the substance. Even though other UV filters (eg. octocrylene) are found in the environment at higher concentrations than 2-ethylhexyl 4-methoxycinnamate, the latter is one that shows toxicity at lower concentrations (see factsheet for references). Furthermore EHMC is a suspected PBT and an endocrine disruptor, and more information on the exposure of aquatic ecosystems is required, and for this reason it is suggested as a good candidate for the Watch list. The available literature indicates that the substance can be measured at sufficiently low concentrations, including in sediment [29].
- **Dichlofluanid**: this substance was short-listed under the last review of PS. Dichlofluanid is currently used as a biocide and has been banned as a PPP since 2003. However, for the PEC calculation, only a pre-banning tonnage was available (from year 1997), and no PEC value was available from the literature related to its current use. For this reason, the available tonnage value was used for the PEC calculation, even though it is likely an overestimation. As suggested at the WG Chemicals meeting 16-17 Oct 2014, a simulation was done to calculate the risk considering a reduction in the tonnage. From this simulation, it could be seen that a 50% reduction in the tonnage would shift the ranking in this exercise from 16th to the 19th position. There is monitoring data from three MS for the time period 2006-2014, and the highest concentration measured is below the derived PNEC, while most measurements were below LOQ, even for sediment. Considering a broader time scale, also the PEC1 and PEC2 calculated from the monitoring data of the substance during the last review of the PS list were similar to more recent values

and still below the PNEC. It was also reported in the Biocide Assessment report that dichlofluanid rapidly degrades in aerobic aquatic systems, and that dichlofluanid does not have the potential to cause long-lasting contamination of surface water or sediment [31]. It is considered that the risk assessment of this substance requires an update in tonnage values relative to its current use. The available literature indicates that the substance can be measured in the low ng/l and µg/kg ranges for water and sediment, respectively [29].

- **Formaldehyde**: The substance was suggested for the WL by a MS. It is used in many different products, EU-wide. A confidential updated tonnage value was used for calculation of the PEC with the ECETOC model. However, in recent years different uses of formaldehyde have been banned (see factsheet). Formaldehyde is reported as a RBSP in three MS, and monitoring data in the period 2006-2014 is available for three MS. The MEC_{95} from all the databases screened in this report is below the PNEC. Furthermore, a decreasing trend in measured concentrations (MEC_{95}) in surface water was apparent (FR: from 17.6 µg/L (2008) to 7.6 µg/L(2012); UK: from 26.2 µg/L(2010) to 21.2 µg/L(2011); SK: from 6.5 µg/L(2007) to 0.2 µg/L(2010)). The PNEC was retrieved from ECHA and was estimated using the probabilistic approach with an AF of 10. A considerable risk was determined for drinking water, with a RQ_{dw} similar to the one for freshwater (25.8 and 28.8, respectively). It is likely that environmental exposure to formaldehyde will occur in many MS but it is less likely that exceedances of the PNEC are detected considering the restricted uses. There seem to be no issues in measuring this substance with the analytical methods available [29].
- **Dimethenamid-P**: dimethenamid was banned in 2006 [32] and since replaced by its active isomer dimethenamid-P. The PNEC values were estimated based on combined data from dimethenamid and dimethenamid-P, since the toxicological reference values established for dimethenamid have been considered applicable to dimethenamid-P by EFSA [33]. The initial assessment of the number of MS for which monitoring data are available was done by considering the monitoring data for dimethenamid and dimethenamid-P separately. In doing so, it was realised that there were monitoring data from four MS for dimethenamid, and only from two MS for dimethenamid-P (NL and IT). However, it was brought to our attention that monitoring data after the latest sales date established for dimethenamid (22 June 2008) can only relate to uses of dimethenamid-P PPPs, even if it has been attributed to "dimethenamid" (stakeholder comment (BASF)). Therefore, monitoring data after 2008 are available from five MS, and is considered sufficient for the evaluation of the substance in the ongoing review of PS. For this reason it is recommended to deselect dimethenamid-P as candidate substance for the Watch list.
- **Triphenyl phosphate**: The use pattern is not clearly stated in the ECHA dossier, but an ERC code related to wide dispersive use is given. An updated tonnage value retrieved from IUCLID was used for the PEC calculation with ECETOC. Triphenyl phosphate is readily biodegradable and the DT50 water < 28 days. The Koc value is very high and its accumulation in sediment is expected. Indeed, the highest risk was calculated for sediment, but no monitoring data were available for this compartment. For the water compartment, monitoring data are available for two MS, in both cases with concentrations lower than the PNEC, and the substance is reported as RBSP in 1 MS. Additional monitoring data could help in the risk

assessment of the substance. There seem to be no issues in measuring this substance with the analytical methods available, even in sediment [29].

- **Acetamiprid**: This neonicotinoid has a widespread use in Europe as PPP and it has not been subject to any restriction. The PEC calculation was done with the application rate considering its use as PPP using FOCUS Step models. Even using the higher-tier Step 3, the calculated PEC exceeded the PNEC. Monitoring data are available from only two MS, in one of which the PNEC has been exceeded. The lowest endpoint retrieved from the EU Review report related to *Chironomus riparius* and an AF of 10 was used to derive the PNEC. It is considered that acetamiprid is a good candidate for the Watch list and there are analytical methods capable of analysing the substance at low concentrations [29].
- **Erythromycin**: This pharmaceutical substance is registered in IUCLID with tonnage value given for an intermediate use (associated code ERC6a). By using ECETOC and ERC6a code to estimate the PEC value, an extremely high PEC_{sed} (50.89 mg/kg), and therefore a considerable risk, would be calculated for this substance, which would become the highest ranked in this exercise. However, the high PEC_{sed} value seemed an overestimated value and therefore, it was decided to use a different ERC code, applicable to pharmaceuticals, i.e. ERC8a. In addition to its intermediate use, erythromycin is a human and veterinary antimicrobial. Sales data were provided by PT, DK and LV and retrieved from the literature for EL [26] and from a report for DE [27]. The PEC calculated considering human use and excretion rates, by using a simplified EMA calculation retrieved from literature (see factsheet) were at least 1-order of magnitude lower than those using ECETOC ERC8a. The substance showed exceedance of the PNEC in measured concentrations in the NORMAN database (since 2002) and in monitoring data from SE, although the concentrations in the NORMAN database in the period 2006-2014 were all <LOQ. No consumption data were available for the use of erythromycin as a veterinary medicine. The risk assessment of this substance would greatly benefit from more information regarding both its use and environmental prevalence, and the inclusion into the Watch List could inform the risk assessors on the latter. There seems to be analytical methods available to analyse the substance at low concentrations, even regarding the LOQ for sediment [29].
- **Clarithromycin**: this pharmaceutical is used both as human and veterinary antimicrobial. Six PEC_{fw} values were calculated by using a simplified EMA calculation retrieved from the literature (see factsheet). According to the information available, only sales data related to human consumption from four MS were used for the PEC calculations, since no consumption values were available for the use of clarithromycin as veterinary medicine. The highest PEC value selected among those calculated was found to be lower than the highest MEC_{95} measured in SE, underlying that there may likely be an underestimation in the PEC calculation, due also to the missing information. The substance showed exceedance of the PNEC both in predicted and in measured concentrations in one MS out of three countries for which monitoring data are available. The risk assessment process of this substance would greatly benefit from more information regarding both its use and environmental prevalence, and the inclusion into the Watch List could inform the risk assessors on the latter. There are analytical methods capable of analysing the substance at low concentrations [29].

- **Ciprofloxacin**: this pharmaceutical is used both as human and veterinary antimicrobial. Five PEC_{fw} values were calculated by using a simplified EMA calculation retrieved from literature (see factsheet). Only sales data relating to human consumption in those MS were available for the PEC calculations. The highest PEC value selected among those calculated was found to be lower than the highest MEC_{95} measured in SE (NORMAN database), underlining that there may likely be an underestimation in the PEC calculation, due at least in part to the missing information. The predicted concentrations and the measured concentrations in the NORMAN database and in SE exceeded the PNEC. Additional monitoring data gained through the inclusion of ciprofloxacin into the Watch List would greatly benefit the risk assessment. There are analytical methods capable of analysing the substance at low concentrations [29].
- **Azithromycin**: this pharmaceutical is used both as human and veterinary antimicrobial. Five PEC_{fw} values were calculated by using a simplified EMA calculation retrieved from literature (see factsheet). Only sales data relating to human consumption in those MS were available for the PEC calculations. The highest PEC value selected among those calculated was found to be lower than the highest dissolved MEC_{95} measured in PT (NORMAN database), underlying that there may likely be an underestimation in the PEC calculation, due at least in part to the missing information. The substance showed exceedance of the PNEC both in predicted and in dissolved measured concentrations in the NORMAN database. Also in the case of azithromycin, it would be advantageous to have additional monitoring data, and the substance is considered a good candidate for the Watch List. There are analytical methods capable of analysing the substance at low concentrations [29].
- **Free cyanides**: Even though there are monitoring available data for total cyanides from > four MS, there appears to be insufficient information with regard to the most bioavailable cyanide species. Furthermore, no tonnage in Europe was available in IUCLID for free cyanides and therefore ECETOC could not be used for PEC calculation. Moreover, no PEC value could be found in the literature. Improved monitoring strategies focused on free cyanide would facilitate the estimation of environmental concentrations, particularly considering the concern for drinking water exposure and the available drinking water standard in Europe for cyanides [22]. Continuous improvements in sampling and analytical methodologies and capabilities in the different MS promise to allow widespread adequate measurement of free cyanides in the near future.
- For **ofloxacin** and **EDTA**, no risk was found in the present assessment (RQ < 1), and consequently they have been excluded from the candidate list.

Table 4. Final ranking after removal of substances with high uncertainty (trichlorfon, cyclododecane), facing a total ban (chromium trioxide), having a RQ < 1 (EDTA and ofloxacin), or with sufficient monitoring data (diflufenican, dimethenamid-P, tolylfluanid). For Cyanide-free, it is expected that appropriate data will soon become available. To be noted that the five substances imidacloprid, thiacloprid, clothianidin, thiamethoxam, and acetamiprid have been grouped together under the "*neonicotinoid class*".

Substance	Cas n.	Critical PNEC value	AF	Critical PEC value	Highest RQ for ranking		Recommended fraction	Analytical method [29]	LOQ (ng/L)	Comment
Oxadiazon	19666-30-9	8.8E-05 mg/L	10	0.039 mg/L	443.18	RQ_{fw}	water	yes	low	
Methiocarb	2032-65-7	1E-05 mg/L	10	0.004 mg/L	395.0	RQ_{fw}	water	yes	low	To determine environmental concentrations post banning as molluscicide.
2,6-ditert-butyl-4-methylphenol	128-37-0	1.290 mg/kg dw	100*	367.640 mg/kg dw	283.24	RQ_{sed}	water/sediment	yes	low	
Tri-allate	2303-17-5	6.7E-04 mg/L	10	0.118	176.12	RQ_{fw}	water	yes	10	
Imidacloprid	105827-78-9/ 138261-41-3	9E-06 mg/L	3	0.008 mg/L	889	RQ_{fw}	water	yes	low	RQs pre-partial restriction. Possible grouping as neonicotinoids. A common analytical method for monitoring is available.
Thiacloprid	111988-49-9	5.0E-05 mg/L	10	0.0109 mg/L	218.0	RQ_{fw}	water	yes	low	
Thiamethoxam	153719-23-4	1.4E-04 mg/L	100	0.0110 mg/L	78.57	RQ_{fw}	water	yes	low	
Clothianidin	210880-92-5	1.3E-04 mg/L	5	0.0080 mg/L	61.54	RQ_{fw}	water	yes	low	
Acetamiprid	135410-20-7/ 160430-64-8	5.0E-04 mg/L	10	0.0050 mg/L	10.0	RQ_{fw}	water	yes	low	
Erythromycin	114-07-8	0.0060 mg/kg dw	10*	0.318 mg/kg dw	52.9	RQ_{sed}	water/sediment	yes	10	ECETOC
		2 E-04 mg/L	10	0.0006 mg/L	3.07	$RQ_{fw/sed}$				Monitoring
2-ethylhexyl 4-methoxycinnamate	5466-77-3	0.2 mg/kg dw	10	8.390 mg/kg dw	41.95	RQ_{sed}	sediment	yes	low	
Dichlofluanid	1085-98-9	0.018 mg/kg dw	10*	0.732 mg/kg dw	40.2	RQ_{sed}	sediment	yes	low	
Formaldehyde	50-00-0	0.47 mg/L	10	13.5 mg/L	28.8	RQ_{fw}	water	yes	low	
Triphenyl phosphate	115-86-6	0.24 mg/kg dw	10*	0.015 mg/kg dw	22.9	RQ_{sed}	sediment	yes	low	

Ciprofloxacin	85721-33-1	8.9E-05 mg/L	50	5.4E-04 mg/L	6.04	$RQ_{fw/sed}$	water/sediment	yes	low	Human consumption
				0.0012 mg/L	13.93					Monitoring
Clarithromycin	81103-11-9	1.3E-04 mg/L	20	4.4E-04 mg/L	3.37	$RQ_{fw/sed}$	water/sediment	yes	low	Human consumption
				6E-04 mg/L	4.96					Monitoring
Azithromycin	83905-01-5	9E-05 mg/L	50	1.3E-04 mg/L	1.42	$RQ_{fw/sed}$	water/sediment	yes	low	Human consumption
				5.83E-04 mg/L	6.48					Monitoring

* The $PNEC_{sed}$ was calculated with the Equilibrium Partitioning method from the $PNEC_{fw}$

It is recommended that in addition to diclofenac, E2 and EE2, already proposed for the Watch List, the list also includes the substances with the highest risk, and not excessive uncertainty in the PEC or PNEC calculation in this exercise.

Even though E1 has been excluded from the list of candidate substances due to availability of monitoring data, it is a transformation product of E2 and is a considerable contributor to estrogenic activity in the aquatic environment. Therefore, it is recommended that E1 be analysed together with E2 to gather data for risk management following the reasons for inclusion of E2 into the Watch List. Both substances may be analysed with the same method in the same run, by GC-MS or LC-MS, without considerable additional burden.

Regarding neonicotinoids, a risk has been predicted for all five substances in the initial list, and their use in Europe is widespread. Even though for imidacloprid and thiamethoxam apparently the monitoring data would be sufficient to consider the substances under the monitoring exercise of the ongoing review of PS, these are related to a period prior to the implementation of the restriction on their use. If the restriction were to be extended, consideration of the substances in the priority substances review would have to be based on updated monitoring data. For thiacloprid, sufficient data also exist, but the exposure situation could also change in the light of the above restrictions. Since the neonicotinoids have similar properties and a similar mode of action and can be measured together with the same method, and without excessive costs, they could be included as a group of substances in the Watch List (Table 4). It is of note that just three of them were restricted due to the risk they pose for bees. However, a risk is identified for all five substances when other insects, relevant to the aquatic environment, are considered.

For substances with more individual uses, the estimation of a more realistic risk is complicated by the lack of sufficient monitoring data. However, the risk estimated for the four antimicrobials was in the same range considering the upper-end measured concentrations in Europe. Erythromycin, clarithromycin, and azithromycin are members of the same class, i.e. macrolide antibiotics, and share the same mode of action. Since it has been suggested by some MS and stakeholders, both in the initial proposal of substances and during the commenting phase of the report, to consider antibiotics as a group for the Watch List, those three substances are now proposed as a group of substances. This proposal is also supported by the availability of a single analytical method [29]. The significant risk estimated for ciprofloxacin would also support its inclusion in the Watch List. However, since it is in a different class of antimicrobials from the above group, it would need to be included as a separate substance.

In conclusion, the ten substances/groups of substances most recommended for the first Watch List are listed below, subject to the availability of the analytical methodology to monitor them:

Diclofenac
17-Beta-estradiol (E2), Estrone (E1)
17-Alpha-ethinylestradiol (EE2)

Oxadiazon

Methiocarb

2,6-ditert-butyl-4-methylphenol

Tri-allate

Imidacloprid, Thiacloprid, Thiamethoxam, Clothianidin, Acetamiprid

Erythromycin Clarithromycin, Azithromycin

2-Ethylhexyl 4-methoxycinnamate

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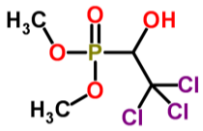
Annex – Substances factsheets

Information on exposure, hazard and risk for the Watch List candidate substances (except for substances with RQ <1)

Trichlorfon (CAS N. 52-68-6)

1. Substance identity

Chemical name (IUPAC)	Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate
EC number	200-149-3
CAS number	52-68-6
Molecular formula	C4H8Cl3O4P
Molecular weight	257.437
Structure	
SMILES	COP(=O)(C(C(Cl)(Cl)Cl)O)OC

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	2.1E-04	EFSA conclusion, 2006[1]
Water solubility (mg/L)	120000	EFSA conclusion, 2006[1]
logK_{ow}	0.43	EFSA conclusion, 2006[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	0	EFSA conclusion, 2006[1]
Biodegradability	NRB	EFSA conclusion, 2006[1]
Bioaccumulation (BCF)	2.74	Consensus between ADMET Predictor v. 7, VEGA Nic software, EPI Suite BCFBAF v. 3.01
BMF	1	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	1050 (year 2000)	From previous exercise
Uses	Insecticide used as veterinary pharmaceutical	
Spatial usage (by MS):	Not known	
Banned uses	PPP	(Commission Decision, C (2007)2096)[3]
ERC code	ERC8a	

Fraction of tonnage to region	0.1	

4.1 Predicted Environmental Concentration

	Description	**Source**
PEC_{fw} (mg/L)	0.034	ECETOC
PEC_{sed} (mg/kg dw)	0.12 (N.R.)	ECETOC
PEC_{biota} (mg/kg)	0.093 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 ECETOC simulation with lower tonnages

From 25 May 2007 no authorisation for plant protection products containing trichlorfon are granted or renewed. Any period of grace granted by Member States shall be as short as possible and shall expire not later than 21 November 2008 (Commission Decision, C (2007)2096)[3].

However, the available tonnage of 1050 relates to the year 2000, which is prior to the banning of the substance as PPP. At the WG Chem meeting 16-17/10/2014, it was suggested to perform a simulation on the PEC calculated with ECETOC using reduced tonnage values of trichlorfon that could be closer to the actual tonnage after the banning, i.e. related to the use as veterinary pharmaceutical only. Since no tonnage value specific for this particular use was available, it was decided to perform the simulation considering a 30%, 50% or 80% decrease in tonnage values. The results of the simulations are compared with the pre-banning tonnage scenario in the following Table.

Tonnes/year	1050	735	525	210
Decrease respective to pre-banning tonnage	-	30%	50%	80%
PEC_{fw} (mg/L)	0.034	0.024	0.017	0.007
RQ_{fw}	35312	24781.25	17697.92	7083.33
Position in the ranking (higher RQ)	1 (RQ_{fw})	1 (RQ_{fw})	1 (RQ_{fw})	2 (RQ_{fw})

4.2 Measured Environmental Concentration

Trichlorfon has been reported as a RBSP, with 6 MS having set EQSs ranging from 0.001 µg/L and 0.01 µg/L[4]. However, monitoring data was available only for 3 MS during the period surveyed (2006-2014).

n. of MS	**Source of monitoring data**	**MEC values**	**RBSP**
3 (FR, NL, IT)	NORMAN DB, 2014[5]	$MEC_{95,\ whole}$: 0.428 µg/L	6 MS[4]
	IT monitoring programme[6]	All values < LOQ	

5. P, B, T, C, M, R, ED properties

Trichlorfon is listed as Endocrine disruptor category 2 in the Endocrine Disruptor's Access Database of the European Commission[7].

In the EFSA Conclusion 2006[1], it was reported that, with respect to gene mutation in vitro, equivocal results were obtained in the cultured mammalian cells (Chinese hamster lung cells). Positive results were obtained for in vitro chromosome aberrations in human lymphocytes, with and without metabolic activation. However the clastogenicity could not have been confirmed *in vivo* for somatic cells (micronucleus test) or germ cells (dominant lethal assay) since the studies were considered as non acceptable due to major deviations from the guidelines. Therefore, the genotoxic potential of trichlorfon *in vivo* could not be concluded in the EFSA Conclusion, 2006[1]. In the same document, it was concluded that trichlorfon is not a carcinogenic compound and has no developmental toxicity.

Trichlorfon is not readily biodegradable (P), and a BCF value of 2.74 L/kg (the mean value of the three results was used) was estimated by using VEGA Nic, ADMET and EPI Suite models.

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorynchus mykiss*, 96 h, LC_{50}	0.7 mg/L	EFSA conclusion, 2006[1]
Aquatic invertebrates	***Daphnia magna*, 48 h, EC_{50}**	**0.00096 mg/L**	**Footprint Pesticides Database[8]**
Algae	*Scenedesmus subspicatus*, 120 h, EC_{50}	10 mg/L	EFSA conclusion, 2006[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute toxicity	Rat, LD50, oral	212 mg/kg bw	EFSA conclusion, 2006[1]
Long term toxicity	**Rat, NOAEL, 2 years, oral [a]**	**4.5 mg/kg bw/day**	**EFSA conclusion, 2006[1]**
Developmental toxicity	Rabbit, NOAEL, maternal toxicity	15 mg/kg bw/day	EFSA conclusion, 2006[1]
Developmental toxicity	Rabbit, NOAEL, offspring toxicity	45 mg/kg bw/day	EFSA conclusion, 2006[1]
Sub-chronic neurotoxicity	Rat, NOAEL, oral, 90 d	6.08 mg/kg bw/day	EFSA conclusion, 2006[1]
Long-term toxicity	Rat, NOAEL	13.2 mg/kg bw/day	EFSA conclusion, 2006[1]
Reproduction toxicity	Rat, NOEL	300 ppm	EFSA conclusion, 2006[1]

[a] Value used for ADI calculation in EFSA conclusion (2006) [1]

6.3 PNEC derivation

The most sensitive endpoint is for acute toxicity in *Daphnia magna.*

To be noted that in the EFSA conclusion (2006)[1], the study on *Daphnia magna* was considered of poor quality. In the U.S. EPA ECOTOX DB[9] the LC50/EC50 values with the same organism ranged from 0.01

µg/L to 750 µg/L. Evidence that *D. magna* showed highest sensitivity among organisms tested with trichlorfon are reported in Coelho et al. (2011)[10].

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna*, 48 h, EC_{50}	0.00096 mg/L	1000 [a]	9.6E-07 mg/L
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI	0.045 mg/kg bw/day	-	0.158 mg/L [b]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] Assessment factor 1000 was chosen because there was just acute toxicity data available for the main trophic levels.

[b] ADI value, retrieved from EFSA Conclusion, 2006[1], used in equation F as $TL_{hh.}$ See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**35312**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.22

8. References

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[3] COMMISSION DECISION of 21 May 2007 concerning the non-inclusion of trichlorfon in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance (notified under document number C(2007) 2096). Available at http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32007D0356

[4] NORMAN database at http://www.norman-network.net/?q=node/24

[5] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report.

[6] Italian Monitoring Programme (data provided directly to the JRC)

[7] Endocrine Disruptor database of the EU Commission), available at: http://ec.europa.eu/environment/chemicals/endocrine/documents/index_en.htm

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[9] http://cfpub.epa.gov/ecotox/

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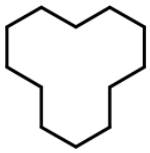
Cyclododecane (CAS N. 294-62-2)

1. Substance identity

Chemical name (IUPAC)	Cyclododecane
EC number	206-033-9
CAS number	294-62-2
Molecular formula	C12H24
Molecular weight	168.32
Structure	
SMILES	C1CCCCCCCCCCC1

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	9.83	ECHA, 2013[1]
Water solubility (mg/L)	0.016	ECHA, 2013[1]
logK_{ow}	7.6	ECHA, 2013[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	6513	ECHA, 2013[1]
Biodegradability	NRB	ECHA, 2013[1]
Bioaccumulation (BCF)	13700	ECHA, 2013[1]
BMF	10	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	Confidential tonnage used for calculation	IUCLID, 2013
Uses	Industrial use resulting in manufacture of another substance (use of intermediates)	IUCLID, 2013
Spatial usage (by MS)	Not known	
Banned uses	-	
ERC code	ERC6a	
Fraction of tonnage to region	0.1	

4.1 Predicted Environmental Concentration

	Description	Source
PEC_{fw} (mg/L)	0.4677	ECETOC
PEC_{sed} (mg/kg dw)	306.44	ECETOC
PEC_{biota} (mg/kg)	**64074.90**	Calculation based on Equation L (Section 3.4.3)

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
None	-	-	-

5. P, B, T, C, M, R, ED properties

Both in the ECHA dossier[1] and in the SVHC report[3], cyclododecane is considered to be not readily biodegradable, according to the biodegradability screening tests available.It was also concluded that cyclododecane has a very high bioaccumulation potential and fulfils the B and vB criteria[3]. The properties of persistency, liability to bioaccumulate and toxicity justified placing cyclododecane in the OSPAR list of Chemical for Priority Action, although no background document was prepared on the grounds that the substance is used as an intermediates in closed systems[4].

The ECHA Member State Committee (MSC) unanimously agreed that there was no sufficient scientific data to justify identification of cyclododecane as a substance of very high concern (SVHC)[5].

6. Hazard assessment

6.1 Ecotoxicology data

No effects found in aquatic organisms at concentrations below water solubility[1].

6.2 QSARS for predicting ecotoxicity values

Software	Endpoint	Endpoint value	Reliability	Conclusions
ADMET predictor v. 7	LC_{50}, fish, 96 h	0.51 mg/L	The substance is within the scope (applicability domain) of the model	The endpoint value is above the water solubility of the substance. Therefore, it cannot be used
ADMET predictor v. 7	$pIGC_{50}$, *Tetrahymena pyriformis*, growth inhibition toxicity	0.247 mg/L	The substance is outside the scope (applicability domain) of the model	The prediction is not reliable.
ADMET predictor v. 7	pLC_{50}, *Daphnia magna*, 48 h	1565.30 g/L	The substance is within the scope (applicability domain) of the model	The endpoint value is above the water solubility of the substance. Therefore, it

				cannot be used
VEGA Nic	*Fathead minnow*, LC50, 96 h (from T.E.S.T tool)	0.78 mg/L	The substance is outside the applicability domain of the model.	The prediction is not reliable
VEGA Nic	*Daphnia magna*, LC50, 48 h (from T.E.S.T tool)	81.25 mg/L	The substance is outside the applicability domain of the model.	The prediction is not reliable
VEGA Nic	*Daphnia magna*, LC50, 48 h (from DEMETRA tool)	1.11 mg/L	The substance is outside the applicability domain of the model.	The prediction is not reliable
VEGA Nic	Fish, classification of toxicity (from SarPy tool)	Tox-2 (toxicity between 1-10 mg/L)	The substance is within the applicability domain of the model.	The prediction should be reliable
ECOSAR v. 1.11	Fish*, Daphnia,* Algae Acute toxicity estimation	< 0.01 mg/L	According to the $logK_{ow}$ value of the substance (log K_{ow} 7.6), no effects at saturation are expected.	The predictions are not reliable.
ECOSAR v. 1.11	Fish*, Daphnia,* Algae chronic toxicity estimation	0.232 µg/L (fish) 0.506 µg/L (*Daphnia*) 6 µg/L (Green algae)	None of the chemical classes used for the models development seems to be representative of the molecular structure of the substance, leading to the consideration that cyclododecane could be outside of the structural domain of the models. Although both molecular weight and $logK_{ow}$ of the substance are below the cut-off values related to the chronic endpoints, the predictions are considered not reliable.	

6.3 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute toxicity	Rat, oral, LD50	>1000 mg/kg bw	ECHA, 2013[1]
Repeated dose toxicity	Rat, oral, 29 d, NOAEL	150 mg/kg bw/day	ECHA, 2013[1]

6.4 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	-	-	-	N.A.
Due to the fact that no effects have been seen at concentrations below solubility, it is not possible to calculate $PNEC_{fw}$ (ECHA, 2013)[1]. No reliable QSARs predictions were found for the substance.				
$PNEC_{sed}$	-	-	-	N.A.
See comment above for $PNEC_{fw}$				
$PNEC_{biota,sec\ pois}$	Rats, repeated dose toxicity, conversion	150 mg/kg bw/day	300	5 mg/kg food [a]

	factor 10, 29 d, NOAEL			
$PNEC_{biota, hh}$	DNEL, from repeated dose toxicity, oral, NOAEL 150 mg/kg bw/day, AF 240 [b]	0.625 mg/kg bw/day	-	38.04 mg/kg bw/day [c]
$PNEC_{dw, hh}$	DNEL, same as above	0.625 mg/kg bw/day	-	2.19 mg/kg bw/day [d]

[a] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of NOAEL (150 mg/kg bw/day) retrieved from ECHA, 2013 into NOEC (1500 mg/kg) by using the conversion factor of 10 (taken from TG n. 27- CIS WFD); b) To the NOEC value (1500 mg/kg), an appropriate AF_{oral} (300) (selected according to the duration test (29 days) (TG n. 27 - CIS WFD) was applied.

[b] At the beginning the DNEL value was estimated from NOAEL value of 150 mg/kg bw/day (repeated dose toxicity study, oral gavage, rat, 29 d), by using an AF of 600. The latter value was calculated by using the default values reported in Table R.8-6 of the ECHA document "Guidance on information requirements and chemical safety assessment Chapter R.8: characterisation of dose [concentration]-response for human health", available at http://echa.europa.eu/documents/10162/13632/information_requirements_r8_en.pdf. Specifically, an AF of 4 was used for the correction of differences in metabolic body weight from rat to human, an AF of 2.5 for remaining interspecies differences, an AF of 10 was chosen for intraspecies differences related to general population, an AF of 6 was used for accounting differences from sub-acute to chronic study, and AF of 1 was selected both for dose-response differences and for issues related to the quality of the whole database. The total multiplication led to a value of 600. However, it was finally decided to remove the AF of 2.5(remaining interspecies differences) leading to a total AF value of 240, with a DNEL value of 0.624 mg/kg bw/day instead of 0.25 mg/kg bw/day. The final ranking would not change. However, the last considerations were included in the final risk assessment of the substance. Therefore, the factsheet has been amended accordingly.

[c] DNEL value used for PNEC calculation according to Equation E (see section 3.3.4)

[d] The DNEL value was calculated from the NOAEL value (ECHA, 2013[1]), and thenused in equation F as TL_{hh} for PNEC calculation. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	N.A.
RQ_{sed}	N.A.
$RQ_{biota,sec\ pois}$	**12814.98**
$RQ_{biota, hh}$	1684.25
$RQ_{dw, hh}$	0.21

8. References

[1] ECHA dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d827c78-e4a2-5770-e044-00144f67d249/DISS-9d827c78-e4a2-5770-e044-00144f67d249_DISS-9d827c78-e4a2-5770-e044-00144f67d249.html

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

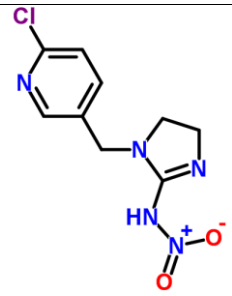
[3] SVHC SUPPORT DOCUMENT, Member State Committee Support document for Agreement on cyclododecane. Adopted on 8 October 2008. Available at http://echa.europa.eu/documents/10162/13638/svhc_supdoc_cyclododecane_en.pdf[4] OSPAR list of Chemicals for Priority Action (Revised 2011). Available at: http://www.google.it/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CCMQFjAA&url=http%3A%2F%2Fwww.ospar.org%2Fdocuments%2Fdbase%2Fdecrecs%2Fagreements%2F04-

12e_list%2520of%2520chemicals%2520for%2520priority%2520action.doc&ei=mw5aVPHZL8TKaOXPgugD&usg=AFQjCNE2l36bdrzuRahZmGXf-2BL5Gt-zA&sig2=4FsSESi1UTzEdCoXlAt0cg&bvm=bv.78677474,d.d2s

[5] ECHA Press Release (ECHA/PR/08/34). ECHA Member State Committee Agrees On The Identification Of 14 Substances Of Very High Concern. Available at: http://www.bssa.org.uk/cms/File/msc_indentification_svhc_20081009%20%282%29.pdf

Imidacloprid (CAS N. 105827-78-9/138261-41-3)

1. Substance identity

EC name	1-(6-chloropyridin-3-ylmethyl)-N-nitroimidazolidin-2-ylidenamine
EC number	428-040-8
CAS number	105827-78-9/138261-41-3
Molecular formula	C9H10ClN5O2
Molecular weight	255.7
Structure	
SMILES	C1CN(C(=N1)N[N+](=O)[O-])CC2=CN=C(C=C2)Cl

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	4E-10	EFSA conclusion, 2008 [1]
Water solubility (mg/L)	610	EFSA conclusion, 2008 [1]
logK_{ow}	0.57	EFSA conclusion, 2008 [1]

3. Environmental fate

Endpoint	**Value**	**Source**
Sorption potential (K_{oc})	225 (mean)	EFSA conclusion, 2008 [1]
Biodegradability	NRB	EFSA conclusion, 2008 [1]
Bioaccumulation (BCF)	0.61	FOOTPRINT PPDB[2]

4. Environmental exposure assessment

	Description	**Source**
Tonnes/year	-	
Uses	Insecticide (PPP and biocide)	
Spatial usage (by MS)	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SE, SI, SK, UK	EU Pesticides DB [3]
Banned uses	Restriction of uses[a]	EU n. 485/2013 [4]
ERC code	ERC8d (N.R.)	

[a] the use as a seed treatment or soil treatment of plant protection products containing imidacloprid is prohibited for crops attractive to bees and for cereals except for uses in greenhouses and for winter cereals. Foliar treatments with plant protection products containing imidacloprid are prohibited for crops attractive to bees and for cereals with the exception of uses in greenhouses and uses after flowering[4].

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.008	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.0.018 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	0.005 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 Comparison of FOCUS Pesticides models

FOCUS Step 1

Crop	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Pome and Stone Fruits (late)	1st appln 70 2nd appln 105 (40 d application interval)	610	225 (mean)	90

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 82 d (although being normalized with the older Q_{10} value of 2.2, this field DT_{50} value was kept for Step1&2 calculations, since the PEC_{fw} value of Step3 retrieved from the EFSA conclusion, 2014[5] was based on the same value), $DT_{50water}$: 90 d [1], $DT_{50sediment}$ 1000 d (conservative value)[1]. In the EFSA Conclusion, 2008[1], an earlier growth stage was reported for apples. However, we have considered a full canopy for FOCUS Step 1-2 calculations, in order to account for the restriction in the uses of the substance (post-flowering application).

FOCUS Step 3 – SWASH Package

No new calculations were made, PEC_{fw} value was retrieved from EFSA Conclusion, 2014[5].

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.054	0.116
FOCUS Step 2	**0.008**	0.018
FOCUS Step 3	0.006187 (single application - R3 stream	NA

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
5 (FR, PT, NL, SE, IT)	NORMAN DB, 2014 [6]	$MEC_{95,\ dissolved}$: 0.114 µg/L	1 MS[10] EQS set (WRc, 2012) [11]
	WATERBASE, 2014 [7]	$MEC_{95,\ whole}$: 0.08 µg/L	
	SE pesticide monitoring programme [8]	MEC_{95}: 0.21 µg/L	
	IT Monitoring Programme [9]	MEC_{95}: 0.099 µg/L	

5. P, B, T, C, M, R, ED properties

No evidence of genotoxic or carcinogenic effects was observed with imidacloprid[1]. Likewise it did not affect the reproductive parameters in rats, or the embryofoetal development in rats and rabbits[1]. In neurotoxicity studies, effects occurred in the functional observational battery, without histopathological findings in the nervous tissues[1]. Imidacloprid is not readily biodegradable (P). It shows a low potential to bioaccumulate in aquatic organism[1].

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Aquatic invertebrates	*Daphnia magna*, NOEC, reproduction	6 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	*Daphnia magna*, NOEC, reproduction	1.8 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	*Daphnia magna*	2 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	*Daphnia magna*	6 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	*Gammarus pulex*, NOEC, swimming/behaviour	0.064 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	***Gammarus pulex*, EC_{10}, immobilisation**	**0.00295 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Hyalella azteca*, NOEC, survival**	**0.00047 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Asellus aquaticus*, EC_{10}, immobilisation**	**0.00171 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	*Chironomus riparius*, EC_{10}, emergence	0.00209 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	*Chironomus riparius*, 10 d, NOEC, recovery after 4 d exposure	< 0.00215 mg/L	EFSA Conclusion, 2014[5]
Aquatic invertebrates	***Chironomus riparius*, NOEC, emergence, growth**	**0.0004 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Chironomus tentans*, EC_{10}, survival**	**0.00042 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Caenis horaria*, EC_{10}, immobilisation**	**0.000024 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**

Aquatic invertebrates	***Chaoborus obscuripes*, EC_{10}, immobilisation**	**0.00457 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Cloeon dipterum*, EC_{10}, immobilisation**	**0.000033 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Sialis lutaria*, EC_{10}, immobilisation**	**0.00128 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**
Aquatic invertebrates	***Plea minutissima*, EC_{10}, immobilisation**	**0.00203 mg/L**	**EFSA Conclusion, 2014[5], RIVM Report[12]**

Values in bold were used in the SSD (EFSA Conclusion, 2014[5])

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	500 mg/kg bw	EFSA Conclusion, 2008[1]
Short-term toxicity (neurotoxicity)	Rat, oral, 90 d, NOAEL	9.3 mg/kg bw/day	EFSA Conclusion, 2008[1]
Short-term toxicity	Dog, oral, 28 d and 90 d, NOAEL	8 mg/kg bw/day	EFSA Conclusion, 2008[1]
Long-term toxicity	**Rat, 2 years, NOAEL. Value used for ADI calculation in the EFSA Conclusion, 2008[1]**	**5.7 mg/kg bw/day**	**EFSA Conclusion, 2008[1]**
Long-term toxicity	Mouse, 2 years, NOAEL	208 mg/kg bw/day	EFSA Conclusion, 2008[1]
Reproductive toxicity	Rat, 2 generation study, a)parent NOAEL b)reproductive NOAEL c)offspring NOAEL	a)20 mg/kg bw/day b)50 mg/kg bw/day c)20 mg/kg bw/day	EFSA Conclusion, 2008[1]
Developmental toxicity	Rat, maternal and developmental NOAEL	30 mg/kg bw/day	EFSA Conclusion, 2008[1]
Developmental toxicity	Rabbit, a)maternal NAOEL b)developmental NOAEL	a)8 mg/kg bw/day b)24 mg/kg bw/day	EFSA Conclusion, 2008[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	HC_5[a]	0.027 µg/L	3	0.009 µg/L [b]
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI [1]	0.06 mg/kg bw/day	-	0.210 mg/L [c]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] HC5 from chronic SSD suggested by NL. Value retrieved from the new EFSA conclusion, 2014[5]

[b] Value retrieved from the new EFSA conclusion, 2014[5], as Tier-2B $RAC_{sw;ch}$

[c] ADI value, retrieved from EFSa conclusion, 2008[1], used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**888.9**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.04

8. References

[1] EFSA Scientific Report (2008) 148, 1-120, Conclusion on the peer review of imidacloprid. Available at http://www.efsa.europa.eu/it/efsajournal/doc/148r.pdf

[2] FOOPRINT Pesticides DataBase, available at http://www.eu-footprint.org/it/ppdb.html

[3]European Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] COMMISSION IMPLEMENTING REGULATION (EU) No 485/2013 of 24 May 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. Available at http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013R0485

[5] EFSA (European Food Safety Authority), 2014. Conclusion on the peer review of the pesticide risk assessment for aquatic organisms for the active substance imidacloprid. EFSA Journal 2014;12(10):3835, 49 pp. doi:10.2903/j.efsa.2014.3835.

[6] NORMAN Database http://www.norman-network.net/?q=node/24

[7] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[8] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

[9] Italian Monitoring Programme (data provided directly to the JRC)

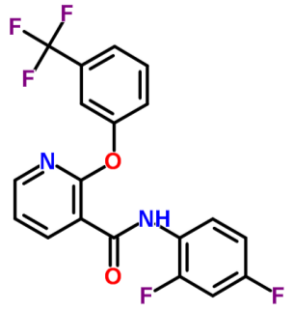
[10] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report

[11]Contract No. 070311/2011/603663/ETU/D1 "Comparative Study of Pressures and Measures in the Major River Basin Management Plans' - Task 2c (Comparison of Specific Pollutants and EQS): Final Report". WRc Ref: UC8981/1 October 2012. Available at: http://ec.europa.eu/environment/archives/water/implrep2007/pdf/P_M%20Task%202c.pdf

[12] Water quality standards for imidacloprid, Proposal for an update according to the Water Framework Directive, RIVM Letter report 270006001/2014, C.E. Smit. Available at http://www.rivm.nl/en/Documents_and_publications/Scientific/Reports/2014/april/Water_quality_standards_for_imidacloprid_Proposal_for_an_update_according_to_the_Water_Framework_Directive

Diflufenican (CAS N. 83164-33-4)

1. Substance identity

EC name	
EC number	
CAS number	83164-33-4
Molecular formula	C19H11F5N2O2
Molecular weight	394.3
Structure	
SMILES	C1=CC(=CC(=C1)OC2=C(C=CC=N2)C(=O)NC3=C(C=C(C=C3)F)F)C(F)(F)F

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	4.25E-06	EFSA conclusion, 2007 [1]
Water solubility (mg/L)	0.05	EFSA conclusion, 2007 [1]
logK_{ow}	4.2	EFSA conclusion, 2007 [1]

3. Environmental fate

Endpoint	**Value**	**Source**
Sorption potential (K_{oc})	1989	EFSA conclusion, 2007 [1]
Biodegradability	NRB	EFSA conclusion, 2007 [1]
Bioaccumulation (BCF)	1596	EFSA conclusion, 2007 [1]
BMF	1	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	**Source**
Tonnes/year	-	
Uses	Herbicide (PPP)	
Spatial usage (by MS)	AT, BE, BG, CZ, DE, DK, EE, EL, ES, FI, FR, HU, IE, IT, LT, LU, LV, NL, PL, PT, RO, SE, SI, SK, UK	EU Pesticides DB [2]

Banned uses	-	
ERC code	ERC8d	
Fraction of tonnage to region		

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.00575	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.112	FOCUS Step 2
PEC_{biota} (mg/kg)	9.18	Calculation based on Equation L (Section 3.4.3)

4.1.1 Comparison of FOCUS Pesticides models

FOCUS Step 1 [a]

Crop[1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Wheat	1 × 120	0.05	1989	214

FOCUS Step 2 [a]

Same parameters and conditions as above, in addition to DT_{50soil} 141.8 d [1], $DT_{50water}$: 31.7 d [1], $DT_{50sediment}$ 338.7 d [1], no crop interception[1].

FOCUS Step 3 – SWASH Package[a]

[a] No new calculations were performed, since PEC value were retrieved from EFSA conclusion, 2007 [1]

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.012	0.218
FOCUS Step 2	0.00575	0.112
FOCUS Step 3	0.000835 D2 ditch	0.0304 R3 stream

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
4 (FR, DE, FI, SE)*	NORMAN DB, 2014 [3]	$MEC_{95, whole}$: 0.09 µg/L $MEC_{95, dissolved}$: 0.152 µg/L	1 MS [6]
	WATERBASE, 2014 [4]	$MEC_{95, whole}$: 0.029 µg/L	
	SE pesticide monitoring programme [5]	MEC_{95}: 0.0314 µg/L	

* Monitoring data for the sediment compartment are available from only 1 MS (FR) from NORMAN and from IPChem, where PNEC exceedances were found.

5. P, B, T, C, M, R, ED properties

There was no concern about the genotoxic properties of diflufenican[1]. Diflufenican did not show any carcinogenic potential in the studies reported in the EFSA Conclusion, 2007[1]. There might be some indications of endocrine disruption at high doses but in view of the potential link with systemic toxicity, no classification for fertility was proposed. In developmental studies with rats and rabbits, there was no evidence of teratogenic activity in the absence of maternal toxicity (EFSA Conclusion, 2007[1]). Diflufenican is not readily biodegradable. Since the BCF in fish was > 1000 and the DT_{90} in sediment was >100 days the risk of bioaccumulation in terrestrial food chains was assessed[1]. The BAF (bioaccumulation factor) was calculated as 0.77. Since the BAF is <1 the risk of bioaccumulation is considered to be low (EFSA Conclusion, 2007[1]).

5.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Cyprinus carpio*, 96 h, LC_{50}	0.098 mg/L	INERIS, 2012[7]
Fish	*Pimephales promelas*, ELS	0.015 mg/L	INERIS, 2012[7]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	>0.24 mg/L	INERIS, 2012[7]
Aquatic invertebrates	*Daphnia magna*, 21 d, NOEC	0.052 mg/L	INERIS, 2012[7]
Algae	*Scenedesmus subspicatus*, 72 h, growth rate, EC_{50}	0.00045 mg/L	INERIS, 2012[7]
Algae	***Scenedesmus subspicatus*, 72 h, NOEC**	**0.0001 mg/L**	**INERIS, 2012[7]**
Aquatic plants	*Lemna gibba*, 14 d, EC_{50}	0.039 mg/L	INERIS, 2012[7]
Sediment dwelling organisms	***Chironomus riparius*, 28 d, NOEC**	**2 mg/kg**	**INERIS, 2012[7]**

5.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	>5000 mg/kg bw	EFSA Conclusion, 2007[1]
Short-term toxicity	**Rat, oral, 13 weeks, NOAEL**	**19.47 mg/kg bw/day**	**EFSA Conclusion, 2007[1]**
Short-term toxicity	Dog, oral, 1 year, NOAEL	100 mg/kg bw/day	EFSA Conclusion, 2007[1]
Long-term toxicity	**Rat, 2 years, NOAEL**	**23.27 mg/kg bw/day (rounded to 25 mg/kg bw/day)**	**EFSA Conclusion, 2007[1] INERIS, 2012[7]**
Long-term toxicity	Mouse, 2 years, NOAEL	62.2 mg/kg bw/day	EFSA Conclusion, 2007[1]
Reproductive toxicity	a)Parental NOAEL b)Reproductive NOAEL c)Offspring NOAEL	a)35.5 mg/kg bw/day b)206.1 mg/kg bw/day c)41.9 mg/kg bw/day	EFSA Conclusion, 2007[1] INERIS, 2012[7]
Developmental toxicity	Rat, a)maternal NOAEL b)developmental NOAEL	a)50 mg/kg bw/day b)500 mg/kg bw/day	EFSA Conclusion, 2007[1]
Developmental toxicity	Rabbit, maternal and developmental NOAEL	350 mg/kg bw/day	EFSA Conclusion, 2007[1]
Reproductive toxicity	*Colinus virginianus*, 20 weeks, NOAEL	91.84 mg/kg bw	EFSA Conclusion, 2007[1] INERIS, 2012[7]

Both studies in bold were used for calculation of the ADI (EFSA Conclusion, 2007[1]).

5.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Scenedesmus subspicatus*, 72 h, NOEC	0.0001 mg/L	10 [a]	1.00E-05 mg/L[7]
$PNEC_{sed}$	*Chironomus riparius*, 28 d, NOEC	2 mg/kg	100 [b]	0.020 mg/kg dw [7]
$PNEC_{biota,sec\ pois}$	Rats, chronic toxicity, conversion factor 20, 2 years, NOAEL	25 mg/kg day	30	16.7 mg/kg food [7]
$PNEC_{biota,\ hh}$	ADI[1]	0.2 mg/kg bw/day	- [c]	12.174 mg/kg food
$PNEC_{dw,\ hh}$	ADI [1]	0.2 mg/kg bw/day	- [d]	0.7 mg/L

[a] Three long term values were available from the main trophic levels
[b] One long term test available
[c] ADI value used in equation B as TL. See section 3.3.4 for calculation
[d] ADI value used in equation C as TL_{hh}. See section 3.3.5 for calculation

6. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**575**
RQ_{sed}	5.6
$RQ_{biota,sec\ pois}$	0.55
$RQ_{biota,\ hh}$	0.75
$RQ_{dw,\ hh}$	0.01

7. References

[1] EFSA Scientific Report (2007) 122, 1-84, Conclusion on the peer review of diflufenican. Available at http://www.efsa.europa.eu/de/scdocs/doc/122r.pdf

[2] European Pesticides Database http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[3] NORMAN Database http://www.norman-network.net/?q=node/24

[4] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

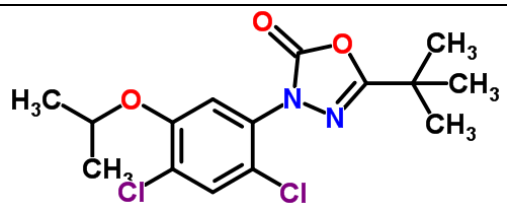
[5] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

[6] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report.

[7] INERIS DIFLUFENICANIL – N° CAS : 83164-33-4 (April, 2012). Available at : http://www.ineris.fr/substances/fr/substance/getDocument/2990

Oxadiazon (CAS N. 19666-30-9)

1. Substance identity

EC name	3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2(3H)-one
EC number	243-215-7
CAS number	19666-30-9
Molecular formula	C15H18Cl2N2O3
Molecular weight	345.22
Structure	
SMILES	CC(C)Oc1cc(c(cc1Cl)Cl)n2c(=O)oc(n2)C(C)(C)C

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	1.035E-04	EFSA Conclusion, 2010 [1]
Water solubility (mg/L)	0.57	EFSA Conclusion, 2010 [1]
logK_{ow}	5.33	EFSA Conclusion, 2010 [1]

3. Environmental fate

Endpoint	**Value**	**Source**
Sorption potential (K_{oc})	1294	EFSA Conclusion, 2010 [1]
Biodegradability	NRB	EFSA Conclusion, 2010 [1]
Bioaccumulation (BCF)	243	EFSA Conclusion, 2010 [1]
BMF	1	Default value, TG n. 27 - CIS WFD [2]

4. Environmental exposure assessment

	Description	**Source**
Tonnes/year	-	
Uses	Herbicide (PPP)	
Spatial usage (by MS)	BE, CY, ES, FR, IT, LU, PT, SK, UK	EU Pesticides DB[3]
Banned uses	-	
ERC code	ERC8d	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.039	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.496	FOCUS Step 2
PEC_{biota} (mg/kg)	9.477	Calculation based on Equation L (Section 3.4.3)

4.11 Comparison of FOCUS Pesticides models

FOCUS Step 1 [a]

Crop [1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Sunflower	1 × 750 g/ha (pre-emergence)	0.57	1294	127

FOCUS Step 2 [a]

Same parameters and conditions as above, in addition to DT_{50soil} 120 d [1], $DT_{50water}$: 127 d [1], $DT_{50sediment}$ 999 d [1], no crop interception [1].

FOCUS Step 3 – SWASH Package [a]

[a] No new calculations were made, since PEC_{fw} and PEC_{sed} values were retrieved from EFSA Conclusion, 2010 [1].

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.0986	1.19
FOCUS Step 2	**0.039**	0.496
FOCUS Step 3	0.0084 (R4 stream)	0.025

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
2 (FR, IT)	NORMAN DB, 2014[4]	$MEC_{95, whole}$: 0.07 µg/L $MEC_{95, dissolved}$: 0.168 µg/L	1 MS [7] EQS set (WRc, 2012)[8]
	IPCheM[5]	MEC_{95}: 12.8 µg/L	
	IT Monitoring Programme[6]	MEC_{95}: 0.376 µg/L	

5 P, B, T, C, M, R, ED properties

Oxadiazon itself did not present genotoxic potential[1]. Liver tumours were observed in both the rat and mouse species; mechanistic studies confirmed that oxadiazon is a peroxisome proliferator[1]. Although peroxisome proliferators are hepatocarcinogens in rodents, the current scientific opinion is that humans are not responsive to this class of non-genotoxic carcinogens and therefore, oxadiazon is unlikely to present a carcinogenic risk to humans[1]. Effects on the reproduction (increase in gestation length and

irregular oestrus cycle) were more prominent in a preliminary dose-range finding study to the multigeneration study where total litter losses were observed at ca. 30 mg/kg bw/day, as the main study was conducted with much lower dose levels. On this basis a classification with the risk phrase R62 "possible risk of impaired fertility" was proposed[1]. Oxadiazon was considered to be very toxic to aquatic organisms, with algae and fish reproduction as the most sensitive endpoints. It is not reaily biodegradable (P).A bioaccumulation study with bluegill sunfish (*Lepomis macrochirus*) gave BCF values of 243 (not B) based on measured oxadiazon residues in fish (EFSA Conclusion, 2010[1]).

6 Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Lepomis macrochirus*, 96 h, LC_{50}	1.2 mg/L	EFSA Conclusion, 2010[1]
Fish	*Oncorhynchus mykiss*, 96 h, LC_{50}	1.2 mg/L	EFSA Conclusion, 2010[1]
Fish	***Oncorhynchus mykiss*, 60 d, ELS NOEC**	**0.00088 mg/L**	**EFSA Conclusion, 2010[1]**
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}, mortality	> 2.4 mg/L	EFSA Conclusion, 2010[1]
Aquatic invertebrates	*Daphnia magna*, 21 d, NOEC, reproduction	0.03 mg/L	EFSA Conclusion, 2010[1]
Algae	*Anabaena flos-aquae,* 120 h, EC_{50}, growth rate	>3.7 mg/L	EFSA Conclusion, 2010[1] DAR, 2006[9]
Algae	*Selenastrum capricornutum,* 120 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)0.0082 mg/L b)0.021 mg/L	EFSA Conclusion, 2010[1]
Algae	*Navicula pelliculosa*, 120 h, a) EC50, b) NOEC	a)0.128 mg/L b) 0.027 mg/L	DAR, 2006[9]
Algae	*Scenedesmus subspicatus*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate, c) NOEC	a)0.00318 mg/L b) 0.00423 c) 0.002 mg/L	EFSA Conclusion, 2010[1] DAR, 2006[9]
Algae with sediment	*Scenedesmus subspicatus*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate, c) NOEC	a)0.0096 mg/L b)0.0108 mg/L c) 0.005 mg/L	EFSA Conclusion, 2010[1] DAR, 2006[9]
Aquatic plants	*Lemna gibba*, 14 d, frond count, a) EC_{50}, b) NOEC	a)0.057 mg/L b) 0.0082 mg/L	EFSA Conclusion, 2010[1] DAR, 2006[9]
Sediment dwelling organisms	*Chironomus riparius*, 28 d, NOEC	5 mg/L	EFSA Conclusion, 2010[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	>5000 mg/kg bw/day	EFSA Conclusion, 2010[1]
Chronic	**Rat, 2 years, NOAEL**	**0.36 mg/kg bw/day**	**EFSA Conclusion, 2010[1]**
Chronic	Mouse, 2 years, NOAEL	0.92 mg/kg bw/day	EFSA Conclusion, 2010[1]
Chronic	Dog, 2 years, NOAEL	1.2 mg/kg bw/day	EFSA Conclusion, 2010[1]
Combined repeated dose and reproduction / developmental screening	Rat, NOAEL	12 mg/kg bw/day	EFSA Conclusion, 2010[1]
Long-term toxicity (reproductive toxicity)	Rat, NOAEL, parental and offspring toxicity	15 mg/kg bw/day	EFSA Conclusion, 2010[1]
Long-term toxicity (reproductive toxicity)	Rat, NOAEL, reproductive toxicity	5 mg/kg bw/day	EFSA Conclusion, 2010[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	***Oncorhynchus mykiss*, 60 d, ELS NOEC**	0.00088 mg/L	10 [a]	0.000088 mg/L
$PNEC_{sed}$	*Chironomus riparius*, 28 d, NOEC	5 mg/L	100 [b]	0.05 mg/L
$PNEC_{biota,sec\ pois}$	Rat, 2 years, NOAEL	0.36 mg/kg bw/day	30 [c]	0.24 mg/kg [d]
$PNEC_{biota,\ hh}$	ADI	0.0036 mg/kg bw/day	-	0.22 mg/kg food [e]
$PNEC_{dw,\ hh}$	ADI	0.0036 mg/kg bw/day	-	0.0126 [f]

[a] Three NOEC values (including algae). AF selected according to ECHA guidance and to TG n.27- CIS WFD, pg 38

[b] Since just 1 NOEC value was available, used an AF of 100. Followed TG n.27- CIS WFD, pg. 96

[c] AF selected based on the duration of the test of 2 y

[d] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of NOAEL (0.36 mg/kg bw/day) value into NOEC (7.2 mg/kg) by using the conversion factor of 20 (taken from TG n. 27- CIS WFD); b) Application of appropriate AF_{oral} (30) to the NOEC value (see Note c).

[e] ADI value retrieved from EFSA Conclusion, 2010 used for PNEC calculation according to Equation E (see section 3.3.4)

[f] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7 Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**443.18**
RQ_{sed}	9.92
$RQ_{biota,sec\ pois}$	39.49
$RQ_{biota,\ hh}$	43.25
$RQ_{dw,\ hh}$	3.10

8 References

[1] EFSA Journal 2010; 8(2):1389, available at http://www.efsa.europa.eu/it/efsajournal/pub/1389.htm

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[3] EU Pesticides Database http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] NORMAN Database http://www.norman-network.net/?q=node/24

[5] IPCheM database at http://ipchem.jrc.ec.europa.eu/

[6]Italian Monitoring Programme (data provided directly to the JRC)

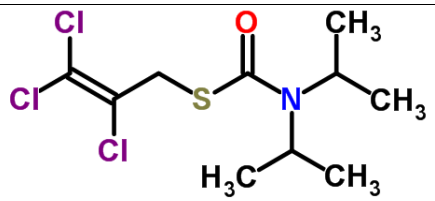
[7]Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report

[8] Contract No. 070311/2011/603663/ETU/D1 "Comparative Study of Pressures and Measures in the Major River Basin Management Plans' - Task 2c (Comparison of Specific Pollutants and EQS): Final Report". WRc Ref: UC8981/1 October 2012. Available at:
http://ec.europa.eu/environment/archives/water/implrep2007/pdf/P_M%20Task%202c.pdf

[9] DAR Oxadiazon, Volume 3 – Annex B.9 (2006).

Tri-allate (CAS N. 2303-17-5)

1. Substance identity

EC name	S-2,3,3-trichloroallyl diisopropylthiocarbamate
EC number	218-962-7
CAS number	2303-17-5
Molecular formula	C10H16Cl3NOS
Molecular weight	304.7
Structure	
SMILES	CC(C)N(C(C)C)C(=O)SCC(=C(Cl)Cl)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	0.012	EFSA conclusion, 2008 [1]
Water solubility (mg/L)	4.1	EFSA conclusion, 2008 [1]
logK_{ow}	4.06	EFSA conclusion, 2008 [1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	4301.4	EFSA conclusion, 2008 [1]
DT50 water	103.8 d	EFSA conclusion, 2008 [1]
Biodegradability	NRB	EFSA conclusion, 2008 [1]
Bioaccumulation (BCF)	1400	EFSA conclusion, 2008 [1]
BMF	1	Default value, TG n. 27 - CIS WFD [2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	10150 (year 2003)	From previous prioritisation exercise
Uses	Herbicide (PPP)	
Spatial usage (by MS)	AT, BE, CZ, FR, IE, IT, NL, UK	EU Pesticides DB [3]
Banned uses	-	
ERC code	ERC8d	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.118	FOCUS Step 2
PEC_{sed} (mg/kg dw)	2.56	FOCUS Step 2
PEC_{biota} (mg/kg)	165.2	Calculation based on Equation L (Section 3.4.3)

4.1.1 Comparison of FOCUS Pesticides models with ECETOC model

FOCUS Step 1

Crop [1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Winter cereals (for Step 1 calculations, used Pome & Stone Fruits late, as reported in the EFSA Conclusion, 2008[1])	1 × 2250 g/ha	4.1	4301.4	68.2

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 58.2 d [1], $DT_{50water}$: 103.8 d [1], $DT_{50sediment}$ 210.9 d [1], no crop interception [1].

FOCUS Step 3 – SWASH Package

No new calculations were made, since PEC_{fw} and PEC_{sed} values were retrieved from EFSA Conclusion, 2008 [1].

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
ECETOC	0.19	86
FOCUS Step 1	0.229	5.49
FOCUS Step 2	**0.118**	2.56
FOCUS Step 3 (D2, ditch-Brimstone)	0.042	0.089

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
2 (FR, NL)	NORMAN DB, 2014 [4]	$MEC_{95, whole}$: 0.1875 µg/L	-

5. P, B, T, C, M, R, ED properties

Tri-allate is unlikely to be genotoxic[1]. Tri-allate is unlikely to pose carcinogenic risk to humans[1]. No evidence of treatment-related oncogenicity was found in either rats, mice or hamsters[1]. No classification proposed for reproductive toxicity[1]. The substance is not readily biodegradable (P). It has a BCF value of 1400[1].

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhynchus mykiss*, 96 h, LC_{50}	0.95 mg/L	EFSA conclusion, 2008[1]
Fish	*Oncorhynchus mykiss*, 88 d , NOEC, growth	0.038 mg/L	EFSA conclusion, 2008[1]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	0.091 mg/L	EFSA conclusion, 2008[1]
Aquatic invertebrates	*Daphnia magna*, 21 d, NOEC, reproduction	0.013 mg/L	EFSA conclusion, 2008[1]
Algae	***Pseudokirchneriella subcapitata*, 96 h,** measured concentrations, a) EC_{50} biomass, b) NOEC biomass, c) EC_{50} growth rate, d) **NOEC growth rate**	a)0.013 mg/L b) 0.0034 mg/L c) 0.036 mg/L **d) 0.0067 mg/L**	EFSA conclusion, 2008[1] **DAR, 2007 [5]**
Algae	*Anabaena flos-aquae*, 96 h, measured concentrations, a) NOEC growth rate, b) EC_{50} growth rate;, c) NOEC biomass, d) EC_{50} biomass	a) 1.6 mg/L b) >3.7 mg/L c) 1.6 mg/L d) 2.6 mg/L	DAR, 2007 [5]
Aquatic plants	*Lemna gibba*, EC_{50}	2.3 mg/L	EFSA conclusion, 2008[1]
Sediment dwelling organisms	*Chironomus riparius*, 28 d, NOEC, EMERGENCE	0.583 mg/L	EFSA conclusion, 2008[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	1100 mg/kg bw	EFSA conclusion, 2008[1]
Short term oral toxicity	Rat, 90 d, NOAEL	6.4 mg/kg bw/day	EFSA conclusion, 2008[1]

(neurotoxicity)			
Short term oral toxicity	Dog, 8-weeks capsule study, NOAEL	12 mg/kg bw/day	EFSA conclusion, 2008[1]
Short term oral toxicity	**Dog, 1-year capsule study, NOAEL**	**2.5 mg/kg bw/day**	**EFSA conclusion, 2008[1]**
Short term oral toxicity	Mice, 8-weeks, NOAEL	11.5 mg/kg bw/day	EFSA conclusion, 2008[1]
Short term oral toxicity	Hamster, 90 d, NOAEL	43.2 mg/kg bw/day	EFSA conclusion, 2008[1]
Chronic	Rat, NOAEL, 2 years	2.5 mg/kg bw/day	EFSA conclusion, 2008[1]
Chronic	Mice, 2-year, NOAEL	12.4 mg/kg bw/day	EFSA conclusion, 2008[1]
Chronic	Hamster, 79-95 weeks, NOAEL	16.2 mg/kg bw/day	EFSA conclusion, 2008[1]
Reproductive toxicity	NOAEL	7.7 mg/kg bw/day	EFSA conclusion, 2008[1]
Developmental toxicity	Rat, NOAEL	30 mg/kg bw/day	EFSA conclusion, 2008[1]
Developmental toxicity	Rabbit, NOAEL a)maternal toxicity b)developmental toxicity	a)15 mg/kg bw/day b)5 mg/kg bw/day	EFSA conclusion, 2008[1]
Long-term toxicity	Rat, NOEL	9 mg/kg bw/day	EFSA conclusion, 2008[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Pseudokirchneriella subcapitata, 96 h,* NOEC[5]	0.0067 mg/L	10	0.00067 mg/L [a]
$PNEC_{sed}$	-	-	-	0.145 mg/kg [b]
$PNEC_{biota,sec\ pois}$	Rats, chronic toxicity, conversion factor 20, 2 years, NOAEL [1]	2.5 mg/kg bw/day	30	1.67 mg/kg food [c]
$PNEC_{biota,\ hh}$	ADI	0.025 mg/kg bw/day	-	1.522 mg/kg food [d]
$PNEC_{dw,\ hh}$	ADI	0.025 mg/kg bw/day	-	0.088 mg/L [e]

[a] Three NOEC values available from the three main trophic levels (fish, aquatic invertebrates, algae)

[b] $PNEC_{sed}$ calculated with the Equilibrium Partitioning Method, where $K_{sed\text{-}water}$= 108.34 m^3m^{-3} (Eq. D), RHO_{sed}= 1300 kg m-3 (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 215.07 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 4301.4 L/kg [1], Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. B (Section 3.3.2).

[c] The AF of 30 was selected according to the duration test (2 years) (TG n. 27 - CIS WFD)[2] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of NOAEL (2.5 mg/kg bw/day) value into NOEC (50 mg/kg) by using the conversion factor of 20 (taken from TG n. 27- CIS WFD); b) Application of appropriate AF_{oral} (30) to the NOEC value (The AF of 30 was selected according to the duration test (2 years) (TG n. 27 - CIS WFD)[4]

[d] ADI value retrieved from EFSA Conclusion, 2008 used for PNEC calculation according to Equation E (see section 3.3.4)

[e] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**176.12**
RQ_{sed}	17.63
$RQ_{biota,sec\ pois}$	99.12
$RQ_{biota,\ hh}$	108.56
$RQ_{dw,\ hh}$	1.35

8. References

[1] EFSA Scientific Report (2008) 181, 1-100 Conclusion on the peer review of tri-allate. Available at http://www.efsa.europa.eu/it/efsajournal/pub/181r.htm

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

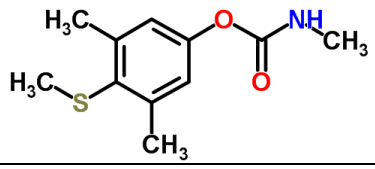
[3] EU Pesticides Database http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] NORMAN Database http://www.norman-network.net/?q=node/24

[5] DAR Tri-allate, Volume 3, Annex B.9 : Ecotoxicology (2007)

Methiocarb (CAS N. 2032-65-7)

1. Substance identity

Chemical name (IUPAC)	Mercaptodimethur/ 3,5-Dimethyl-4-methylthiophenyl N-methylcarbamate
EC number	217-991-2
CAS number	2032-65-7
Molecular formula	C11H15NO2S
Molecular weight	225.3
Structure	
SMILES	CNC(=O)Oc1cc(C)c(SC)c(C)c1

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	1.5E-05	EFSA Conclusion, 2010[1]
Water solubility (mg/L)	27	EFSA Conclusion, 2010[1]
logK_{ow}	3.18	EFSA Conclusion, 2010[1]

3. Environmental fate

Endpoint	**Value**	**Source**
Hydrolysis (DT50)	9.1 d	EFSA Conclusion, 2010[1]
Sorption potential (K_{oc})	Highest value: 1000 Mean value: 660 (used for PEC calculation Step 2)	EFSA Conclusion, 2010[1]
Biodegradability	NRB	EFSA Conclusion, 2010[1]
Bioaccumulation (BCF)	75.86	Source: Experimental value retrieved from VegaNIC vers. 1.0.8
BMF	1	Default value, TG n. 27 - CIS WFD [2]

4. Environmental exposure assessment

	Description	**Source**
Tonnes/year	1500 (year 2000)	From previous prioritisation exercise
Uses	Insecticide, Repellant (PPP)	
Spatial usage (by MS):	Authorised in: AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HU, IE, IT, LV, NL, PL, PT, RO, SI, SK, UK	EU Pesticides DB [3]

Banned uses	Moluscicide	Commission Implementing Regulation (EU) No 187/2014 [4]
ERC code	ERC8d	
Fraction of tonnage to region	0.1	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.00395	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.026	FOCUS Step 2
PEC_{biota} (mg/kg)	0.30	Calculation based on Equation L (Section 3.4.3)

4.1.1 Comparison of FOCUS Pesticides models with ECETOC model

FOCUS Step 1

Crop[1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} (d) [1]
Maize	2×150 (seed treatment)	27	660 (mean)	15.3 d (whole system)

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 2.8 d [1], $DT_{50water}$: 8.5 d [1], $DT_{50sediment}$ 20.1 d [1], no crop interception (seed treatment) [1].

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
ECETOC	0.044	4.54
FOCUS Step 1	0.0266	0.176
FOCUS Step 2	0.00395	0.026

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
5 (FR, NL, UK, SE, IT)	NORMAN DB, 2014 [5]	$MEC_{95, whole}$: 0.0585 µg/L	1 MS [9]
	IPCheM [6]	MEC_{95}: 0.095 µg/L	
	SE pesticide monitoring programme [7]	All values < LOQ	
	IT Monitoring Programme [8]	MEC_{site}: 0.02 µg/L	

5. P, B, T, C, M, R, ED properties

Methiocarb is acutely very toxic by oral route and toxic after inhalation in rats[1]. Methiocarb was clastogenic in the chromosomal aberration assay in CHO cells but this was not confirmed in cytological

analysis in a micronucleus test[1]. There was no evidence of genotoxicity in other in vitro and in vivo studies[1]. There was no evidence of carcinogenicity in mice and rats[1]. Methiocarb did not affect reproductive and developmental parameters and did not show any potential to cause delayed neurotoxicity[1]. The substance is not readily biodegradable.It has a BCF that ranges from 60 to 90 in fish[1]. However, also a BCF experimental value of 75.86 L/kg was reported in the VEGA Nic software, and used for calculations.

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Lepomis macrochirus*, 96 h, LC_{50}	0.65 mg/L	EFSA Conclusion, 2006[1] DAR, 2004[10]
Fish	*Oncorhyncus mykiss*, 56 d, NOEC	0.05 mg/L	EFSA Conclusion, 2006[1] DAR, 2004[10]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	0.0077 mg/L	EFSA Conclusion, 2006[1] DAR, 2004[10]
Aquatic invertebrates	***Daphnia magna*, 21 d NOEC, reproduction**	**0.0001 mg/L**	**EFSA Conclusion, 2006[1] DAR, 2004[10]**
Algae	*Scenedesmus subspicatus*, 72 h, a) EC_{50} growth rate, b) EC_{50} biomass, c) NOEC based on biomass	a)2.2 mg/L b)0.82 mg/L c) 0.052 mg/L	EFSA Conclusion, 2006[1] DAR, 2004[10]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD50	19 mg/kg bw (based on weight of evidence of published data with range of 13–135 mg/kg bw).	EFSA Conclusion, 2006[1]
Sub-chronic toxicity (with investigation of neurofunction)	**Dog, 90 d, dietary study, NOAEL. Value also used for ADI calculation in the EFSA Conclusion.**	**1.33 mg/kg bw/day**	**EFSA Conclusion, 2006[1]**
Long term toxicity	Dog, 2-year, dietary study, NOAEL	2.2 mg/kg bw/day	EFSA Conclusion, 2006[1]
Reproductive toxicity	NOAEL	4.3 mg/kg bw/day	EFSA Conclusion, 2006[1]
Developmental toxicity	NOAEL, a) parental toxicity, b) developmental toxicity	a)0.5 mg/kg bw/day b)10 mg/kg bw/day	EFSA Conclusion, 2006[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna*, 21 d, NOEC	0.0001 mg/L	10 [a]	1.00E-05 mg/L [a]
$PNEC_{sed}$	-	-	-	0.001 mg/kg dw [b]
$PNEC_{biota,sec\ pois}$	Dog, 90 d, conversion factor 40, NOAEL	1.33 mg/kg bw/day	90	0.591 mg/kg food [c]
$PNEC_{biota,\ hh}$	ADI	0.013 mg/kg bw/day	-	0.791 mg/kg food [d]
$PNEC_{dw,\ hh}$	ADI	0.013 mg/kg bw/day	-	0.046 mg/L [e]

[a] An AF of 10 was chosen based on the availability of three NOEC values from the three main trophic levels (fish, aquatic invertebrates, algae), according to TG n. 27- CIS WFD

[b] $K_{sed\text{-}water}$= 25.8 m^3m^{-3} (calculated with eq. D), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 50 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 1000 L/kg, Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. B (Section 3.3.2).

[c] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of NOAEL (1.33 mg/kg bw/day) value into NOEC (53.2 mg/kg) by using the conversion factor of 40 (taken from TG n. 27- CIS WFD); b) Application of appropriate AF_{oral} (90) to the NOEC value. The AF was selected according to the duration of the test (90 d)

[d] ADI value retrieved from EFSA Conclusion, 2006 used for PNEC calculation according to Equation E (see section 3.3.4)

[e] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**395**
RQ_{sed}	50.39
$RQ_{biota,sec\ pois}$	0.51
$RQ_{biota,\ hh}$	0.38
$RQ_{dw,\ hh}$	0.09

8. References

[1] EFSA Scientific Report (2006) 79, 1-82, Conclusion on the peer review of methiocarb. Available at http://www.efsa.europa.eu/it/efsajournal/doc/79r.pdf

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[3] European Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] Commission Implementing Regulation (EU) No 187/2014 of 26 February 2014 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance methiocarb. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32014R0187&from=EN

[5] NORMAN database at http://www.norman-network.net/?q=node/24

[6] IPCheM database at http://ipchem.jrc.ec.europa.eu/

[7] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

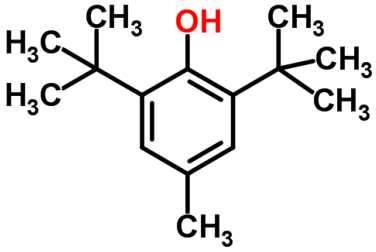
[8] Italian Monitoring Programme (data provided directly to the JRC)

[9] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report

[10] DAR Methiocarb – Volume 3, Annex B, Ecotoxicology (June 2004)

2,6-di-tert-butyl-4-methylphenol (CAS N. 128-37-0)

1. Substance identity

EC name	2,6-di-tert-butyl-p-cresol
EC number	204-881-4
CAS number	128-37-0
Molecular formula	C15H24O
Molecular weight	220.350
Structure	
SMILES	c1(c(O)c(C(C)(C)C)cc(c1)C)C(C)(C)C

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	1.1	ECHA, 2013[1]
Water solubility (mg/L)	0.76	ECHA, 2013[1]
logK_{ow}	5.1	ECHA, 2013[1]

3. Environmental fate

Endpoint	**Value**	**Source**
Sorption potential (K_{oc})	8183	ECHA, 2013[1]
Biodegradability	NRB	ECHA, 2013[1]
Bioaccumulation (BCF)	2500	ECHA, 2013[1]
BMF	2	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	**Source**
Tonnes/year	A confidential and recent tonnage value was used for calculation	IUCLID, 2013[3]
Uses	Industrial uses, use in plastics, rubber products, adhesives, coatings, dyes, fuel (biodiesel), use for the formulation of PPP	ECHA, 2013[1]

	and biocides, use as laboratory reagent. Used as antioxidant in food. Uses in Europe (UK communication): Stabiliser for rubber (largely during polymerisation) (50%) Stabiliser for oils, lubricants and fuels (25%) Stabiliser for plastics (10%) Food additive/others (15%)	
Spatial usage (by MS)	Not known	
Banned uses	**-**	
ERC code	ERC8d	
Fraction of tonnage to region	0.1	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.423	ECETOC
PEC_{sed} (mg/kg dw)	367.64	ECETOC
PEC_{biota} (mg/kg)	2115	Calculation based on Equation L (Section 3.4.3)

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
None[a]	-	**-**	-

[a] Monitoring data was provided by SE but only regarding sewage treatment effluent and sludge

5. P, B, T, C, M, R, ED properties

IARC classification: group 3, as not classifiable as to its carcinogenicity to humans. There is limited evidence for the carcinogenicity of butylated hydroxytoluene in experimental animals.[3]

Not genotoxic[1]. The substance is not readily biodegradable. The highest BCF value was of 2500 L/kg (ECHA, 2013[1]).

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Danio rerio*, 96 h, LC_0	≥0.57 mg/L	ECHA, 2013[1]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}, mobility	0.48 mg/L	ECHA, 2013[1]
Aquatic invertebrates	***Daphnia magna*, 21 d, NOEC, reproduction**	**0.316 mg/L**	**ECHA, 2013[1]**
Algae	*Scenedesmus subspicatus*, 72 h, EC_{50}, biomass and growth rate	>0.4 mg/L	ECHA, 2013[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute toxicity	Rat, oral, LD_{50}	>2930 mg/L	ECHA, 2013[1]
Repeated dose toxicity, carcinogenicity	**Rat, oral, two-generation carcinogenicity study, 22 months, NOAEL. The effect value was used for DMEL calculation in the ECHA report.**	**25 mg/kg bw/day**	**ECHA, 2013[1]**
Repeated dose toxicity, carcinogenicity	Rat, oral, two-generation carcinogenicity study, 22 months, NOAEL	100 mg/kg bw/day	ECHA, 2013[1]
Reproductive toxicity	Rat, oral, 22 months, NOAEL	500 mg/kg bw/day	ECHA, 2013[1]
Developmental toxicity	Rat, oral, 22 months, NOAEL	100 mg/kg bw/day	ECHA, 2013[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna*, 21 d, NOEC	0.316 mg/L	100 [a]	3.16E-03 mg/L [b]
$PNEC_{sed}$	-	-	-	1.290 mg/kg dw [c]
$PNEC_{biota,sec\ pois}$	-	-	30	16.7 mg/kg food [d]
$PNEC_{biota,\ hh}$	DMEL	0.25 mg/kg bw/day	-	15.217 mg/kg food [e]
$PNEC_{dw,\ hh}$	DMEL	0.25 mg/kg bw/day	-	0.875 mg/L [f]

[a] One long term value from *Daphnia magna* was available.

[b] In IUCLID, three different $PNEC_{fw}$ were reported. Two were calculated from QSAR estimations, and the third one was calculated from the same study on *Daphnia magna*, as reported in the table above, with the same AF.

[c] Equilibrium partitioning method used in the ECHA dossier[1]. No new calculations were performed.

[d] All values used were retrieved from ECHA dossier[1]

[e] DMEL value, retrieved from ECHA, 2013[1], used for PNEC calculation according to Equation E (see section 3.3.4)

[f] DMEL value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	133.86
RQ_{sed}	**283.24**
$RQ_{biota,sec\ pois}$	126.65
$RQ_{biota,\ hh}$	138.99
$RQ_{dw,\ hh}$	0.48

8. References

[1]ECHA Dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d82f461-e7b6-3a89-e044-00144f67d249/AGGR-7097be3d-db74-4fb0-9968-20bfdf833cb2_DISS-9d82f461-e7b6-3a89-e044-00144f67d249.html#AGGR-7097be3d-db74-4fb0-9968-20bfdf833cb2

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

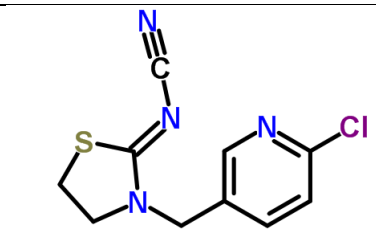
[3] Complete IUCLID dossier of 2,6-di-tert-butyl-4-methylphenol

[4] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

[5] IARC monograph Vol. 40 available at http://monographs.iarc.fr/ENG/Monographs/vol1-42/mono40.pdf, and http://monographs.iarc.fr/ENG/Classification/ClassificationsCASOrder.pdf).

Thiacloprid (CAS N. 111988-49-9)

1. Substance identity

EC name	(Z)-3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidenecyanamide
EC number	
CAS number	111988-49-9
Molecular formula	C10H9ClN4S
Molecular weight	252.7
Structure	
SMILES	S1C(\N(CC1)Cc1ccc(nc1)Cl)=N\C#N

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	3×10^{-10}	EU List of Endpoints (LoE), 2002[1]
Water solubility (mg/L)	184	EU List of Endpoints (LoE), 2002[1]
logK_{ow}	1.26	EU List of Endpoints (LoE), 2002[1]

3. Environmental fate

Endpoint	Value	Source
DT50 (whole system) (d)	27	EU List of Endpoints (LoE), 2002[1]
Sorption potential (K_{oc})	615	EU List of Endpoints (LoE), 2002[1]
Biodegradability	NRB	
Bioaccumulation (BCF)	3.15	EPI Suite, BCFBAF vers. 3.01
BMF	1	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	-	
Uses	Insecticide (Biocide and PPP)	
Spatial usage (by MS)	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HU, IE, IT, LT, LU, LV, MT, NL, PL, PT, RO, SE, SI, SK, UK	EU Pesticides DB [3]

Banned uses	-	
ERC code	ERC8d (N.R.)	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.0109	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.042 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	0.034 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.11 Comparison of FOCUS Pesticides models

FOCUS Step 1

Crop [1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Pome & Stone Fruits late	2 × 180 (14 d application interval full canopy)	184	615	27

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 1.30 d [1], $DT_{50water}$: 31 d [1], $DT_{50sediment}$ 62 d[1]

FOCUS Step 3 – SWASH Package

In addition to the input values listed above, it was considered an application window of 44 d (30+(n. of applications-1) x application interval)[4], with a foliar application and a pre-harvest interval (PHI) of 14 d. In accordance both with the FOCUS SW Appendix C and D[5] and with the BBCH of 54-75 related to pome and stone fruits (as reported in the GAP table of the LoE, 2002[3]). The following application windows were selected for the relevant crop scenarios for the PAT calculator: D3, D4, D5, R1, R2 from 18/06 to 31/07; R3 and R4 from 24/06 to 07/08. Just for the runs of D4 and D5 scenarios, no results were achieved due to some software errors.

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.085	0.457
FOCUS Step 2	**0.0109**	0.042
FOCUS Step 3	0.0057 (R3 stream)	0.004 (D3 pond)

It is acknowledged that even though thiacloprid is used as PPP and biocide, the application rate approach is not suitable for the calculation of PEC values for biocides, but just for PPP, so the results could be an underestimation. However, the tonnage values related to the biocide use were not available and therefore, no further calculation could be done.

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
4(FI, SE, IT, NL)	WATERBASE, 2014 [6]	All values < LOQ	-
	SE pesticide monitoring programme [7]	MEC_{95}: 0.116 µg/L	
	IT Monitoring Programme [8]	MEC_{95}: 0.03 µg/L	
	NORMAN DB, 2014[9]	MEC_{95}: 0.14 µg/L (NL)	

5 P, B, T, C, M, R, ED properties

Increase in malignant uterine adenocarcinomas and thyroid adenomas in rats and ovarian luteomas in mice was observed for thiacloprid[9]. Tumours occur by a non-genotoxic mechanism and a threshold can be identified for the onset of tumours. Based on the T25 estimate of carcinogenic potency, thiacloprid is considered to be of medium potency within the EU. Classified as Carc Cat 3. (R40)[9]. Data indicate that thiacloprid is not mutagenic in vitro. In addition, a negative result was obtained in a standard in vivo micronucleus test. No classification was proposed for reproductive toxicity[9]. The substance is not readily biodegradable, however data presented in the Biocide Assessment Report show that thiacloprid did degrade reasonably rapidly in the aquatic environment with DT_{50} values of 31 and 62 d derived from an outdoor microcosm for the water and sediment compartments respectively. Thiacloprid has a low potential for bioconcentration with a mean measured log P_{ow} = 1.26 (Biocide Assessment Report[9]). An estimated BCF value of 3.16 was provided by Episuite. Finally, according to the available data, the most sensitive chronic endpoint for thiacloprid is that derived for a 28 d Chironomus study (NOEC of 0.0005 mg L^{-1}). This means that the trigger of < 0.01 mg l-1 given in the TGD is exceeded and thiacloprid fulfils the toxic criterion[9].

6 Hazard assessment

6.11 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Lepomis macrochirus*, 96 h, LC_{50}	25.2 mg/L	Biocide Assessment Report, 2008[9]
Fish	*Oncorhyncus mykiss*, 97 d, a)LC_{50}, b) NOEC	a)>3.91 mg/L b)0.24 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Daphnia magna*, 48 h, immobility, EC_{50}	≥85.1 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Daphnia magna*, 48 h, immobility, EC_{50}	≥100 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Hyalella azteca*, 96 h, immobility, EC_{50}	0.0407 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Hyalella azteca*, 96 h, immobility, EC_{50}	≥47 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Asellus aquaticus*, 48h, mortality & immobility,	0.0758 mg/L	Biocide Assessment Report, 2008[9]

	EC_{50}		
Aquatic invertebrates	*Gammarus pulex*, 48 h, mortality & immobility, EC_{50}	0.027 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Ecydonurus pulex*, 48 h, immobility, EC_{50}	0.0077 mg/L	Biocide Assessment Report, 2008[9]
Aquatic invertebrates	*Daphnia magna*, 21 d, survival, reproduction and growth, NOEC	0.58 mg/L	Biocide Assessment Report, 2008[9]
Algae	*Scenedesmus subspicatus,* 72 h, growth inhibition, a) NOEC, b) EC_{50} biomass, c) EC_{50} growth rate	a)32 mg/L b)44.7 mg/L c)96.7 mg/L	Biocide Assessment Report, 2008[9]
Aquatic plants	*Lemna gibba,* 15 d, reduced frond number, EC_{50}	>95.4 mg/L	Biocide Assessment Report, 2008[9]
Sediment dwelling organisms	***Chironomus riparius,* 28 d, a) NOEC number and time of emergence**, b)EC_{50} emergence rate c) EC_{50} development	**a)0.0005 mg/L** b)0.00218 mg/L c)≥0.0018 mg/L	**Biocide Assessment Report, 2008[9]**
Insects, sediment dwellers, zooplankton, phytoplankton	Outdoor microcosm study. Most sensitive group: Ceratopogonidae (insects), e.g. increase of number of species, 98 d, EAC	0.0016 mg/L	Biocide Assessment Report, 2008[9]

6.12 Mammalian toxicology data

Type of test	**Endpoint**	**Value**	**Reference**
Acute oral toxicity	Rat, LD_{50}	444 mg/kg	Biocide Assessment Report, 2008[9]
Repeated oral dose toxicity	**Rat, 2 year, NOAEL. Values used for ADI calculation in the EU Review Report, 2003[8]**	**1.2 mg/kg/day**	**Biocide Assessment Report, 2008[9]**
Carcinogenicity	Rat, lowest dose with tumours	25 mg/kg bw/day	Biocide Assessment Report, 2008[9]
Reproductive toxicity	NOAEL	2 mg/kg/day 3.7 mg/kg/day (dystocia)	Biocide Assessment Report, 2008[9]
Developmental toxicity	NOAEL	2 mg/kg/day	Biocide Assessment Report, 2008[9]

6.13 PNEC derivation

PNEC	**Endpoint**	**Endpoint value**	**AF**	**PNEC value**
$PNEC_{fw}$	*Chironomus riparius*, 28 d, NOEC [a], number and	0.0005 mg/L	10 [b]	5E-05 mg/L [9]

	time of emergence			
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI [c]	0.01 mg/kg bw/day	-	0.035 mg/L

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota

[a] Due to the mode of action of neonicotinoids, the lowest endpoint from the aquatic species tested corresponds to the midge *Chironomus riparius*. Therefore, it was selected for $PNEC_{fw}$ calculation.

[b] Three long term values were available.

[c] ADI value, retrieved from EU Review Report 2003[10], used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7 Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**218.00**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.31

8 References

[1] List of end points, 2002 (based on doc 1654/VI/94, Rev. 7, 22 Apr 1998)

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[3] European Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] Generic guidance for FOCUS surface water scenarios 2011. Available at: http://focus.jrc.ec.europa.eu/sw/index.html

[5] Appendix C and D for parameterisation of drainage and run-off inputs, respectively. Available at: http://focus.jrc.ec.europa.eu/sw/index.html

[6] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[7] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

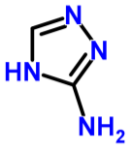
[8] Italian Monitoring Programme (data provided directly to the JRC)

[9] Directive 98/8/EC concerning the placing of biocidal products on the market. Inclusion of active substances in Annex I or IA to Directive 98/8/EC. Assessment report, THIACLOPRID Product-type 8 (Wood Preservative) 2008. Available at: http://dissemination.echa.europa.eu/Biocides/factsheet?id=0053-08

[10] EUROPEAN COMMISSION Review report for the active substance thiacloprid SANCO/4347/2000 – Final. Available at http://ec.europa.eu/food/plant/protection/evaluation/newactive/thiacloprid.pdf

Aminotriazole (CAS N. 61-82-5)

1. Substance identity

Chemical name (IUPAC)	1H-1,2,4-Triazol-3-amine
EC number	200-521-5
CAS number	61-82-5
Molecular formula	C2H4N4
Molecular weight	84.08
Structure	
SMILES	n1nc(N)nc1

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	3.3E-05	INERIS, 2011 [1]
Water solubility (mg/L)	2.6E+05	EC Review report, 2001 [2]
logK_{ow}	-0.97	INERIS, 2011 [1]

3. Environmental fate

Endpoint	Value	Source
Hydrolysis (DT50)	86.1	EC Review report, 2001 [2]
Sorption potential (K_{oc})	94	EFSA conclusion, 2014 [3]
Biodegradability	NRB	INERIS, 2011 [1]
Bioaccumulation (BCF)	2.38 (whole fish) in 7 days	EC Review report, 2001 [2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	22550 (year 1994)	From previous prioritisation exercise
Uses	Herbicide (PPP)	
Spatial usage (by MS):	BE, EL, ES, FR, HU, IT, LU, NL, PT, UK	EU Pesticides DB [4]
Banned uses	-	
ERC code	ERC8d	
Fraction of tonnage to region	0.1	

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.501	FOCUS Step 2 (see Table below for details)
PEC_{sed} (mg/kg dw)	0.459 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	1.192 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 Comparison of FOCUS Pesticides models with ECETOC model

FOCUS Step 1

Crop [3]	Application Rate (g/ha)[3]	Water solubility (mg/L)[3]	K_{oc} (L/kg) [3]	DT_{50} whole system (d) [3]
Orchards (citrus fruits, pome and stone fruits, assorted fruits-edible or inedible peel, tree nuts). For calculations, use of pome and stone fruits (early)	1 x 2977 g/ha	264000	94	86.1

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 7.4 d [3], $DT_{50water}$: 86.1 d [3], $DT_{50sediment}$ 86.1 d [3], no crop interception [3].

FOCUS Step 3 – SWASH Package

No new calculations were made, since PEC_{fw} and PEC_{sed} values were retrieved from EFSA Conclusion, 2014 [3]. The worst case values were from orchards, Autumn application.

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
ECETOC	0.73	9.27
FOCUS Step 1	1.17	1.06
FOCUS Step 2	**0.501**	0.459
FOCUS Step 3 (D3 stream)	0.0176	0.0028

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
1 (FR)	NORMAN DB, 2014 [5]	$MEC_{95, whole}$: 0.873 µg/L	-

5. P, B, T, C, M, R, ED properties

Aminotriazole is classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as toxic for reproduction category 2 and toxic effects were observed in endocrine organs[3]. It is also listed in the Endocrine Disruptor's Database of the European Commission[6] as an ED Category 1 (Cat. 1 Human Health, Cat. 3 Wildlife, Cat. 1 overall). No evidence of a genotoxic potential relevant to humans[3]. The substance is not readily biodegradable (P). It has a low BCF of 2.38 (not B) (EFSA conclusion, 2014)[3].

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhyncus mykiss*, 96 h, LC_{50}	>1000 mg/L	EFSA Conclusion, 2014[3]
Fish	*Oncorhyncus mykiss*, 21 d, NOEC	100 mg/L	EFSA Conclusion, 2014[3]
Fish	*Oncorhyncus mykiss*, 96 h, LC_{50}	>22.15 mg/L	EFSA Conclusion, 2014[3]
Aquatic invertebrate	*Daphnia magna*, 48 h, EC_{50}	6.1 mg/l	EFSA Conclusion, 2014[3]
Aquatic invertebrate	*Mysidopsis bahia,* 96 h, EC_{50}	2.8 mg/l	EFSA Conclusion, 2014[3]
Aquatic invertebrate	***Daphnia magna*, 21 d, NOEC**	**0.32 mg/L**	**EFSA Conclusion, 2014[3]**
Aquatic invertebrate	*Daphnia magna*, 48 h, EC_{50}	2.66 mg/L	EFSA Conclusion, 2014[3]
Algae	*Scenedesmus subspicatus*, 72 h, growth, EC_{50}	2.3 mg/L	EFSA Conclusion, 2014[3]
Algae	*Anabaena flos-aquae*. 120 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)3.9 mg/L b)>4.8 mg/L	EFSA Conclusion, 2014[3]
Algae	*Selenastrum capricornutum*, 72 h growth, a) EC_{50} biomass, b) EC_{50} growth rate	a)1.6 mg/L b)>5.1 mg/L	EFSA Conclusion, 2014[3]
Algae	*Selenastrum capricornutum*, 8 d, a) EC_{50} growth rate and biomass, b)NOEC	a)>6 mg/L b)6 mg/L	EFSA Conclusion, 2014[3]
Algae	*Navicula pelliculosa*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)1.3 mg/L b)>5.1 mg/L	EFSA Conclusion, 2014[3]
Algae	*Selenastrum capricornutum*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)1.5 mg/L b)>3.1 mg/L	EFSA Conclusion, 2014[3]
Algae	*Selenastrum capricornutum*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)27.9 mg/L b)>44.3 mg/L	EFSA Conclusion, 2014[3]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	>5000 mg/kg bw	EFSA Conclusion, 2014[3]
Short term oral toxicity	**Rat, 90 d, NOAEL. Use for ADI calculation in the EFSA conclusion.**	**0.1 mg/kg bw/day**	**EFSA Conclusion, 2014[3]**
Short term oral toxicity	Dog, 1 year, NOAEL	0.3 mg/kg bw/day	EFSA Conclusion, 2014[3]
Long term toxicity	Rat, 2 year, NOAEL	0.5 mg/kg bw/day	EFSA Conclusion, 2014[3]
Long term toxicity	Mice, 18 months, NOAEL	1.5 mg/kg bw/day	EFSA Conclusion, 2014[3]
Long term toxicity	Hamster, 18 months, NOAEL	1 mg/kg bw/day	EFSA Conclusion, 2014[3]
Reproductive toxicity	Rat, 2 generation, NOAEL, a)parental toxicity, b) Reproductive toxicity, c) Offspring toxicity	a)0.12 mg/kg bw/day b)0.9 mg/kg bw/day c)0.9 mg/kg bw/day	EFSA Conclusion, 2014[3]
Developmental toxicity	Rabbit, NOAEL	3 mg/kg bw/day	EFSA Conclusion, 2014[3]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna,* chronic study, 21 d, NOEC	0.32 mg/L	10	0.032 mg/L [a]
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI [b]	0.001 mg/kg bw/day	-	0.004 mg/L [c]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] An AF of 10 was chosen based on the availability of three NOEC values from the three main trophic levels (fish, aquatic invertebrates, algae).

[b] ADI retrieved from the European review report (2001)

[c] ADI value used in equation F as TLhh. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	15.66
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	**143.14**

8. References

[1] INERIS Aminotriazole n. CAS: 61-82-5 (June, 2011). Available at: http://www.ineris.fr/substances/fr/substance/getDocument/3068

[2] EUROPEAN COMMISSION Review report for the active substance amitrole 6839/VI/97-final (2001). Available at http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.detail

[3] EFSA (European Food Safety Authority), 2014. Conclusion on the peer review of the pesticide risk assessment of the active substance amitrole. EFSA Journal 2014;12(7):3742, 84 pp. doi:10.2903/j.efsa.2014.3742

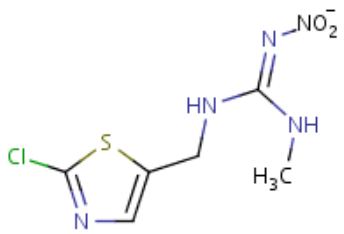
[4] EU Pesticides Database http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[5] NORMAN database at http://www.norman-network.net/?q=node/24

[6] Endocrine Disruptor database of the EU Commission), available at: http://ec.europa.eu/environment/chemicals/endocrine/documents/index_en.htm

Clothianidin (CAS N. 210880-92-5)

1. Substance identity

EC number	433-460-1
CAS number	210880-92-5
Molecular formula	C6H8Cl N5O2S
Molecular weight	249.7
Structure	
SMILES	CN/C(=N\N(=O)[O-])/NCc1cnc(s1)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	1.3E-10	Biocide Assessment Report, 2007[1]
Water solubility (mg/L)	327	Biocide Assessment Report, 2007[1]
logK_{ow}	0.7	Biocide Assessment Report, 2007[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	160	EU Review Report, 2005[2]
Biodegradability	NRB	Biocide Assessment Report, 2007[1]
Bioaccumulation (BCF)	a) BCF_{fish} 0.78 b) BCF 3.16 (estimated)	a) Biocide Assessment Report, 2007[1] b) EPI Suite, BCFBAF V3.01[3]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	-	
Uses	Insecticide (PPP and biocide)	
Spatial usage (by MS)	AT, BE, BG, CZ, DE, DK, EL, ES, FI, FR, HU, IE, IT, LT, NL, PL, PT, RO, SK, UK	EU Pesticides DB[4]

Banned uses	Restriction of uses	EU n. 485/2013[5]
ERC code	ERC8d (N.R.)	
PEC_{fw} (mg/L)	0.008	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.014 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	0.025 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 Comparison of FOCUS Pesticides models

FOCUS Step 1

Crop [2]	Application Rate (g/ha)[2]	Water solubility (mg/L)[2]	K_{oc} (L/kg) [2]	DT_{50} whole system (d) [2]
Sugar beet (seed treatment)	1 × 78	327	160	64.8

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 274 d (geometric mean from field studies)[1], $DT_{50water}$: 64.8 d[2], $DT_{50sediment}$ 1000 d (conservative value), no crop interception (seed treatment). The crop used is not listed in the restricted uses.

FOCUS Step 3 – SWASH package

Due the type of treatment, soil incorporation method was selected for all scenarios in SWASH and the chemical application method (CAM) was set to 8, "soil incorporation at one depth" with a depth of 3 cm for the PRZM scenarios, as specified in comments from a stakeholder. The definition of the application window was made by using the default application dates given by FOCUS SWASH, since the first useful date corresponded to a growth stage of zero, prior to the emergence date for each specific crop scenario (D3, D4, R1, R3). Since drift is assumed to be zero and surface runoff is assumed to be negligible due to the soil depth at which the seeds are drilled, only drainage scenarios were taken into account. Just for D4 run, no results were achieved due to some software error.

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.021	0.034
FOCUS Step 2	**0.008**	0.014
FOCUS Step 3	0.000248 D3 pond	0.0018 D3 pond

It is acknowledged that even though clothianidin is used as PPP and biocide, the application rate approach is not suitable for the calculation of PEC values for biocides, but just for PPP, so the results could be an underestimation. However, the tonnage values related to the biocide use were not available and therefore, no further calculation could be done.

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
1 (SE)	SE monitoring programme[6]	All value < LOQ	

5. P, B, T, C, M, R, ED properties

Based on the results of in vitro and in vivo genotoxicity tests, clothianidin is unlikely to pose a genotoxic risk to humans[7]. Clothianidin is unlikely to pose a carcinogenic risk to humans, and is unlikely to pose a teratogenic risk to humans at doses below those inducing toxic effects in the mother[7]. Clothianidin is also unlikely to affect fertility and developmental parameters in humans at doses below a range that elicits other toxic effects in adults[7]. The substance is not readily biodegradable (P) [7]. The low P_{ow} indicates that clothianidin has low potential to bioaccumulate in organisms[7]. Both estimated bioconcentration factors for the aquatic (BCF_{fish} = 0.78) and the terrestrial compartment ($BCF_{earthworm}$ = 0.9) can be classified as low. (Biocide Assessment Report, 2007[1]). An estimated BCF value of 3.16 L/kg was retrieved from EPI Suite[3].

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhyncus mykiss*, 96 h, mortality, LC_{50}	>100 mg/L	Biocide Assessment Report, 2007[1]
Fish	*Pimephales promelas*, 33 d, hatching, mortality and growth, NOEC	≥20 mg/L	Biocide Assessment Report, 2007[1]
Aquatic invertebrates	*Daphnia magna*, 48 h, immobility, EC_{50}	26 mg/L	Biocide Assessment Report, 2007[1]
Aquatic invertebrates	*Daphnia magna*, 21 d, mortality and reproduction, NOEC	0.12 mg/L	Biocide Assessment Report, 2007[1]
Algae	*Selenastrum capricornutum*, 96 h, growth inhibition a) NOEC b)EC_{50}	a)15 mg/L b)56 mg/L	Biocide Assessment Report, 2007[1]
Sediment dwelling organisms	***Chironomus riparius*, 28 d, emergence and development, EC_{10}**	**0.00065 mg/L**	**Biocide Assessment Report, 2007[1]**
Sediment dwelling organisms, phytoplankton and zooplankton	Mesocosm study, 14 weeks, NOEC	0.001 mg/L	Biocide Assessment Report, 2007[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	523 mg/kg bw	Biocide Assessment

			Report, 2007[1]
Short-term oral repeated dose toxicity	Dog, 90 d, NOAEL	20 mg/kg bw/day	Biocide Assessment Report, 2007[1]
Short-term oral repeated dose toxicity	Mouse, 90 d, mortality, NOEL	16 mg/kg bw/day	Biocide Assessment Report, 2007[1]
Long-term toxicity	**Rat, 2 year, dermal, NOAEL. Value used for ADI calculation in the EU Review Report, 2005[2].**	**9.7 mg/kg bw/day**	**EU Review Report, 2005[2] Biocide Assessment Report, 2007[1]**
Reproductive toxicity	Rat, NOAEL, a) parental, b) reproduction, c) offspring	a)31 mg/kg bw/day b) 31 mg/kg bw/day c) 10 mg/kg bw/day	Biocide Assessment Report, 2007[1]
Developmental toxicity	Rabbit, NOAEL, a) maternal, b) foetal	a)25 mg/kg bw/day b)25 mg/kg bw/day	Biocide Assessment Report, 2007[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Chironomus riparius*, 28 d, EC_{10}	0.00065 mg/L	5[a]	1.3E-04 mg/L[b]
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI	0.097 mg/kg bw/day	-	0.340 mg/L[c]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] Due to the mode of action of neonicotinoids, the lowest endpoint from the aquatic species tested corresponds to the midge *Chironomus riparius*. Therefore, it was selected for $PNEC_{fw}$ calculation. A lower AF of 5 was selected, due to the availability of the mesocosm study. The same PNEC value was used in the Biocide Assessment Report[1].

[b] Three long term values were available.

[c] ADI value, retrieved from EU Review Report 2005[2], used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**61.54**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.02

8. References

[1] Directive 98/8/EC concerning the placing of biocidal products on the market Inclusion of active substances in Annex I to Directive 98/8/EC Assessment Report CLOTHIANIDIN Product-Type 8 (Wood Preservative) 13 September 2007 Annex I. Available at http://dissemination.echa.europa.eu/Biocides/factsheet?id=0015-08

[2] EUROPEAN COMMISSION Review report for the active substance clothianidin. SANCO/10533/05 – Final Available at http://ec.europa.eu/food/plant/protection/evaluation/newactive/list_clothianidin.pdf

[3] EPI Suite, BCFBAF v3.01

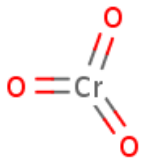
[4]European Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[5] the use as a seed treatment or soil treatment of plant protection products containing clothianidin is prohibited for crops attractive to bees and for cereals except for uses in greenhouses and for winter cereals. Foliar treatments with plant protection products containing clothianidin are prohibited for crops attractive to bees and for cereals with the exception of uses in greenhouses and uses after flowering, from COMMISSION IMPLEMENTING REGULATION (EU) No 485/2013 of 24 May 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. Available at http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013R0485.

[6] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

Chromium trioxide (CAS N. 1333-82-0)

1. Substance identity

EC name	
EC number	
CAS number	**1333-82-0**
Molecular formula	**CrO3**
Molecular weight	**99.99**
Structure	
SMILES	**[Cr](=O)(=O)=O**

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	Not available (inorganic ionic compound)	EU-RAR, 2005[1]
Water solubility (mg/L)	1667 mg/L	EU-RAR, 2005[1]
logK_{ow}	Not available (inorganic ionic compound)	EU-RAR, 2005[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential K_{oc}	Not available	EU-RAR, 2005[1]
Partition coefficient solid-water in sediment Kp_{sed} (L/kg)	1000	EU-RAR, 2005[1]
Biodegradability	NRB	EU-RAR, 2005[1]
Bioaccumulation (BCF)	2.8	EU-RAR, 2005[1]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	-	**-**
Uses	Manufacture of substances and of	ECHA, 2013[2]

	preparations, formulation of preparations and materials, industrial use resulting in inclusion into or onto a matrix, use as laboratory reagent.	
Spatial usage (by MS)	Not known	-
Banned uses	All consumer uses, and all professional uses except as laboratory substance[2]. Chromium trioxide meets the criteria for inclusion in Annex XIV to Regulation (EC) N. 1906/2006[3]. Furthermore, the latest application date expected for chromium trioxide is 21 March 2016, and the sunset date is 21 September 2017[4].	ECHA, 2013[2] Regulation (EC) N. 1906/2006[3] COMMISSION REGULATION (EU) No 348/2013[4]
ERC code	-	-
PEC_{fw} (mg/L)	0.35	EU-RAR, 2005[1]
PEC_{sed} (mg/kg dw)	0.152	EU-RAR, 2005[1]
PEC_{biota} (mg/kg)	0.98 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on BCF value not reaching the trigger value required for biota assessment

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
None	-	-	-

5. P, B, T, C, M, R, ED properties

The evidence clearly indicates that highly water-soluble Cr(VI) compounds can produce significant mutagenic activity in vitro and in vivo[1]. The Cr (VI) compounds under consideration are therefore regarded as in vivo somatic cell mutagens[1]. In addition, toxicokinetic and dominant lethal data suggest that water-soluble Cr (VI) has the potential to be an in vivo germ cell mutagen[1]. Chrome plating workers exposed to chromium (VI) trioxide in aqueous solution have shown a clear excess in mortality from lung cancer[1]. Therefore chromium (VI) trioxide should be regarded as a human carcinogen[1]. Adverse effects on fertility have been found in studies in mice following repeated oral exposure[1]. In addition, adverse effects on the testes have been seen following repeated oral exposure in the rat (EU-RAR, 2005[1]). The substance is not readily biodegradable (P). It shows a low potential to bioaccumulate in aquatic organism[1].

6. Hazard assessment

6.1 Ecotoxicology data

Since chromium trioxide is recommended for deselection as candidate substance for the Watch List, because of the imminent ban (Last application date in 2016, sunset date 2017), no ecotoxicity data are reported at this stage.

6.2 Mammalian toxicology data

No mammalian toxicity data are reported at this stage due to imminent ban and deselection as Watch List candidate.

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	$HC_{5\text{-}50\%}$	0.0102 mg/L	3	3.40E-03 mg/L[a]
$PNEC_{sed}$	-	-	-	6.80 mg/kg dw[b]
$PNEC_{biota,sec\ pois}$	Mouse, oral, conversion factor 8.3, NOAEL	20 mg/kg bw/day	10	17 mg/kg food[c] (N.R.)
$PNEC_{biota,\ hh}$	TDI	0.0009 mg/kg bw/day	-	0.055 mg/kg food[d] (N.R.)
$PNEC_{dw,\ hh}$	TDI	0.0009 mg/kg bw/day	-	0.003 mg/L[e]

N.R. Not required based on BCF value not reaching the trigger value required for biota assessment

[a]No new calculations were made. PNEC value retrieved from EU-RAR, 2005[1] with a probabilistic approach.

[b] Equilibrium partitioning method used, with the following values: $K_{sed\text{-}water}$= 500 m^3m^{-3} (from EU-RAR, 2005), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value). Conversion from wet weight to dry weight was done with eq. B of section 3.3.2

[c]No new calculations were performed. Value retrieved from EU-RAR, 2005[1]

[d] TDI value, retrieved from WHO Report 2013[6], used for PNEC calculation according to Equation E (see section 3.3.4)

[e] TDI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	102.94
RQ_{sed}	0.045
$RQ_{biota,sec\ pois}$	0.058
$RQ_{biota,\ hh}$	17.89
$RQ_{dw,\ hh}$	**111.11**

8. References

[1]European Risk Assessment Report on Chromium Trioxide, Sodium chromate, Sodium dichromate, Ammonium dichromate and Potassium dichromate (2005) EUR 21508 EN, and Brussels, C7/VR/csteeop/Cr/100903 D(03) Available at http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0348&from=EN

[2] ECHA dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9c7ac228-b090-229d-e044-00144f67d249/DISS-9c7ac228-b090-229d-e044-00144f67d249_DISS-9c7ac228-b090-229d-e044-00144f67d249.html

[3] REGULATION (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 December 2006, Official Journal of the European Union. Available at http://faolex.fao.org/docs/pdf/eur68317.pdf

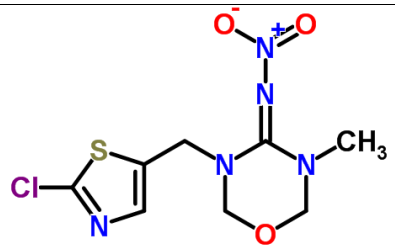
[4] COMMISSION REGULATION (EU) No 348/2013 of 17 April 2013 amending Annex XIV to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), 2013

[5] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[6]Inorganic chromium(VI) compounds; Concise International Chemical Assessment Document 78. IPCS, WHO (2013)

Thiamethoxam (CAS N. 153719-23-4)

1. Substance identity

EC name	Thiamethoxam
EC number	428-650-4
CAS number	153719-23-4
Molecular formula	C8H10ClN5O3S
Molecular weight	291.71
Structure	
SMILES	CN1COCN(C1=N[N+](=O)[O-])Cc2cnc(s2)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	6.6 E-09	Biocide Assessment Report[1]
Water solubility (mg/L)	4100	Biocide Assessment Report[1]
logK_{ow}	-0.13	Biocide Assessment Report[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	56.2	Biocide Assessment Report[1]
Biodegradability	NRB	Biocide Assessment Report[1]
Bioaccumulation (BCF)	3.16 (estimated)	EPI Suite BCFBAF, v.3.01
BMF	1	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

	Description	Source
Tonnes/year		
Uses	Insecticide (PPP and biocide)	
Spatial usage (by MS):	AT, BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HU, IT, LT, LV, MT, NL, PL, PT, RO, SE, SI, SK, UK	EU Pesticides DB[3]
Banned uses	Restriction of uses[a]	EU n. 485/2013[4]
ERC code	ERC8d	

Fraction of tonnage to region	-	

[a]The use as a seed treatment or soil treatment of plant protection products containing thiamethoxam is prohibited for crops attractive to bees and for cereals except for uses in greenhouses and for winter cereals. Foliar treatments with plant protection products containing thiamethoxam are prohibited for crops attractive to bees and for cereals with the exception of uses in greenhouses and uses after flowering[4].

4.1 Predicted Environmental cCncentration

	Value	**Source**
PEC_{fw} (mg/L)	0.011	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.0074 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	0.035 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 Comparison between FOCUS Pesticides models

FOCUS Step 1

Crop	**Application Rate (g/ha)**[5]	**Water solubility (mg/L)**[5]	**K_{oc} (L/kg)** [5]	**DT_{50} whole system (d)**[5]
Pome & Stone Fruits (late)	2 × 100 (14 d application interval)	4100	56.2	46.4

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 156 d (mean)[5], $DT_{50water}$ 38.2 d[5], $DT_{50sediment}$ 1000 d (conservative value), full crop interception.

FOCUS Step 3 – SWASH package

In addition to the input values listed above, foliar application, and a pre-harvest interval (PHI) of 14 days. In accordance both with the FOCUS SW Appendix C and D[6], and with a growth stage corresponding to post blosson (approximately BBCH 70) related to pome and stone fruits (as reported in the GAP table of the EU LoE[5]), the following application windows were selected for the relevant scenarios for the PAT calculator: D3 and D4 from 15.06 to 16.10, D5 from 15.06 to 26.09, R1 from 15.06 to 16.10, R2 from 15.07 to 16.09, R3 and R4 from 15.07 to 01.10. Just for runs of D4 and D5 no results were achieved due to some software error.

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.071	0.048
FOCUS Step 2	0.011	0.007
FOCUS Step 3	0.005 D3 pond	0.008 D3 pond

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
4 (FI, SE, IT, NL)	WATERBASE, 2014[7]	$MEC_{95, whole}$: 0.03 µg/L	-
	SE pesticide monitoring programme [8]	$MEC_{95:}$ 0.014 µg/L	
	IT monitoring programme[9]	MEC_{95}: 0.0365 µg/L	
	NORMAN DB, 2014[10]	MEC_{95}: 0.35 µg/L (NL)	

5. P, B, T, C, M, R, ED properties

Thiamethoxam is not clastogenic or aneugenic[1]. On the basis of the absence of genotoxicity in vivo, the absence of carcinogenicity in rats and the mode of action by which liver tumours arise in mice, it was concluded that thiamethoxam is unlikely to pose a carcinogenic risk at human dietary exposure levels[11]. The Joint FAO/WHO Meeting concluded that thiamethoxam can cause fetotoxicity and skeletal anomalies (malformations and variants), but only at maternally toxic doses[11]. Thiamethoxam is not a neurotoxin in mammals at the tested dose levels, although it is a member of the neonicotinoid chemical class[11]. The substance is not readily biodegradable (P). It shows a low potential for bioaccumulation (not B) (Biocide Assessment Report, 2008[1]). A BCF value of 3.16 L/kg was also estimated with EPI Suite.

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	96 h, LC_{50}	>125 mg/L	Biocide Assessment Report, 2008[1]
Fish	88 d, NOEC	20 mg/L	Biocide Assessment Report, 2008[1]
Aquatic invertebrates	Ostracoda, 48 h, EC_{50}	0.18 mg/L	Biocide Assessment Report, 2008[1]
Aquatic invetebrates	*Gammarus* sp., 48 h, EC_{50}	2.8 mg/L	Biocide Assessment Report, 2008[1]
Aquatic invetebrates	*Daphnia magna,* 21 d, NOEC	100 mg/L	Biocide Assessment Report, 2008[1]
Aquatic invetebrates	*Lymnea stagnalis*, 48 h, EC50	100 mg/L	Biocide Assessment Report, 2008[1]
Aquatic invetebrates	***Cloeon* Sp., 48 h, EC_{50}**	**0.014 mg/L**	**Biocide Assessment Report, 2008[1]**
Sediment dwelling organisms	*Chironomus riparius*, 30 d, NOEC	0.01 mg/L	Biocide Assessment Report, 2008[1]
Algae	*Selenastrum capricornutum*, 72 h, NOEC	81.8 mg/L	Biocide Assessment Report, 2008[1]
Aquatic plants	*Lemna gibba*, 7 d, EC_{50}	>90.2 mg/L	Biocide Assessment Report, 2008[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD50	1563 mg/kg bw	Biocide Assessment Report, 2008[1]
Repeated oral dose toxicity	Mouse, 90 d, NOAEL	1.41 mg/kg bw/day	Biocide Assessment Report, 2008[1]
Repeated oral dose toxicity	Dog, 1 year, NOAEL	4.05 mg/kg bw/day	Biocide Assessment Report, 2008[1]
Carcinogenicity	**Mouse, 18 months, NOAEL. Value used for ADI calculation in the EU Review Report[4]**	**2.63 mg/kg bw/day**	Biocide Assessment Report, 2008[1]
Reproductive toxicity	Rat, 2 generation study, NOAEL	62 mg/kg bw/day	Biocide Assessment Report, 2008[1]
Developmental toxicity	Rabbit, NOAEL	50 mg/kg bw/day	Biocide Assessment Report, 2008[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Cleon* sp. (Ephemeroptera), 48 h, EC_{50}	0.014 mg/L	100	0.00014 mg/L[a]
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI[b]	0.026 mg/kg bw/day	-	0.091 mg/L[c]

N.R. Not required based on Koc and BCF value not reaching the trigger values required for sediment and biota assessment

[a] Due to the mode of action of neonicotinoids, the lowest endpoint from the aquatic species tested corresponds to *Cleon* species. Therefore, it was selected for $PNEC_{fw}$ calculation. An assessment factor of 100 was used instead of the TGD (Technical Guidance Document) recommended 1000 because this taxa was regarded with high probability as being the most sensitive and a further long-term NOEC from different taxonomic group would not be lower than the data already available (Biocide Assessment Report, 2008)[1].

[b] ADI from EU LoE, 2006[5].

[c] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**78.57**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.

$RQ_{dw, hh}$	0.12

8. References

[1] Assessment Report Thiamethoxam Product-type 8 (Wood preservative) (2008) - Directive 98/8/EC concerning the placing of biocidal products on the market Inclusion of active substances in Annex I or IA to Directive 98/8/EC

[2] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[3] EU Pesticides Database http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[4] COMMISSION IMPLEMENTING REGULATION (EU) No 485/2013 of 24 May 2013 amending Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. Official Journal of the European Union. Available at http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013R0485

[5] EUROPEAN COMMISSION Review report for the active substance thiamethoxam, SANCO/10390/2002 - rev. final. Available at http://ec.europa.eu/food/plant/protection/evaluation/newactive/thiamethoxam_en.pdf

[6] Appendix C and D for parameterisation of drainage and run-off inputs, respectively. Available at: http://focus.jrc.ec.europa.eu/sw/index.html

[7] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[8] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

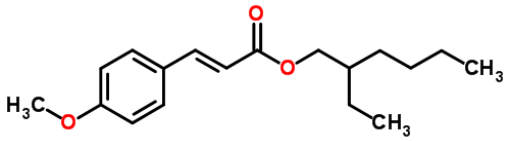
[9] Italian Monitoring Programme (data provided directly to the JRC)

[10] NORMAN Database http://www.norman-network.net/?q=node/24

[11] Joint FAO/WHO Meeting report. Available at http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report10/Thiamethoxam.pdf.

2-Ethylhexyl 4-methoxycinnamate (CAS N. 5466-77-3)

1. Substance identity

Chemical name (IUPAC)	2-Ethylhexyl 4-methoxycinnamate
EC number	226-775-7
CAS number	5466-77-3
Molecular formula	C18H26O3
Molecular weight	290.4
Structure	
SMILES	CCCCC(CC)COC(=O)/C=C/C1=CC=C(C=C1)OC

2. Reasons for proposal as candidate for the Watch list and suspected environmental risk

2-Ethylhexyl 4-methoxycinnamate (EHMC) is an organic sun-blocking agent derived from cinnamic acid that absorbs ultraviolet radiation in the UV-B range. It is used in sunscreen lotions to protect human skin from solar radiation, and to protect cosmetics and personal care products from photodegradation.

It is part of the Draft Community Rolling Action Plan (CoRAP)[1], where the initial grounds of concerned are listed as environment/suspected PBT, potential endocrine disruptor, possible risk; exposure/wide dispersive use, consumer use environmental exposure and high (aggregated) tonnage.

UV filters may enter the aquatic environment directly, as a result of bathing and washing activities in seas, rivers, lakes and swimming pools, as well as industrial discharges. Alternatively, they can enter the aquatic environment indirectly via domestic wastewater discharges and via wastewater treatment plants (Giokas, 2007)[2]. The Cosmetics Directive 92/8/EEC restricts the use of EHMC at a maximum concentration of 10%[3].

EHMC is a ubiquitous sunscreen filter in European environment, having been detected in surface waters, sediment and biota[4-10]. Due to its physic-chemical properties, EHMC is expected to accumulate in sediments. This substance has been measured up to 4 µg/kg in river sediments[5] and 34 µg/kg in lake sediments in Germany[10] and 79 µg/kg in Tokyo bay sediments[11]. A seasonal variation in the concentrations of UV filters (including EHMC) in the aquatic environment has been observed in many cases with a peak during the summer period.

3. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	30	ECHA, 2014[12]
Water solubility (mg/L)	0.75	ECHA, 2014[12]
logK_{ow}	>6	ECHA, 2014[12]

4. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	13290	ECHA, 2014[12]
Biodegradability	RB	ECHA, 2014[12]
Bioaccumulation (BCF)	433	ECHA, 2014[12]
BMF	1	Default value, TG n. 27 - CIS WFD[13]

5. Environmental exposure assessment

	Description	Source
Tonnes/year	7500 (year 2000)	Previous prioritisation exercise
Uses	Sunscreen ingredient in personal care products	
Spatial usage (by MS):	Widespread use (worldwide)	Sunscreen Ingredients, 2006[14]
Banned uses	-	
ERC code	ERC8a	
Fraction of tonnage to region	0.1	

5.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.0063	ECETOC
PEC_{sed} (mg/kg dw)	8.39	ECETOC
PEC_{biota} (mg/kg)	2.73 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required because readily biodegradable.

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
2(DE, SE)	NORMAN DB, 2014 [15]	$MEC_{95, whole}$: 3.98E-04 mg/L (DE)	-
	SE National Screening Programme 2009: UV-filters[4]	MEC_{95}: 3.03E-05 mg/L (surface water) MEC_{95}: 0.043 mg/kg dw (sediment) MEC_{95}: 7.8E-04 mg/kg ww (biota)	

6. P, B, T, C, M, R, ED properties

Endocrine disruptor-Category 1 both for human health and aquatic organisms[16]. In the latter case, an increase in plasma VTG + and increased mRNA expression levels of estrogen receptor (ER) alpha, among sex hormone receptors in the liver (Endocrine Disruptor database of the EU Commission)[16].

EHMC has been reported to display low but multiple hormonal activities in fish including vitellogenin induction, histological changes in gonads and effects on the expression of genes involved in different hormonal pathways in fathead minnows[17]. EHMC has also caused toxic effects on reproduction in snails[18].

Negative results for genotoxicity[12]. The substance is readily biodegradable (not P),. It has a BCF value of 433 L/kg (ECHA, 2014[12]).

7. Hazard assessment

7.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Algae	*Selenastrum capricornutum*, 72 h, growth rate, EC_{50}	32 mg/L	ECHA, 2014[12]
Algae	*Selenastrum capricornutum*, 72 h, growth rate, NOEC	>100000 µg/L	ECHA, 2014[12]
Aquatic invertebrates	***Melanoides tuberculata*, 28 d, number of embryos per snail, sediment toxicity test, NOEC**	**2 mg/kg**	**Kaiser et al. (2012)[18] (R2)**
Aquatic invertebrates	*Potamopyrgus antipodarum*, 56 d, number of embryos per snail, sediment toxicity test, NOEC	0.08 mg/kg [a]	Kaiser et al. (2012)[18] (R2)
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	>0.0271 mg/L	ECHA, 2014[12]
Fish	*Danio rerio*, 48 h, sediment contact test, sublethal effects, NOEC	100 mg/kg	Kaiser et al. (2012)[18] (R2)
Fish	*Cyprinus carpio*, 96 h, LC_{50}	>100000 µg/L	ECHA, 2014[12]

[a] Even though this value was lower, it was not selected for the risk assessment, because no dose-effect curve was seen, in contrast with the one chosen (in bold).

(R2) Relevance and reliability were assessed using a literature evaluation tool (LET) based on the CRED system (Kase et al, unpublished). Assessed to be reliable with restrictions (Klimisch score 2).

7.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Repeated dose toxicity	Rat, oral, min 90 d, NOAEL	450 mg/kg bw/day	ECHA, 2014[12]
Reproductive toxicity	Rat, oral, 2 generation study, NOAEL	450 mg/kg bw/day	ECHA, 2014[12]
Developmental toxicity	Rabbit, oral, NOAEL	500 mg/kg bw/day	ECHA, 2014[12]

7.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value	Comment
$PNEC_{fw}$	-	-	-	-	[a]
$PNEC_{sed}$	*Melanoides tuberculata*, 28 d, sediment toxicity	2 mg/kg	10 [b]	0.2 mg/kg	Kaiser et al. (2012)[18]

	test, NOEC				
$PNEC_{biota,sec\ pois}$	N.R.	-	-	-	RB
$PNEC_{biota,\ hh}$	N.R.	-	-	-	RB
$PNEC_{dw,\ hh}$	DNEL, repeated dose toxicity, oral	2.25 mg/kg bw/day	-	7.875 mg/kg bw/day	ECHA, 2014[12] (for DNEL & AF)[c]

N.R. Not required because the substance is readily biodegradable.

[a] The substance showed no inhibitory effect in the range of the water solubility (ECHA, 2014)[12]

[b] Two long-term endpoints were available for two snail species *Potamopyrgus antipodarum* and *Melanoides tuberculata* at concentrations below water solubility. For *P. antipodarum*, although the NOEC was lower (0.08mg/kg), there was no clear dose response, and for this reason it was not selected. Additionally, for *Chironomus riparius* and *Lumbriculus variegatus* no effects were observed over 28 days for concentrations up to 50 mg/kg dw. Thus, there is data for three long-term tests with species representing different living and feeding conditions and an AF of 10 was selected.

[c] DNEL, retrieved from ECHA, 2014[12], used in equation F as TL_{hh} (see section 3.3.5)

8. Risk Quotient (PEC/PNEC)

RQ	**Value**
RQ_{fw}	-
RQ_{sed}	**41.95**
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	8E-04

9. References

[1] ECHA Draft Community Rolling Action Plan (CoRAP) update for years 2015-2017. Available at: http://echa.europa.eu/documents/10162/13628/corap_2015_2017_en.pdf

[2] Giokas DL, Salvador A, Chisvert, A. (2007) UV filters: From sunscreens to human body and the environment. Trends in Analytical Chemistry, 26(5): 360-374.

[3] http://ec.europa.eu/consumers/cosmetics/cosing/index.cfm?fuseaction=search.details&id=31273

[4] Remberger M, Lilja K, Kaj L, Viktor T, Brorström-Lundén E. (2011) Results from the Swedish National Screening Programme 2009. Subreport 3: UV-filters. IVL Swedish Environmental Research Institute. http://www.ivl.se/download/18.7df4c4e812d2da6a416800088960/B1971.pdf

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[12] ECHA dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9ea053bf-39e2-163b-e044-00144f67d031/DISS-9ea053bf-39e2-63b-e044-00144f67d031_DISS-9ea053bf-39e2-163b-e044-00144f67d031.html

[13] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[14] Update of Sunscreen Ingredients Nomination to NTP. Imogene Sevin, Ph.D. Technical Resources International, Inc., 2006

[15] NORMAN database at http://www.norman-network.net/?q=node/24

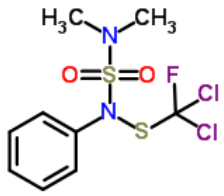
[16]Endocrine Disruptor database of the EU Commission), available at http://ec.europa.eu/environment/chemicals/endocrine/documents/index_en.htm

[17] Christen V, Zucchi S, Fent K. (2011) Effects of the UV-filter 2-2thyl-4-trimethoxycinnamate (EHMC) on expression of genes involved in hormonal pathways in fathead minnows (Pimephales promelas) and link to vitellogenic induction and histology. Aq. Tox. 102:167-176.

[18] Kaiser D, Sieratowicz A, Zielke H, Oetken M, Hollert H, Oehlmann J. (2012) Ecotoxicological effect characterisation of widely used organic UV filters. Environmental Pollution 163:84-90.

Dichlofluanid (CAS N. 1085-98-9)

1. Substance identity

EC name	
EC number	214-118-7
CAS number	1085-98-9
Molecular formula	C9H11Cl2FN2O2S2
Molecular weight	333.2
Structure	
SMILES	CN(C)S(=O)(=O)N(c1ccccc1)SC(F)(Cl)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	2.15E-05 Pa at 20 °C	Biocide Assessment Report, 2006[1]
Water solubility (mg/L)	1.58 mg/l at 20 °C	Biocide Assessment Report, 2006[1]
logK_{ow}	3.5	Biocide Assessment Report, 2006[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	1344	Biocide Assessment Report, 2006[1]
Biodegradability	NRB	Biocide Assessment Report, 2006[1]
Bioaccumulation (BCF)	72	Biocide Assessment Report, 2006[1]
DT_{50} water/sediment systems	< 1 d Dichlofluanid was very rapidly degraded in aerobic aquatic systems to DMSA. DMSA stayed mainly in the water phase.	Biocide Assessment Report, 2006[1]
BMF	1	Default value, TG n. 27 - CIS WFD[2]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	10000 (year 1997)	From previous prioritization exercise
Uses	Wood preservative (Biocide)	

Spatial usage (by MS):	Not known	
Banned uses	Fungicide- PPP	(Commission Regulation (EC) No 2076/2002)[3]
ERC code	ERC8b	
Fraction of tonnage to region	0.1	
PEC_{fw} (mg/L)	0.0053	ECETOC
PEC_{sed} (mg/kg dw)	0.732	ECETOC
PEC_{biota} (mg/kg)	0.38	Calculation based on Equation L (Section 3.4.3)

4.1.1 ECETOC simulation with lower tonnages

Authorisations for plant protection products containing the active substance dichlofluanid were withdrawn by 25 July 2003[3]. However, the available tonnage of 10000 relates to the year 1997, which is prior to the banning of the substance as PPP.

At the WG Chem meeting 16-17/10/2014 it was suggested to perform a simulation on the PEC calculated with ECETOC using reduced tonnage values of dichlofluanid that could be closer to the actual tonnage after the banning, i.e. related to the use as biocide only. Since no tonnage value specific for this particular use was available, it was decided to perform the simulation considering a 20%, and 30% or 50% decrease in tonnage values. The results of the simulations are compared with the pre-banning tonnage scenario in the following Table.

Tonnes/year	10000	8000	7000	5000
Decrease respective to pre-banning tonnage	-	20%	30%	50%
PEC_{sed} (mg/kg dw)	0.732	0.586	0.513	0.366
RQ_{sed}	40.17	32.14	28.14	20.07
Position in the ranking (higher RQ)	16 (RQ_{sed})	16 (RQ_{sed})	17 (RQ_{sed})	19 (RQ_{sed})

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
3 (FR, FI, IT)	NORMAN DB, 2014[4]	MEC_{site}: 0.03 µg/L MEC_{sed}: < LOD	-
	WATERBASE, 2014[5]	Values below LOQ	
	Italian Monitoring Programme[6]	Values below LOQ	

5. P, B, T, C, M, R, ED properties

Following repeated oral administration of dichlofluanid, the most prominent finding was fluorosis caused by the release of fluoride from the dichlofluanid molecule during its metabolism[1]. This resulted in skeletal osteosclerosis, observed in lifetime dietary studies in both rats and mice[1]. Chronic nephropathy was also observed following repeated oral administration, but in dogs only[1]. The mode of action for the nephropathy is uncertain and possible explanations include direct nephrotoxicity of the active substance or a secondary consequence of elevated systemic fluoride levels[1]. Dichlofluanid is not genotoxic in vivo[1]. In terms of carcinogenicity, dichlofluanid induced thyroid tumours in rats at high doses, but by a mechanism not considered to be relevant for human health[1]. No increase in tumour incidence was observed in mice. Overall, dichlofluanid does not show any carcinogenic potential of relevance to human health[1]. In experimental animal studies dichlofluanid did not affect fertility and did not cause developmental toxicity[1]. The evidence suggests that this substance does not possess significant potential with respect to toxicity for reproduction[1].

The substance is not readily biodegradable (P). It has a low BCF of 72 L/kg (not B) [1].

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Salmo gairdneri*, 96 h, LC_{50}	0.01 mg/L	Biocide Assessment Report, 2006[1]
Fish	*Salmo gairdneri*, 21 d, NOEC	0.00455 mg/L	Biocide Assessment Report, 2006[1]
Fish	*Pimephales promelas*, 33 d, body length and weight, NOEC	0.00407 mg/L	Biocide Assessment Report, 2006[1]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	0.42 mg/L	Biocide Assessment Report, 2006[1]
Aquatic invertebrates	***Daphnia magna*, 21 d, NOEC**	**0.00265 mg/L**	Biocide Assessment Report, 2006[1]
Algae	*Scenedesmus subspicatus*, 96 h, growth rate, a)NOEC, b)72 h EC_{50}	a)1 mg/L b)15 mg/L	Biocide Assessment Report, 2006[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	>5000 mg/kg	Biocide Assessment Report, 2006[1]
Repeated dose toxicity	Dog, 90 d NOAEL, subchronic	20 mg/kg/day	Biocide Assessment Report, 2006[1]

Repeated dose toxicity	**Dog, 365 d, NOAEL, chronic. Value used for DNEL calculation**	**2.5 mg/kg/day**	Biocide Assessment Report, 2006[1]
Reproductive toxicity	Rat, NOAEL	16 mg/kg/day	Biocide Assessment Report, 2006[1]
Developmental toxicity	Rat, NOAEL	30 mg/kg/day	Biocide Assessment Report, 2006[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna*, 21 d, reproduction, NOEC	0.00265 mg/L	10[a]	2.65E-04 mg/L
$PNEC_{sed}$	-	-	-	0.018 mg/kg dw[b]
$PNEC_{biota,sec\ pois}$	Dog, repeated dose toxicity, 365 d, oral, conversion factor 40, NOAEL	2.5 mg/kg bw/day	30[c]	3.3 mg/kg food[d]
$PNEC_{biota,\ hh}$	ADI	0.35mg/kg bw/day	-	21.304 mg/kg food[e]
$PNEC_{dw,\ hh}$	ADI	0.35mg/kg bw/day	-	1.225 mg/L[f]

[a]Three long term values available from the three main trophic levels.

[b]Equilibrium partitioning method used for $PNEC_{sed}$ calculation, with the following values: $K_{sed\text{-}water}$= 34.4 m^3m^{-3} (calculated with eq. D of section 3.3.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 67.2 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 1344 L/kg[4], Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. B of section 3.3.2.

cThe AF of 30 was selected according to the duration of the test (365 days). See TG n. 27- CIS WFD[2]

[d] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of the NOAEL (2.5 mg/kg bw/day) value retrieved from the Biocide Assessment Report (2006)[1], into NOEC (100 mg/kg) by using the conversion factor of 40 (taken from TG n. 27- CIS WFD, and it depends both on species tested and age/study); b) Application of appropriate AF_{oral} (30) to the NOEC value.

[e] ADI value retrieved from Biocide Assessment Report (2006) [1], used for PNEC calculation according to Equation E (see section 3.3.4)

[f] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	20.04
RQ_{sed}	**40.17**
$RQ_{biota,sec\ pois}$	0.11
$RQ_{biota,\ hh}$	0.02
$RQ_{dw,\ hh}$	0.004

8. References

[1] Directive 98/8/EC concerning the placing of biocidal products on the market - Inclusion of active substances in Annex I to Directive 98/8/EC. Assessment Report, DICHLOFLUANID PT8 (2006). Available at http://dissemination.echa.europa.eu/Biocides/factsheet?id=0025-08

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[3] COMMISSION REGULATION (EC) No 2076/2002 of 20 November 2002 extending the time period referred to in Article 8(2) of Council Directive 91/414/EEC and concerning the non-inclusion of certain active substances in Annex I to that Directive and the withdrawal of authorisations for plant protection products containing these substances. Available at http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.detail

[4] NORMAN database at http://www.norman-network.net/?q=node/24

[5] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[6] Italian Monitoring Programme (data provided directly to the JRC)

Formaldehyde (CAS N. 50-00-0)

1. Substance identity

EC name	Formaldehyde
EC number	200-001-8
CAS number	50-00-0
Molecular formula	CH2O
Molecular weight	30.03
Structure	
SMILES	C=O

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	131.87 Pa	ECHA, 2014[1]
Water solubility (mg/L)	550000	ECHA, 2014[1]
logK_{ow}	0.35	ECHA, 2014[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	15.9	ECHA, 2014[1]
Biodegradability	RB	ECHA, 2014[1]
Bioaccumulation (BCF)	<1	ECHA, 2014[1]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	A confidential tonnage value was used for calculation	IUCLID, 2014[2]
Uses	Manufacture of substances, formulation in preparations and in materials, such as wood-based materials, paper, impregnated paper, bonded fibers or fiber mats, bonded	ECHA, 2014[1]

	particulates, rubber, leather foam, firelighters, fertilized granules, cleaning agents (ECHA, 2014)[1].	
Spatial usage (by MS)	Not known	
Banned uses	As biocide (PT1 Human hygiene, PT4 Food and feed area, PT5 Disinfection of Drinking water, PT6 Preservatives for products during storage, PT9 Fibre, leather, rubber and polymerised materials preservatives, PT11 Preservatives for liquid-cooling and processing systems, PT12 Slimicides, PT13 Working or cutting fluid preservatives, PT18 Insecticides, acaricides and products to control other arthropods, PT21 Antifouling products, PT23 Control of other vertebrates)	Consolidated list of non-inclusion decisions, 2013[3]
ERC code	ERC8d	
Fraction of tonnage to region	0.1	
PEC_{fw} (mg/L)	13.53	ECETOC
PEC_{sed} (mg/kg dw)	70.2 (N.R.)	ECETOC
PEC_{biota} (mg/kg)	13.53 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
3 (FR, UK, SK)	NORMAN DB, 2014[4]	$MEC_{95, whole}$: 7.165 µg/L	3 MS[7] EQS set (WRc, 2012)[8]
	WATERBASE, 2014[5]	$MEC_{95, whole}$: 22.75 µg/L	
	IPCheM[6]	MEC_{95}: 119.75 µg/L	

5. P, B, T, C, M, R, ED properties

In the ECHA dissemination website, formaldehyde is classified as: Carc. 2 H351: Suspected of causing cancer <state route of exposure if it is conclusively proven that no other routs of exposure cause the hazard> Route of exposure: inhalation (ECHA, 2014[1]).

Potential mechanisms underlying formaldehyde-induced reproductive and developmental toxicities, including chromosome and DNA damage (genotoxicity), oxidative stress, altered level and/or function of

enzymes, hormones and proteins, apoptosis, toxicogenomic and epigenomic effects (such as DNA methylation), were identified (Duong A. et al.[9]).

Formaldehyde is not persistent, and not bioaccumulative (BCF < 1) (ECHA, 2014[1]).

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Morone saxatilis*, 96 h, LC_{50}	6.7 mg/L	ECHA, 2014[1]
Fish	*Rasbora heteromorpha*, LC_{50}, 48 h	50 mg/L	ECHA, 2014[1]
Fish	*Ictalurus punctatus*, LC_{50}, 96 h	25.5 mg/L	ECHA, 2014[1]
Fish	*Ictalurus melas*, LC_{50}, 96 h	25 mg/L	ECHA, 2014[1]
Fish	*Leuciscus idus melanotus*, LC_{50}, 48h	15 mg/L	ECHA, 2014[1]
Fish	*Pimephales promelas*, LC_{50}, 96 h	24.1 mg/L	ECHA, 2014[1]
Fish	*Danio rerio*, LC_{50}, 96 h	41 mg/L	ECHA, 2014[1]
Fish	*Oryzias latipes*, NOEC, 28 d, mortality, target organ pathologies	≥48 mg/L	ECHA, 2014[1]
Fish	*Danio rerio*, 144 h, LC_{50}, Mortality of embryos/larvae	6.9 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Daphnia pulex*, 48 h, mobility, a) EC_{10} b) EC_{50}	a)1.9 mg/L b)5.8 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Pinctada fucata martensii* 96 h, LC_{50}	5.3 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Ceriodaphnia cf. dubia*, 48 h, mobility, EC_{50}	12.98 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Daphnia magna*, 24 h, LC_{50}	52 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Streptocephalus seali*, EC_{10}	25 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Daphnia magna*,48 h, EC_{50}	29 mg/L	ECHA, 2014[1]
Aquatic invertebrates	*Daphnia magna*, 24h, EC_{50}	14.7 mg/L	ECHA, 2014[1]
Algae	*Scenedesmus subspicatus*, 72 h, a) EC_{50} biomass, b) EC_{50} growth rate	a)3.48 mg/L b)4.89 mg/L	ECHA, 2014[1]
Algae	*Pseudokirchnerella subcapitata*, 48 h, a) EC_{50} growth rate, b) EC_{50} dissolved oxygen production	a)2.49 mg/L b)2.627 mg/L	ECHA, 2014[1]

Studies assigned as not reliable, or not assignable in the ECHA, 2013[1] were not reported.

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity (weight of evidence)	Rat, LD_{50}	460 mg/kg bw	ECHA, 2014[1]
Acute oral toxicity (weight of evidence)	Rat, LD_{50}	800 mg/kg bw	ECHA, 2014[1]
Repeated oral toxicity	Rat, oral drinking water, 105 weeks, NOAEL	15 mg/kg bw/day	ECHA, 2014[1]
Carcinogenicity	Rat, oral, 13 weeks, 1) LOAEC toxicity, 2)LOAEC carcinogenicity	a)17 ppm b)20ppm	ECHA, 2014[1]

Carcinogenicity	Rat, inhalation, 13 weeks, 1)NOAEC toxicity, 2)LOAEC toxicity, 2)LOAEC carcinogenicity	a)1 ppm b)10ppm c)10ppm	ECHA, 2014[1]
Carcinogenicity	Rat, oral drinking water, 32 weeks non-effective dose level for promoting activity	5000 mg/L drinking water	ECHA, 2014[1]
Reproductive toxicity	Mouse, oral, a) NOAEL, b) LOAEL maternal toxicity	a)185 mg/kg bw/day b)74 mg/kg bw/day	ECHA, 2014[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	-	-	10[a]	0.47mg/L[b]
$PNEC_{sed}$	-	-	-	2.440 mg/kg dw[c] (N.R.)
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	TDI	0.15 mg/kg bw	-	0.525 mg/L[d]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] The AF was reported in the ECHA dossier, 2014[1]

[b] In the ECHA report, the PNEC value was estimated with a probabilistic approach. No new calculations were performed.

[c] No new calculations were done, because value was retrieved from ECHA, 2014[1]. The equilibrium partitioning method was used in the dossier.

[d] TDI value, retrieved from EFSA opinion, 2006[11], was used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**28.79**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	25.77

8. References

[1] ECHA dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9daa7594-c409-0ed0-e044-00144f67d249/DISS-9daa7594-c409-0ed0-e044-00144f67d249_DISS-9daa7594-c409-0ed0-e044-00144f67d249.html

[2] Complete IUCLID dossier of formaldehyde.

[3] Existing active substances for which a decision of non-inclusion into Annex I or Ia of Directive 98/8/EC has been adopted - In accordance with Article 4(2) of Regulation (EC) No 2032/2003, biocidal products containing active substances for which a non-inclusion decision was taken shall be removed from the market within 12 months of the entering into force of such decision; unless otherwise stipulated in that non-inclusion decision-Dates by which

products containing these active substances shall no longer be placed on the market for the relevant product-types (February 2013). Available at: http://ec.europa.eu/environment/chemicals/biocides/pdf/list_dates_product_2.pdf

[4] NORMAN database at http://www.norman-network.net/?q=node/24

[5] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[6] IPCheM database at http://ipchem.jrc.ec.europa.eu/

[7] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report.

[8] Contract No. 070311/2011/603663/ETU/D1 "Comparative Study of Pressures and Measures in the Major River Basin Management Plans' - Task 2c (Comparison of Specific Pollutants and EQS): Final Report". WRc Ref: UC8981/1 October 2012. Available at http://ec.europa.eu/environment/archives/water/implrep2007/pdf/P_M%20Task%202c.pdf

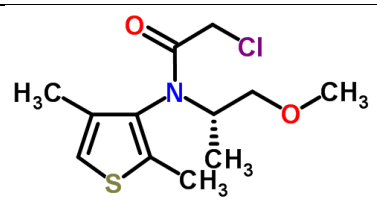
[9] Reproductive and Developmental Toxicity of Formaldehyde: A Systematic Review by Anh Duong, Craig Steinmaus, Cliona M. McHale, Charles P. Vaughan, and Luoping Zhanga. Available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3203331/).

[10] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[11] EFSA Journal (2006) 415, 1-10. Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to Use of formaldehyde as a preservative during the manufacture and preparation of food additives. Available at http://www.efsa.europa.eu/it/efsajournal/doc/415.pdf

Dimethenamid-P (CAS N. 163515-14-8)

1. Substance identity

Chemical name (IUPAC)	2-Chloro-N-(2,4-dimethyl-3-thienyl)-N-[(2S)-1-methoxy-2-propanyl]acetamide
EC number	
CAS number	163515-14-8
Molecular formula	C12H18ClNO2S
Molecular weight	275.798
Structure	
SMILES	Cc1csc(c1N([C@@H](C)COC)C(=O)CCl)C

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	$2.5 \cdot 10^{-3}$	EC Review, 2001 [1]
Water solubility (mg/L)	1449	EC Review, 2001 [1]
logK_{ow}	1.89	EC Review, 2001 [1]

3. Environmental fate

Endpoint	Value	Source
Hydrolysis (DT50)	33.40 d	EC Review, 2001 [1]
Sorption potential (K_{oc})	170.16	INERIS, 2011 [2]
Biodegradability	NRB	Consensus between EPISUITE (Biowin), ADMET Predictor v.7
Bioaccumulation (BCF)	58	CLP Report, 2013[3]

4. Environmental exposure assessment

	Description	Source
Tonnes/year	-	
Uses	Herbicide	
Spatial usage (by MS)	AT, BE, BG, CZ, DE, EL, ES, FR, HU, IE, IT, LU, NL, PL, PT, RO, SI, SK, UK	EU Pesticides DB [4]
Banned uses	Dimethenamid banned as Herbicide, PPP Replaced as herbicide by its active isomer,	Commission Decision (2006) [5]

	dimethenamid-P	
Crop	Maize	
Application rate (g/ha)	1 × 864 [a]	Stakeholder's comment (BASF)

[a] New maximum application rates have been reported by the stakeholder (up to 0.864 kg a.s./ha), based on recent authorization conditions for dimethenamid-P, which will decrease the PEC estimated using the FOCUS Step models based on the previous application rate of 1 × 1000 g/ha (EC Review, 2001) [1].

4.1 Predicted Environmental Concentration

PEC_{fw} (mg/L)	0.0657	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.109	FOCUS Step 2
PEC_{biota} (mg/kg)	3.81	Calculation based on Equation L (Section 3.4.3)

4.1.1 Comparison of FOCUS Pesticides models with ECETOC model

FOCUS Step 1

Crop [1]	Application Rate (g/ha)[1]	Water solubility (mg/L)[1]	K_{oc} (L/kg) [1]	DT_{50} whole system (d) [1]
Maize	1 × 864 g/ha	1449	170.16	33.16

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 16.3 d [1], $DT_{50water}$: 28 d [1], $DT_{50sediment}$ 33 d [1], Minimal crop interception [1].

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.243	0.402
FOCUS Step 2	0.0656	0.109

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
5 (IT, FR, DE, LU, NL)	IPCheM[6]	$MEC_{95:}$ 0.072 µg/L (dimethenamid) $MEC_{site:}$ 0.01 µg/L (dimethenamid-P)	-
	NORMAN DB[7]	$MEC_{whole:}$ 0.2 µg/L (dimethenamid)	
	WATERBASE, 2014[8]	$MEC_{site:}$ 0.012 µg/L (dimethenamid)	
	IT monitoring programme[9]	$MEC_{95:}$ 0.524 µg/L (dimethenamid, IT) $MEC_{95:}$ 0.1315 µg/L (dimethenamid-P, IT)	

5. P, B, T, C, M, R, ED properties

Results from genotoxicity studies do not indicate that dimethenamid-P or racemic dimethenamid possess a genotoxic potential[3]. No evidence of a carcinogenic potential in rats and mice could be established[3]. Dimethenamid-P does not show any adverse effects on sexual function and fertility in adult males and females or developmental toxicity in the offspring. Dimethenamid-P has not to be classified as reproductive toxicant[3]. Dimethenamid-P is considered not readily/ rapidly biodegradable (a degradation > 70 % within 28 days) for purposes of classification and labelling. Dimethenamid-P has a log Kow of 1.89[3]. The experimentally derived steady state BCF value of 58 L/kg ww (without lipid normalization) is below the trigger of 100 (criterion for bioaccumulating potential conform Directive 67/548/EEC) and is also below the trigger of 500 criterion for bioaccumulating potential conform Regulation EC 1272/2008).[3]

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhyncus mykiss*, 96 h, LC_{50}	6.3 mg/L	Data provided from Stakeholder's comment (BASF)*
Fish	*Lepomis macrochirus*, 96 h, LC_{50}	10.4 mg/L	Data provided from Stakeholder's comment (BASF)*
Fish	*Cyprinodon variegatus*, 96 h, LC_{50}	12 mg/L	Data provided from Stakeholder's comment (BASF)*
Fish	*Oncorhyncus mykiss,* 21 d, NOEC	0.630 mg/L	Data provided from Stakeholder's comment (BASF)*
Fish	*Oncorhyncus mykiss,* 90 d, NOEC (ELS)	0.120 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic invertebrates	*Daphnia magna,* 48 h, EC_{50}	12 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic invertebrates	*Americamysis bahia,* 48 h, LC_{50}	>9.2 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic invertebrates	*Daphnia magna,* 21 d, NOEC	1.36 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic invertebrates	*Daphnia magna,* 21 d, NOEC	0.680 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Pseudokirchneriella subcapitata,* 72 h, growth rate, arithmetic mean: a)EC_{50} b)EC_{10}	a)0.0588 mg/L b)0.0159 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Anabaena flos-aquae*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)1.340 mg/L b)0.073 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Ankistrodesmus bibraianus*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.0370 mg/L b)0.00367 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Chlamydomonas reinhardtii*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.2245 mg/L b)0.0620 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Desmodesmus subspicatus*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.0857 mg/L b)0.00927 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Dictyococcus varians*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.1498 mg/L b)0.0049 mg/L	Data provided from Stakeholder's comment (BASF)*

Algae	*Monoraphidium griffithii*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.0250 mg/L b)0.0026 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Navicula pelliculosa*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.287 mg/L b)0.082 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Neochloris aquatica*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)>1000 mg/L b)0.0871 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Pandorina morum*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.9238 mg/L b)0.0329 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Planktosphaeria botryoides*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.9120 mg/L b)0.0517 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Schroederia setigera*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)>0.4055 mg/L b)0.0287 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Skeletonema costatum*, 72 h, growth rate, a)EC_{50} b)EC_{10}	a)0.309 mg/L b)0.060 mg/L	Data provided from Stakeholder's comment (BASF)*
Algae	*Staurastrum punctulatum,* 72 h, growth rate, a)EC_{50} b)EC_{10}	a)>1000 mg/L b)0.0227 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Lemna gibba,* 7 d, growth rate, arithmetic mean: a)EC_{50} b)EC_{10}	a)0.0543 mg/L b)0.0089 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Glyceria maxima,* 14 d, growth rate, a)EC_{50} b)EC_{10}	a)0.184 mg/L b)0.027 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Lemna gibba* (with sediment), 7 d, growth rate, a)EC_{50} b)EC_{10}	a)0.0990 mg/L b)0.0152 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Acorus calamus,* 13 d, growth rate, a)EC_{50} b)NOEC	a)>1.324 mg/L b)≥1.324 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Iris pseudacorus,* 13 d, growth rate, a)EC_{50} b)NOEC	a)0.229 mg/L b)0.022 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Ludwigia palustris,* 13 d, growth rate, a)EC_{50} b)NOEC	a)0.047 mg/L b)0.011 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Mentha aquatica,* 13 d, growth rate, a)EC_{50} b)NOEC	a)0.278 mg/L b)0.124 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Sparganium erectum,* 13 d, growth rate, a)EC_{50} b)NOEC	a)0.278 mg/L b)0.124 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Veronica beccalunga,* 13 d, growth rate, a)EC_{50} b)NOEC	a)0.129 mg/L b)0.011 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Ceratophyllum demersum,* 9 d, growth rate, a)EC_{50} b)NOEC	a)0.0157 mg/L b)0.0019 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Crassula recurva,* 12 d, growth rate, a)EC_{50} b)NOEC	a)0.0995 mg/L b)0.0400 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Elodea densa,* 12 d, growth rate, a)EC_{50} b)NOEC	a)0.2044 mg/L b)0.0400 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Myriophyllum spicatum,* 9 d, growth rate, a)EC_{50} b)NOEC	a)0.0972 mg/L b)0.0092 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Potamogeton crispus,* 9 d, growth rate, a)EC_{50} b)NOEC	a)0.2839 mg/L b)0.0400 mg/L	Data provided from Stakeholder's comment (BASF)*
Aquatic plants	*Vallisneria spiralis,* 12 d, growth rate, a)EC_{50} b)NOEC	a)>0.3360 mg/L b)≥0.3360 mg/L	Data provided from Stakeholder's comment (BASF)*

* and submitted during Annex I inclusion and Recent Renewal Process (2014)

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	429 mg/kg bw	EC Review, 2001 [1]
Short-term oral toxicity	Rat, 90 d, NOAEL	10 mg/kg bw/day	EC Review, 2001 [1]
Short-term oral toxicity	Dog, 90 d, NOAEL	4.3 mg/kg bw/day	EC Review, 2001 [1]
Short-term oral toxicity	**Dog, 1 year, NOAEL. Value used for ADI calculation in the EC Review, 2001[1]**	**2 mg/kg bw/day**	**EC Review, 2001 [1]**
Long-term toxicity	Rat, 105 weeks, NOAEL	<5 mg/kg bw/day	EC Review, 2001 [1]
Long-term toxicity	Mouse, 94 weeks, NOAEL	3.8 mg/kg bw/day	EC Review, 2001 [1]
Reproductive toxicity	NOAEL	36 mg/kg bw/day	EC Review, 2001 [1]
Developmental toxicity	Rat, NOAEL	50 mg/kg bw/day	EC Review, 2001 [1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value	Reference
$PNEC_{fw}$	HC_5	0.0027 mg/L	[1]	0.0027 mg/L[a]	
$PNEC_{sed}$	-	-	-	0.005 mg/kg dw[b]	
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.	-
$PNEC_{biota,\ hh}$	-	-	-	N.R.	-
$PNEC_{dw,\ hh}$	ADI [c]	0.02 mg/L	-	0.070 mg/L	INERIS, 2011[2]

[a] Probabilistic approach used. Stakeholder's comment.

[b] Value provided from Stakeholder's comment.

[c] Same ADI for dimethenamid and dimethenamid-P. No new calculations were performed.

To be noted that a further $PNEC_{fw}$ value of 0.2 µg/L (AF 10) was derived by UBA (DE), which was also suggested by NORMAN. All the available ecotoxicity data related to dimethenamid-P will be further assessed in the current monitoring-based prioritisation exercise.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**24.33**
RQ_{sed}	21.8
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.94

8. References

[1] EUROPEAN COMMISSION Review Report for the active substance dimethenamid-p, SANCO/1402/2001-Final. Available at http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.detail

[2] INERIS, DIMETHENAMIDE – N° CAS : 87674-68-8 & DIMETHENAMID-P – N° CAS : 163515-14-8 (October 2011). Available at http://www.ineris.fr/substances/fr/substance/getDocument/3077

[3] CLH Report (2012) Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2 Substance Name: Dimethenamid-P. Available at: http://echa.europa.eu/documents/10162/7307455a-5bcf-4e09-aea3-755346b9769a

[4] European Pesticides Database: http://ec.europa.eu/sanco_pesticides/public/?event=homepage

[5] COMMISSION DECISION of 22 December 2006 concerning the non-inclusion of dimethenamid in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance (notified under document number C(2006) 6895). Available at http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32006D1009

[6] IPCheM database at http://ipchem.jrc.ec.europa.eu/

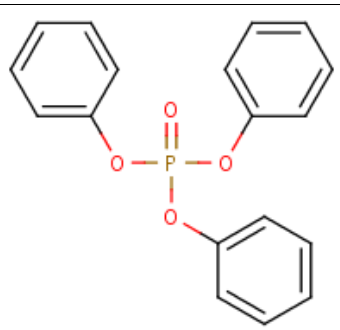
[7] NORMAN Database http://www.norman-network.net/?q=node/24

[8] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[9]Italian Monitoring Programme (data provided directly to the JRC)

Triphenyl phosphate (CAS N. 115-86-6)

1. Substance identity

CAS number	**115-86-6**
Molecular formula	**C18-H15-O4-P**
Molecular weight	**326.29**
Structure	
SMILES	P(Oc1ccccc1)(Oc1ccccc1)(Oc1ccccc1)=O

2. Physico-chemical Properties

Endpoint	**Value**	**Source**
Vapour Pressure (Pa)	0.000835	ECHA, 2013[1]
Water solubility (mg/L)	1.9	ECHA, 2013[1]
logK_{ow}	4.63	ECHA, 2013[1]

3. Environmental fate

Endpoint	**Value**	**Source**
Sorption potential K_{oc}	3561	ECHA, 2013[1]
Biodegradability	RB	ECHA, 2013[1], EPA Report[2]
Bioaccumulation (BCF)	144	ECHA, 2013[1]
BMF	1	Default value, TG n. 27 - CIS WFD[3]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	**Source**
Tonnes/year	A confidential and recent tonnage value was used for calculation	IUCLID, 2014[4]
Uses	Manufacture of substances,	ECHA, 2013[1]

	formulation in materials (plastics and rubber), formulation of flame retardant/plasticizer in preparations and cosmetics	
Spatial usage (by MS)	Not known	
Banned uses	-	
ERC code	ERC8a	
Fraction of tonnage to region	0.1	
PEC_{fw} (mg/L)	0.015	ECETOC
PEC_{sed} (mg/kg dw)	5.49	ECETOC
PEC_{biota} (mg/kg)	2.169 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required because the substance is readily biodegradable.

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
2 (DE, NL)	NORMAN DB, 2014[5]	$MEC_{95,\ whole}$: 0.07 µg/L	1 MS (RBSP EQS ECOSTAT – UBA report)[6]

5. P, B, T, C, M, R, ED properties

Triphenyl-phosphate is neither carcinogenic nor toxic to reproduction[2]. In the ECHA report, it is not classified as carcinogenic (category 1 or 2), mutagenic (category 1 or 2), or toxic for reproduction (category 1, 2 or 3) according to Directive 67/548/EEC, or carcinogenic (category 1A or 1B), germ cell mutagenic (category 1A or 1B), or toxic for reproduction (category 1A, 1B or 2) according to Regulation EC No 1272/2008. The substance is not classified as CMR[1]. No other evidence of chronic toxicity, as identified by the classifications T, R48 or Xn, R48 according to Directive 67/548/EEC or specific target organ toxicity after repeated exposure (STOT RE category 1 or 2) according to Regulation EC No 1272/2008 was reported[1].

Triphenyl phosphate is readily biodegradable (not P). It has a BCF value of 144 L/kg (not B) (EPA report[2], and ECHA, 2013[1]).

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhynchus mykiss*, 96 h, EC_{50}	0.310 mg/L	NORMAN, 2014[7]
Fish	***Oncorhynchus mykiss*, 30 d, EC_{10}**	**0.037mg/L**	**NORMAN, 2014[7]**
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	1 mg/L	NORMAN, 2014[7]
Aquatic invertebrates	*Daphnia magna*, 21 d, NOEC	0.052 mg/L	NORMAN, 2014[7]
Algae	*Desmodesmus subspicatus*, 72 h, EC_{50} growth rate	1.547 mg/L	NORMAN, 2014[7]
Algae	*Ankistrodesmus falcatus,* 72 h, NOEC	0.1 mg/L	NORMAN, 2014[7]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, feed, LD_{50}	>20000 mg/kg bw	ECHA, 2013[1]
Repeated dose toxicity	**Rat, feed, 4 weeks, NOAEL. Value used for DNEL calculation in the ECHA, 2013[1]**	**23.5 mg/kg bw/day**	**ECHA, 2013[1]**
Reproductive toxicity	Rat, feed, 3 months, NOEL	690 mg/kg bw/day	ECHA, 2013[1]
Developmental toxicity	Rat, feed, 91 d, NOAEL	≥690 mg/kg bw/day	ECHA, 2013[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Oncorhyncus mykiss*, 30 d, EC_{10}	0.037 mg/L	10[a]	3.70E-03 mg/L[b]
$PNEC_{sed}$	-	-	-	0.240 mg/kg dw[c]
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.[d]
$PNEC_{biota,\ hh}$	-	-	-	N.R.[d]
$PNEC_{dw,\ hh}$	DNEL, oral, repeated dose toxicity	0.04 mg/kg bw/day	-	0.140 mg/L[e]

N.R. Not required because the substance is readily biodegradable.

[a]AF of 10, because three long term values were available.

[b]No new calculations were made, since PNEC value was retrieved from ECHA, 2013[1] and NORMAN, 2014[1].

[c]No new calculations were made, since PNEC value was retrieved from ECHA, 2013[1]. Equilibrium partitioning method was used in the dossier.

[d]Not required because the substance is readily biodegradable.

[e]DNEL value, retrieved from ECHA, 2013[1], used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	4.05
RQ_{sed}	**22.90**
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.11

8. References

[1] ECHA dissemination website: http://apps.echa.europa.eu/registered/data/dossiers/DISS-9c823fa6-50fe-0b74-e044-00144f67d249/DISS-9c823fa6-50fe-0b74-e044-00144f67d249_DISS-9c823fa6-50fe-0b74-e044-00144f67d249.html

[2]EPA report, available at http://www.epa.gov/dfe/pubs/flameret/altrep-v2/altrept-v2-section1a.pdf

[3] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

[4]Complete IUCLID dossier of triphenyl phosphate.

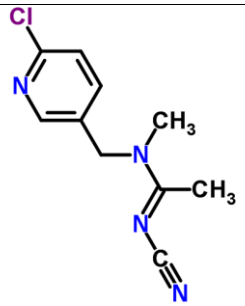
[5]NORMAN database at http://www.norman-network.net/?q=node/24

[6] Irmer U, Rau F, Arle J, Claussen U, Mohaupt V. (2013) Ecological Environmental Quality Standards of "River Basin Specific Pollutants" in Surface Waters - Update and Development Analysis of a European Comparison between Member States. ECOSTAT- UBA report.

[7] NORMAN factsheet on triphenyl phosphate, version of 31.08.2014 (available on CIRCA BC).

Acetamiprid (CAS N. 135410-20-7/160430-64-8)

1. Substance identity

EC name	
EC number	
CAS number	135410-20-7/160430-64-8
Molecular formula	C10-H11-Cl-N4
Molecular weight	222.6779
Structure	
SMILES	C/C(=N\C#N)/N(C)Cc1ccc(nc1)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	1E-06Pa (25°C)	EU Review Report, 2004[1]
Water solubility (mg/L)	2950 (pH 7, 25°C)	EU Review Report, 2004[1]
logK_{ow}	0.8 (25°C)	EU Review Report, 2004[1]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	106.5	EU Review Report, 2004[1]
Biodegradability	NRB	EU Review Report, 2004[1]
Bioaccumulation (BCF)	3.16 (estimated)	EPisuite, BCFBAF v3.01[2]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	-	
Uses	Insecticide (Plant Protection Product)	EU Pesticides DB[3]
Spatial usage (by MS)	AT, BE, BG, CY, CZ, DE, DK, EL, ES, FI, FR, HU, IE, IT, LT, LU, MT, NL, PL, PT, RO, SE, SI, SK, UK	EU Pesticides DB[3]

Banned uses	-	
ERC code	ERC8d (N.R.)	
PEC_{fw} (mg/L)	0.0050	FOCUS Step 2
PEC_{sed} (mg/kg dw)	0.0045 (N.R.)	FOCUS Step 2
PEC_{biota} (mg/kg)	0.016 (N.R.)	Calculation based on Equation L (Section 3.4.3)

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

4.1.1 FOCUS Pesticides models

FOCUS Step 1

Crop [3]	Application Rate (g/ha)[3]	Water solubility (mg/L)[3]	K_{oc} (L/kg) [3]	DT_{50} water (d) [3]
Citrus	2 x 100 (30 d interval of application)	2950	106.5	5.8

FOCUS Step 2

Same parameters and conditions as above, in addition to DT_{50soil} 2.6 d (mean) [3], $DT_{50water}$: 5.8 d[3], $DT_{50sediment}$ 1000 d (conservative value), minimal crop interception (application on young citrus)[3].

FOCUS Step 3 - SWASH package

Foliar interception and an application window of 60 days were considered. Thus, an application window from 15/01 to 16/03 was chosen for D3 and R4 crop-specific scenarios for the PAT calculator.

Results

Tier	PEC_{fw} (mg/L)	PEC_{sed} (mg/kg)
FOCUS Step 1	0.034	0.032
FOCUS Step 2	**0.005 (higher value with single appln)**	0.0045 (higher value with single appln)
FOCUS Step 3	0.0029 D6 ditch	0.0016 D6 ditch

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
2 (SE, IT)	WATERBASE (2014)[4]	Values < LOD	-
	SE pesticide monitoring programme[5]	$MEC_{95,}$ SE: 0.0054 µg/L	
	IT monitoring programme[6]	$MEC_{95,}$ IT: 0.1745 µg/L	

5. P, B, T, C, M, R, ED properties

Acetamiprid is not readily biodegradable (P)[1]. The potential for bioaccumulation is low, and has been estimated with EPISuite to be of 3.16 (not B). Also in the EU Review Report[1] the bioaccumulation potential was considered to be not relevant.

Evidence of clastogenic potential in vitro[1]. This event was found to be not relevant for the in vivo situation with a negative mouse micronucleus assay and metaphase analysis in rat bone marrow[1]. No carcinogenic potential, treatment related mammary glands hyperplasia was found at 1000 ppm[1]. No teratogenicity or fetotoxicity was observed at the tested doses, but in the reproductive toxicity study reduced postnatal survival and decreased pup weight was found at parental toxic doses (EU Review Report, 2004[1]).

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	*Oncorhyncus mykiss*, 96 h , mortality, EC_{50}	>100 mg/L	EU Review Report, 2004[1]
Fish	*Pimephales promelas,* 35 d , growth, NOEC	19.2 mg/L	EU Review Report, 2004[1]
Aquatic invertebrates	*Daphnia magna,* 48 h , mortality, EC_{50}	49.8 mg/L	EU Review Report, 2004[1]
Aquatic invertebrates	*Daphnia magna,* 21 d , reproduction, NOEC	5 mg/L	EU Review Report, 2004[1]
Algae	*Scenedesmus subspicatus,* 72 h, biomass, EC_{50}	>98.3 mg/L	EU Review Report, 2004[1]
Sediment dwelling organisms	***Chironomus riparius*, 28 d, emergence and developmental rate, NOEC**	**0.005 mg/L**	EU Review Report, 2004[1]
Aquatic plants	*Lemna gibba*, 14 d, frond, EC_{50}	1 mg/L	EU Review Report, 2004[1]

6.2 Mammalian toxicology data

Type of test	Endpoint	Value	Reference
Acute oral toxicity	Rat, LD_{50}	417 mg/kg bw	EU Review Report, 2004[1]
Short term oral toxicity	Rat, 90 d, NOAEL	12.4 mg/kg bw/day	EU Review Report, 2004[1]
Long term toxicity	**Rat, 2 year, NOAEL.**	**7 mg/kg bw/day**	EU Review Report,

	Value used for ADI calculation in the EU Review Report[1], with the NOAEL from thereproductive study below.		2004[1]
Reproductive toxicity	**Rat, NOAEL**	**6.5 mg/kg bw/day**	EU Review Report, 2004[1]
Developmental toxicity	Rabbit, NOAEL	15 mg/kg bw/day	EU Review Report, 2004[1]

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Chironomus riparius*, 28 d, emergence and developmental rate, NOEC[a]	0.005 mg/L[b]	10[c]	5.00E-04 mg/L
$PNEC_{sed}$	-	-	-	N.R.
$PNEC_{biota,sec\ pois}$	-	-	-	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI	0.07mg/kg bw/day	-	0.245 mg/L[d]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] Due to the mode of action of neonicotinoids, the lowest endpoint from the aquatic species tested corresponds to the midge *Chironomus riparius*. Therefore, it was selected for $PNEC_{fw}$ calculation.

[b] Value retrieved from EU Review Report, 2004[1]

[c] Three long-term values were available.

[d]ADI value, retrieved from EU Review Report, 2004[1], used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**10**
RQ_{sed}	N.R.
$RQ_{biota,sec\ pois}$	N.R.
$RQ_{biota,\ hh}$	N.R.
$RQ_{dw,\ hh}$	0.02

8. References

[1] EUROPEAN COMMISSION Review report for the active substance acetamiprid, SANCO/1392/2001– Final. Available at http://ec.europa.eu/food/plant/protection/evaluation/newactive/acetamiprid.pdf

[2] EPISuite BCFBAF v3.01 (2012)

[3] http://ec.europa.eu/sanco_pesticides/public/?event=homepage

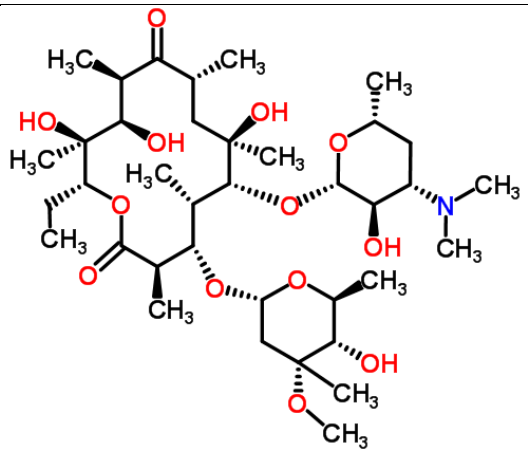
[4] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

[5] Swedish National Screening Programme Pesticides (data provided directly to the JRC)

[6] Italian Monitoring Programme (data provided directly to the JRC)

Erythromycin (CAS N. 114-07-8)

1. Substance identity

EC name	Erythromycin
EC number	204-040-1
CAS number	114-07-8
Molecular formula	C37H67NO13
Molecular weight	733.94
Structure	
SMILES	CC[C@@H]1[C@@]([C@@H]([C@H](C(=O)[C@@H](C[C@@]([C@@H]([C@H]([C@@H]([C@H](C(=O)O1)C)O[C@H]2C[C@@]([C@H]([C@@H](O2)C)O)(C)OC)C)O[C@H]3[C@@H]([C@H](C[C@H](O3)C)N(C)C)O)(C)O)C)O)(C)O

2. Reason for proposal as candidate for the Watch list and suspected environmental risk

Erythromycin is a macrolide antibiotic produced by Streptomyces erythreus. It inhibits bacterial protein synthesis by binding to bacterial 50S ribosomal subunits; binding inhibits peptidyl transferase activity and interferes with translocation of amino acids during translation and assembly of proteins. Erythromycin may be bacteriostatic or bactericidal depending on the organism and drug concentration.[1] Erythromycin has been classified as Category 2 according to the NORMAN Prioritisation Methodology[2]. In the NORMAN factsheet[3] it was reported a frequency of exceedance of 12% and an extent of exceedance of 3.74-fold of the lowest PNEC[3], considering monitoring data from 2002-2011 in the NORMAN database[4]. Besides the use as human and veterinary medicine, it is reported to have Industrial use resulting in manufacture of another substance (use of intermediates)[5].

3. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	3.04E-25	ChemIDPlus [6]
Water solubility (mg/L)	2000	Drugbank [1]
logK_{ow}	3.06	ChemIDPlus [6]

4. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	570	PubChem [7]
Biodegradability	NRB	NORMAN, 2014 [3]
Bioaccumulation (BCF)	48.5	NORMAN, 2014 [3]
BMF	1	Default value, TG n. 27 - CIS WFD [8]

5. Environmental exposure assessment

	Description	Source
Tonnes/year	Confidential tonnage used for calculation	IUCLID, 2013[9]
Uses	Industrial use resulting in manufacture of another substance (use of intermediates)	ECHA, 2014[5]
	Pharmaceutical (human and veterinary medicine)	-
Spatial usage (by MS)	Widespread use	Drugs.com [10]
Banned uses	-	
ERC code	ERC6a *	ECHA, 2014 [5]
Fraction of tonnage to region	0.1	

* This substance is registered as having an environmental release category ERC6a. However, the PEC sediment calculated using this code with ECETOC seemed unrealistically high and it was decided to use instead ERC8a in this exercise, suitable for pharmaceuticals (see section 4.1.1 of this factsheet).

5.1 Predicted Environmental Concentration

	ECETOC[a]	Human consumption (Eq. G)[b]	MEC[c]
PEC_{fw} (mg/L)	**0.00526**	**0.0002**	**0.000613**
PEC_{sed} (mg/kg dw)	0.3185[d]	0.006[e]	0.0185[e]
PEC_{biota} (mg/kg)	0.255[f]	0.010[f]	0.030[f]

[a] Intermediate use

[b] Besse et al., 2008 11 (Equation G) for PEC equation, human consumption data from DK.

[c] MEC_{95} (SE)

[d] PEC_{sed} calculated from ECETOC

[e] PEC_{sed} calculated with the Equilibrium Partitioning Method, where $K_{sed\text{-}water}$= 15.05 m^3m^{-3} (calculated with eq. K of section 3.4.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 28.5 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 570 L/kg (from PubChem[7]), Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. I (see section 3.4.2).

[f] Calculation with Equation L (Section 3.4.3)

5.1.1 PEC calculation considering different uses or sales data from MS

Uses	Calculation tool/ equation	Country	PEC_{fw} (µg/L)
Intermediate use	ECETOC tool ERC8a was used, instead of ERC6a	Europe (use of tonnes registered in IUCLID)	5.26
Human use	PEC_b[a]	Portugal	0.150
Human use	PEC_b[a]	Latvia	0.073
Human use	PEC_b[a]	Greece	0.0614
Human use	PEC_b[a]	Germany	0.132
Human use	PEC_b[a]	Denmark	**0.2**
Veterinary use	No sales data	-	-

[a] PEC_b equation was retrieved from Besse et al, 2008 [11]

PEC_b equation: $PEC_{fw} = (consumption \times F_{excreta}) / (WWinhab \times hab \times dilution \times 365)$

where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants in the respective country (retrieved from PT[12], LV[13], EL[14], DE[15], and DK[16] official sources). $F_{excreta}$ is the excretion factor of the active substance[11], *dilution* is the dilution factor (default value of 10), *consumption* is the quantity (mg/year) of active ingredient consumed by the population during 1 year. Consumption data were taken from Besse et al, 2008[11] for FR, from Iatrou et al, 2014[17] for EL, from UBA report[18] for DE, and directly provided to the JRC for PT, LV, and DK[19].

5.2 Measured Environmental Concentration

From an analysis of pharmaceutical datasets in river systems worldwide collected from the literature, Hughes et al. (2013)[20] report that erythromycin has a mean detection frequency worldwide of 55.5% (from all the records for that particular substance), and median and maximum concentration worldwide of 0.05 and 90 µg/L, respectively. The frequency of quantification of erythromycin in the NORMAN database is 15%[3]. However, considering just the monitoring data for the period 2006-2014, all values were < LOQ.

n. of MS	Source of monitoring data	MEC values	RBSP
3 (NL, CH, SE)	NORMAN DB, 2014 [4]	All values < LOQ (CH, NL)*	-
	SE Screening Programme Pharmaceuticals [21]	MEC_{95}: 0.613 µg/L (SE)	

* To be noted that these values correspond to the period 2006-2014, while higher MEC values were available if monitoring data since 2002 were considered.

6. P, B, T, C, M, R, ED properties

Reported to be not carcinogenic and genotoxic, as erythromycin stearate form[22].

Utilizing the Closed Bottle Test, -3% of the theoretical BOD was reported in 4 weeks, indicating that biodegradation is not an important environmental fate process in water (P) (PubChem)[7]. A pKa of 8.9 indicates erythromycin will exist almost entirely in the cation form at pH values of 5 to 9 and therefore volatilization from water surfaces is not expected to be an important fate process. An estimated BCF of 49 suggests the potential for bioconcentration in aquatic organisms is moderate (Not B) (PubChem)[7].

7. Hazard assessment

7.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference [a]
Fish	*Morone saxatilis*, 96 h, LC_{50}	349 mg/L	Bills et al. 1993[23](NORMAN, 2014[3] and UBA, 2014[24])
Fish	*Danio rerio*, 96 h, mortality, LC_{50}	>1000 mg/L	Isidori et al. 2005[27] (UBA, 2014[24])
Fish	*Pimephales promelas*, 96 h, mortality, LC_{50}	61 mg/L	Sanderson et al. 2003[26] (UBA, 2014[24])
Fish	*Oryzias latipes*, 10 d, hatchability, time to hatch, NOEC	1000 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Fish	*Oryzias latipes*, 40 d, juvenile survival, NOEC	100 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Fish	*Oryzias latipes*, 40 d, juvenile growth, NOEC	1000 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Fish	*Oryzias latipes*, 100 d, adult survival growth, NOEC	10 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Aquatic invertebrates	*Ceriodaphnia dubia*, 48 h, EC_{50}	10.23 mg/L	Isidori et al. 2005[25] (NORMAN, 2014[3] and UBA, 2014[24])
Aquatic invertebrates	*Brachionus calyciflorus*, 24 h, mortality, LC_{50}	27.53 mg/L	Isidori et al. 2005[25] (UBA, 2014[24])
Aquatic invertebrates	*Brachionus calyciflorus*, 48 h, mortality, EC_{50}	0.940 mg/L	Isidori et al. 2005[25] (UBA, 2014[24])
Aquatic invertebrates	*Daphnia magna,* 24 h, mobility, EC_{50}	22.45 mg/L	Isidori et al. 2005[25] (UBA, 2014[24])
Aquatic invertebrates	*Thamnocephalus platyrus,* 24 h, mobility, EC_{50}	17.68 mg/L	Isidori et al. 2005[25] (UBA, 2014[24])

Aquatic invertebrates	*Daphnia magna,* 48 h, mobility, EC_{50}	207.8 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Aquatic invertebrates	*Moina macrocopa,* 48 h, mobility, EC_{50}	135.5 mg/L	Ji et al. 2012[27](UBA, 2014[24])
Aquatic invertebrates	*Penaeus vannamei,* 48 h, mobility, EC_{50}	0.0227 mg/L	Williams et al. 1992[28] (UBA, 2014[24])
Aquatic invertebrates	*Daphnia magna*, 21 d, reproduction, NOEC	0.248 mg/L	Meinertz et al. 2010[29] (NORMAN, 2014[3] and UBA, 2014[24])
Aquatic invertebrates	*Ceriodaphnia dubia*, 7 d, population growth, EC_{50}	0.220 mg/L	Isidori et al. 2005[25] (UBA, 2014[23])
Aquatic invertebrates	*Moina macrocopa*, 7 d, survival reproduction, NOEC	50 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Aquatic invertebrates	*Daphnia magna*, 21 d, survival, NOEC	33.3 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Aquatic invertebrates	*Daphnia magna*, 21 d, reproduction growth, NOEC	11.1 mg/L	Ji et al. 2012[27] (UBA, 2014[24])
Algae	*Pseudokirchneriella subcapitata,* 72 h, growth, EC_{50}	0.020 mg/L	Isidori et al. 2005[25] (NORMAN, 2014[3] and UBA, 2014[24])
Algae	*Pseudokirchneriella subcapitata,* 72 h, biomass, NOEC	0.0103 mg/L	Eguchi et al. 2004[30] (NORMAN, 2014[3] and UBA, 2014[24])
Algae	*Pseudokirchneriella subcapitata,* 72 h, biomass, EC_{50}	0.0366 mg/L	Eguchi et al. 2004[30] (NORMAN, 2014[3] and UBA, 2014[24])
Algae	*Pseudokirchneriella subcapitata,* 72 h, growth (chlorophyll fluorescence), EC_{50}	0.350 mg/L	Gonzaléz-Pleiter et al. 2013[31] (UBA, 2014[24])
Algae	*Chlorella vulgaris,* 72 h, biomass, EC_{50}	33.8 mg/L	Eguchi et al. 2004[30] (NORMAN, 2014[3] and UBA, 2014[24])
Algae	*Pseudokirchneriella subcapitata,* 72 h, growth (chlorophyll fluorescence), EC_{10}	0.036 mg/L	Gonzaléz-Pleiter et al. 2013[31] (UBA, 2014[24])
Algae	*Chlorella vulgaris,* 72 h, biomass, NOEC	12.5 mg/L	Eguchi et al. 2004[30] (UBA, 2014[24])
Cyanobacteria	*Microcystis wesenbergii*	0.023 mg/L	Ando et al. 2007[32]

	NIES-107, 144 h, EC_{50}		(NORMAN, 2014[3] and UBA, 2014[24])
Cyanobacteria	***Synechococcus leopoldensis IAM-M6*, 144 h, biomass, NOEC**	**0.002 mg/L**	**Ando et al. 2007[32] (NORMAN, 2014[3] and UBA, 2014[24])**
Cyanobacteria	*Nostoc sp* PCC 7120, 144 h, biomass, EC_{50}	0.2 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena variabilis NIES-23,* 144 h, biomass, EC_{50}	0.430 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Microcystis wesenbergii NIES-107,* 144 h, biomass, NOEC	0.0047 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Synechococcus sp. PCC 7002,* 144 h, biomass, EC_{50}	0.230 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Synechococcus leopoldensis IAM-M6*, 144 h, EC_{50}	0.160 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Microcystis aeruginosa NIES-44,* 144 h, biomass, EC_{50}	0.023 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae ATCC 29413,* 144 h, biomass, EC_{50}	0.270 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena cylindrica NIES-19,* 144 h, biomass, EC_{50}	0.035 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae*, 72 h, yield, EC_{50}	0.140 mg/L	Förster et al. 2013[33] (UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae*, 72 h, growth rate, EC_{50}	0.348 mg/L	Förster et al. 2013[33] (UBA, 2014[24])
Cyanobacteria	*Anabaena sp.*, 72 h, growth (inhibition of constitutive luminescence), EC_{50}	0.022 mg/L	Gonzaléz-Pleiter et al. 2013[31] (UBA, 2014[24])
Cyanobacteria	*Anabaena variabilis NIES-23,* 144 h, biomass, NOEC	0.047 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Nostoc sp* PCC 7120, 144 h, biomass, NOEC	0.1 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Synechococcus sp. PCC 7002,* 144 h, biomass, NOEC	0.0078 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Microcystis aeruginosa*	0.010 mg/L	Ando et al. 2007[32]

	NIES-44, 144 h, biomass, NOEC		(UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae ATCC 29413,* 144 h, biomass, NOEC	0.047 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena cylindrica NIES-19,* 144 h, biomass, NOEC	0.0031 mg/L	Ando et al. 2007[32] (UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae*, 72 h, yield, growth rate, NOEC	0.030 mg/L	Förster et al. 2013[33] (UBA, 2014[24])
Cyanobacteria	*Anabaena flos-aquae*, 72 h, yield. growth rate, LOEC	0.090 mg/L	Förster et al. 2013[33] (UBA, 2014[24])
Cyanobacteria	*Anabaena sp.*, 72 h, growth (inhibition of constitutive luminescence), EC_{10}	0.005 mg/L	Gonzaléz-Pleiter et al. 2013[31] (UBA, 2014[24])
Cyanobacteria	*Synechocystis sp.,* 5 d, growth, NOEC	0.010 mg/L	Pomati et al. 2004[34] (UBA, 2014[24])
Aquatic plants	*Lemna minor*, frond number, 7 d, EC_{50}	5.62 mg/L	Pomati et al 2004[34] (UBA, 2014[24])
Aquatic plants	*Lemna minor*, frond number, 7 d, NOEC	0.010 mg/L	Pomati et al 2004[34] (UBA, 2014[24])

[a] The references were taken from the NORMAN factsheet on erythromycin[3], and from the UBA report[24], where the reliability of the studies have been assessed and considered reliable.

7.2 Mammalian toxicology data

No information retrieved

7.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Synechococcus leopoldensis* IAM-M6, 144 h, NOEC	0.002 mg/L	10	0.0002 mg/l [23]
$PNEC_{sed}$	-	-	-	0.0060 mg/kg dw [a]
$PNEC_{biota,sec\ pois}$	-	-	-	- [b]
$PNEC_{biota,\ hh}$	ADI	0.0007 mg/kg bw/day	-	0.043 mg/kg food [c]
$PNEC_{dw,\ hh}$	ADI	0.0007 mg/kg bw/day	-	0.002 mg/L [d]

[a] Calculated with the Equilibrium partitioning method. $K_{sed\text{-}water}$= 15.05 m^3m^{-3} (calculated with eq. D), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 28.5 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 570 L/kg, Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. B (Section 3.3.2).

[b] Mammalian toxicity values lacking.

[c] ADI value retrieved from the WHO report (see reference 35) used in equation E as TL. See section 3.3.4 for calculation

[d] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation

8. Risk Quotient (PEC/PNEC)

RQ	ECETOC[a]	Human consumption (Eq. G)[b]	MEC[c]
RQ_{fw}	26.3	**1.00**	**3.07**
RQ_{sed}	**52.9**	**1.00**	**3.07**
$RQ_{biota,sec\ pois}$	No info	No info	No info
$RQ_{biota,\ hh}$	5.99	0.23	0.7
$RQ_{dw,\ hh}$	2.15	0.08	0.25

9. References

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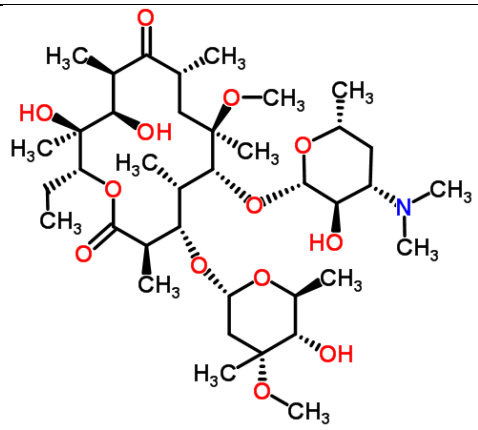
Clarithromycin (CAS N. 81103-11-9)

1. Substance identity

Chemical name (IUPAC)	2R,3R,4S,5R,8R,9S,10S,11R,12R,14R)-11-[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-5-ethyl-3,4-dihydroxy-9-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4, 6-dimethyloxan-2-yl]oxy-12-methoxy-2,4,8,10,12,14-hexamethyl-6-oxacyclotetradecan-1,7-dione
EC number	-
CAS number	81103-11-9
Chemical class	Aazalide, a subclass of macrolide antibiotics
Molecular formula	C38H69NO13
Molecular weight	747.95
Structure	
SMILES	CC[C@@H]1[C@@]([C@@H]([C@H](C(=O)[C@@H](C[C@@]([C@@H]([C@H]([C@@H]([C@H](C(=O)O1)C)O[C@H]2C[C@@]([C@H]([C@@H](O2)C)O)(C)OC)C)O[C@H]3[C@@H]([C@H](C[C@H](O3)C)N(C)C)O)(C)OC)C)C)O)(C)O

2. Reason for proposal as candidate for the Watch list and suspected environmental risk

Clarithromycin, a semisynthetic macrolide antibiotic derived from erythromycin, inhibits bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit. Binding inhibits peptidyl transferase activity and interferes with amino acid translocation during the translation and protein assembly process[1].

Clarithromycin has been classified as Category 2 according to the NORMAN Prioritisation Methodology[2], with a frequency of exceedance of 15% and an extent of exceedance of 2.33-fold of the lowest PNEC[1], considering monitoring data from 2002-2011in the NORMAN database[3].

A significant ecotoxicological risk due to the presence of clarithromycin in treated waste water in EL was estimated from acute and chronic toxicity data in algae [4]. In addition, a risk indicator considered adverse to ecosystems was calculated for clarithromycin [5] considering the presence of this substance in the Llobregat river in ES[6]. Furthermore, clarithromycin was considered to pose a potential risk to the environment considering the predicted exposure in Turkey [7].

3. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (mm Hg)	2.32E-25	PubChem, 2014[8]
Water solubility (mg/L)	0.33	Drugbank, 2014[9]
logK_{ow}	3.16	Drugbank, 2014 [9]

4. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	150	PubChem, 2014[8]
Biodegradability	NRB	PubChem, 2014[8]
Bioaccumulation (BCF)	56.49 (estimated)	PubChem, 2014[8]
BMF	1	Default value, TG n. 27 - CIS WFD [10]

5. Environmental exposure assessment

	Description	Source
Tonnes/year	-	
Uses	Pharmaceutical	
Spatial usage (by MS)	Wide dispersive use (diffuse sources, present in urban wastewater)	Drugbank, 2014 [9]
Banned uses	-	
ERC code	-	
Fraction of tonnage to region	-	

5.1 Predicted Environmental Concentration

	Human consumption (Eq. G)[a]	MEC[b]
PEC_{fw} (mg/L)	0.000438	0.000645
PEC_{sed} (mg/kg dw)	0.0040[c]	0.0059[c]
PEC_{biota} (mg/kg)	0.025[d]	0.036[d]

[a]Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from EL.

[b] MEC_{95} (SE)

[c] PEC_{sed} calculated with the Equilibrium Partitioning Method, where $K_{sed\text{-}water}$= 4.55 m^3m^{-3} (calculated with eq. K of section 3.4.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 7.5 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 150 L/kg (from PubChem[8]), Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. I (see section 3.4.2).

[d] Calculation with Equation L (Section 3.4.3)

5.1.1 PEC calculation considering different uses or sales data from MS

Uses	Calculation tool/ equation	Country	PEC_{fw} (µg/L)
Human use	PEC_b[a]	France	0.062
Human use	PEC_b[a]	Portugal	0.080
Human use	PEC_b[a]	Latvia	0.073
Human use	PEC_b[a]	Greece	**0.438**
Human use	PEC_b[a]	Germany	0.0406
Human use	PEC_b[a]	Denmark	0.0188
Veterinary use	No sales data	-	-

[a] PEC_b equation was retrieved from Besse et al, 2008 [11]

PEC_b equation: $PEC_{fw} = (consumption \times F_{excreta}) / (WWinhab \times hab \times dilution \times 365)$

where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants in the respective country (retrieved from PT[12], LV[13], EL[14], DE[15] and DK[16] official sources). $F_{excreta}$ is the excretion factor of the active substance[11], *dilution* is the dilution factor (default value of 10), *consumption* is the quantity (mg/year) of active ingredient consumed by the population during 1 year. Consumption data were taken from Besse et al, 2008[11] for FR, from Iatrou et al, 2014[4] for EL, from UBA report for DE[17], and directly provided to the JRC for PT, LV, and DK[18].

5.2 Measured Environmental Concentration

From an analysis of pharmaceutical datasets in river systems worldwide collected from the literature, Hughes et al. (2013)[19] report that clarithromycin has a mean detection frequency worldwide of 54% (from all the records for that particular substance), median and maximum concentration worldwide of 0.016 and 0.260 µg/L, respectively, while median and maximum concentrations in European studies were around 0.05 and 0.5 µg/L, respectively (exact values for European studies could not be retrieved from the plot in the publication). The frequency of detection of clarithromycin in the NORMAN database is 33% (NORMAN, 2014)[1].

n. of MS	Source of monitoring data	MEC values	RBSP
3 (NL, CH, SE)	NORMAN DB, 2014[3]	$MEC_{95,\ whole}$: 0.096 µg/L (CH, NL*) $MEC_{site,\ dissolved}$: 0.001 µg/L (CH)	-
	SE Screening Programme Pharmaceuticals[20]	MEC_{95}: 0.645 µg/L (SE)	

* All values <LOD

6. P, B, T, C, M, R, ED properties

Clarithromycin failed to exhibit mutagenic potential in several in vitro tests, including the Salmonella mammalian microsome test, bacterial induced mutation frequency test, rat hepatocyte DNA synthesis assay, mouse lymphoma assay, mouse dominant lethal test, and mouse micronucleus test[8].

P: Very persistent (DT50 sediment = 379 days) NORMAN, 2014[1]

B: Not Bioaccumulative ($BCF_{estimated}$ = 56.49) NORMAN, 2014[1]

7. Hazard assessment

7.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference[a]
Algae	*Pseudokirchneriella subcapitata,* 72 h, biomass, EC_{50}	2 µg/L	Isidori et al. 2005[21]
Algae	*Pseudokirchneriella subcapitata,* 96 h, NOEC	4 µg/L	Yamashita et al. 2006[22]
Algae	***Anabaena flos-aquae*, 72 h, growth rate, EC_{10}**	**2.6 µg/L**	**UBA, 2014[23]**
Invertebrates	*Ceriodaphnia dubia,* 48 h, LC_{50} (static)	18 660 µg/L	Isidori et al. 2005[21]
Invertebrates	*Daphnia magna,* 21 d, NOEC (semi-static)	3.1 µg/L	Yamashita et al. 2006[22]
Rotifera	*Brachionus calyciflorus,* 24 h, LC_{50}	35 460 µg/L	Isidori et al. 2005[21]
Fish	*Oryzias latipes,* 96 h, EC_{50}	> 100,000	Kim et al. 2009[24]

[a] The references were taken from the NORMAN factsheet on clarithromycin[1], and the studies were considered reliable.

7.2 Mammalian toxicology data

No studies found.

7.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Anabaena flos-aquae,* 72 h, EC_{10}	2.6 µg/L	10*2	0.13 µg/L [a]
$PNEC_{sed}$	-	-	-	0.0012 mg/kg dw[b]
$PNEC_{biota,sec\ pois}$	-	-	-	Info missing
$PNEC_{biota,\ hh}$	ADI	0.0002 mg/kg bw/day	-	0.012 mg/kg food [c]

$PNEC_{dw, hh}$	ADI	0.0002 mg/kg bw/day	-	0.001 mg/L [d]

[a] The PNEC value was retrieved from the UBA factsheet[23] on the substance. The additional AF of 2 was used because the toxic metabolite 14-Hydroxy-Claritromycin occur up to about 50% in surface water and is equivalent toxic.
[b] Calculated using the equilibrium partitioning method. The following values were used: $K_{sed\text{-}water}$= 4.55 m^3m^{-3} (calculated with eq. D of section 3.3.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 7.5 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 150 L/kg (from PubChem[8]), Foc_{sed}= 0.05 kg kg-1 (default value). Conversion from wet weight to dry weight was done with eq. B (see section 3.3.2).
[c]ADI value retrieved from Leung et al., 2013 (see reference 25) used in equation E as TL. See section 3.3.4 for calculation
[d] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation

8. Risk Quotient (PEC/PNEC)

RQ	Human consumption (Eq. G)[a]	MEC[b]
RQ_{fw}	**3.37**	**4.96**
RQ_{sed}	**3.37**	**4.96**
$RQ_{biota,sec\ pois}$	No info	No info
$RQ_{biota, hh}$	2.03	2.99
$RQ_{dw, hh}$	0.63	0.92

[a]Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from EL.
[b] MEC_{95} (SE)

9. References

[1] NORMAN factsheet on clarithromycin, version of 31.08.2014 (available on CIRCA BC).

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[17] Pharmaceuticals in the Environment – A first Compilation of German Monitoring Data. Federal Environment Agency, 01.10.2013. Available at http://www.umweltbundesamt.de/sites/default/files/medien/377/dokumente/compilation-pharmaceuticalsintheenvironment_uba.pdf[18] National human consumption data of antibiotics in Portugal, Latvia, and Denmark directly provided to the JRC by the respective MS.

[19] Hughes SR, Kay P, Brown LE. (2013) Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ Sci Technol. 47(2):661-77.

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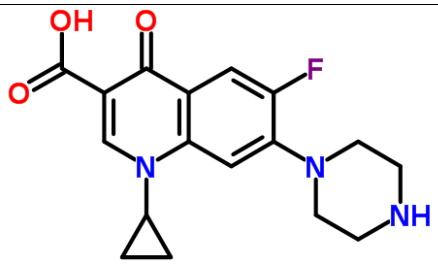
Ciprofloxacin (CAS N. 85721-33-1)

1. Substance identity

Chemical name (IUPAC)	1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid
EC number	-
CAS number	85721-33-1
Chemical class	Carboxy-fluoroquinoline
Molecular formula	C17H18FN3O3
Molecular weight	331.3
Structure	
SMILES	c1c2c(cc(c1F)N3CCNCC3)n(cc(c2=O)C(=O)O)C4CC4

2. Reason for proposal as candidate for the Watch list and suspected environmental risk

Ciprofloxacin is a broad-spectrum antiinfective agent of the fluoroquinolone class. Ciprofloxacin has in vitro activity against a wide range of gram-negative and gram-positive microorganisms[1]. Ciprofloxacycin has been classified as Category 2 according to the NORMAN Prioritisation Methodology[2], with a frequency of exceedancy of 18% and an extent of exceedancy of 7.53-fold of the lowest PNEC[3], considering monitoring data from 2002-2011 in the NORMAN database[4].

Ciprofloxacin has been classified as posing moderate risk from the Stockholm County Council[5]. In addition, a risk quotient greater than 1 was determined for this substance by performing an environmental risk assessment according to the guideline recommended by the European Medicines Agency (EMA), and measured concentrations confirmed that the release of ciprofloxacin from wastewater treatment works may potentially be of environmental concern in NO[6]. A significant ecotoxicological risk due to the presence of ciprofloxacin in treated waste water in EL was estimated from acute toxicity data in algae[7].

In addition, a risk indicator considered adverse to ecosystems was calculated for ciprofloxacin[8] considering the presence of this substance in the Llobregat river in ES[9]. Ciprofloxacin is one of the most frequently detected fluoroquinolone antibiotics in hospital wastewater and concentrations in surface water are potentially hazardous to the environment[10].

3. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (mm Hg)	2.85E-13	Pubchem, 2014[11]
Water solubility (mg/L)	30000	Pubchem, 2014[11]
logK_{ow}	0.28	Pubchem, 2014[11] , Schwab et al. 2005[12]

4. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	61000	Pubchem, 2014[11]
Biodegradability	NRB	Pubchem, 2014[11]
Bioaccumulation (BCF)	3.2 L/kg	Schwab et al. 2005[12]
BMF	1	Default value, TG n. 27 - CIS WFD [13]

5. Environmental exposure assessment

	Description	Source
Tonnes/year	-	
Uses	Pharmaceutical	
Spatial usage (by MS)	Wide dispersive use (diffuse sources, present in urban wastewater)	NORMAN, 2014[3]
Banned uses	-	
ERC code	-	
Fraction of tonnage to region	-	

5.1 Predicted Environmental Concentration

	Human consumption (Eq. G)[a]	MEC[b]
PEC_{fw} (mg/L)	0.000538	0.00124
PEC_{sed} (mg/kg dw)	1.6418[c]	3.78[c]
PEC_{biota} (mg/kg)	0.0017[d]	0.004[d]

[a]Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from PT.

[b] MEC_{95} (SE – NORMAN DB)

[c] PEC_{sed} calculated with the Equilibrium Partitioning Method, where $K_{sed\text{-}water}$= 1525.8 m^3m^{-3} (calculated with eq. K of section 3.4.2), RHO_{sed}= 1300 kg m-3 (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 3050 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 61000 L/kg (from PubChem[11]), Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. I (see section 3.4.2).

[d] Calculation with Equation L (Section 3.4.3)

5.1.1 PEC calculation considering different uses or sales data from MS

Uses	Calculation tool/ equation	Country	PEC_{fw} (µg/L)
Human use	PEC_b[a]	France	0.139
Human use	PEC_b[a]	Portugal	**0.540**
Human use	PEC_b[a]	Latvia	0.210
Human use	PEC_b[a]	Greece	0.530
Human use	PEC_b[a]	Denmark	0.166
Veterinary use	No sales data	-	-

[a] PEC_b equation was retrieved from Besse et al, 2008[14]

PEC_b equation: $PEC_{fw} = (consumption \times F_{excreta}) / (WWinhab \times hab \times dilution \times 365)$

where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants in the respective country (retrieved from PT[16], LV[17], EL[18], DK[19] official sources). $F_{excreta}$ is the excretion factor of the active substance retrieved from Besse et al, 2008[14], *dilution* is the dilution factor (default value of 10), *consumption* is the quantity (mg/year) of active ingredient consumed by the population during 1 year. Consumption data were taken from Besse et al, 2008[14] for FR, from Iatrou et al, 2014[7] for EL, and directly provided to the JRC for PT[15]. DK and LV[20].

5.2 Measured Environmental Concentration

From an analysis of pharmaceutical datasets in river systems worldwide collected from the literature, Hughes et al. (2013)[21] report that ciprofloxacin has a mean detection frequency worldwide of 33.4% (from all the records for that particular substance) and median and maximum concentrations worldwide of 164 and 6500 µg/L, respectively. The frequency of quantification of ciprofloxacin in the NORMAN database is 18% (NORMAN, 2014)[3].

n. of MS	Source of monitoring data	MEC values	RBSP
2 (SE, PT)	NORMAN DB, 2014[4]	$MEC_{95, whole}$: 1.24 µg/L (SE) $MEC_{95, dissolved}$: 0.22 µg/L[a] (PT)	-
	SE Screening Programme Pharmaceuticals[22]	MEC_{95}: 0.206 µg/L (SE)	

[a] outlier has been removed from the monitoring dataset.

6. P, B, T, C, M, R, ED properties

Eight in vitro mutagenicity tests have been conducted with Ciprofloxacin, 2 of the 8 tests were positive, but results of the further 3 in vivo test systems gave negative results[23]. Long-term carcinogenicity studies in rats and mice resulted in no carcinogenic or tumorigenic effects[23]. Fertility studies performed in rats at oral doses of Ciprofloxacin up to 100 mg/kg (approximately 0.7-times the highest recommended therapeutic dose based upon mg/m2) revealed no evidence of impairment[23].

An estimated BCF of 3 (SRC), from its log Kow of 0.28, suggests the potential for bioconcentration in aquatic organisms is low (SRC)[11]. Using the OECD closed bottle biodegradation study, 0% degradation

over a 40-day incubation period was observed indicating that biodegradation is not an important environmental fate process in water[11].

7. Hazard assessment

7.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference[a]
Fish	*Oncorhyncus mykiss*, 96 h, LC_{50}	>9.4 mg/L	Gagliano & McNamara 1996[24]
Aquatic invertebrates	*Daphnia magna*, 48 h, EC_{50}	58.8 mg/L	Martins et al. 2012[25]
Aquatic invertebrates	*Daphnia magna*, 21 d, NOEC	4.67 mg/L	Martins et al. 2012[25]
Cyanobacteria	*Anabaena flos-aquae*, 72 h, EC_{50}	0.036 mg/L	Ebert et al. 2011[26]
Cyanobacteria	***Anabaena flos-aquae*, 72 h, EC_{10}**	**0.00447 mg/L**	Ebert et al. 2011[26]
Aquatic plants	*Lemna minor*, 7 d, EC_{50}	0.499 mg/L	Ebert et al. 2011[26]
Aquatic plants	*Lemna minor*, 7 d, EC_{10}	0.149 mg/L	Brain et al. 2004[27]

[a] The references were taken from the NORMAN factsheet on ciprofloxacin[13], and the studies were considered reliable.

7.2 Mammalian toxicology data

No information retrieved

7.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Anabaena flos-aquae*, 72 h, EC_{10}	0.00447 mg/L	50	8.9E-05 mg/L [a]
$PNEC_{sed}$	-	-	-	0.272 mg/kg dw [b]
$PNEC_{biota,sec\ pois}$	**-**	**-**	**-**	N.R.
$PNEC_{biota,\ hh}$	-	-	-	N.R.
$PNEC_{dw,\ hh}$	ADI	0.0016 mg/kg day	-	0.006 mg/L [c]

N.R. Not required based on BCF value not reaching the trigger value required for biota assessment

[a] Two long-term values available from the main trophic levels. No new calculations were performed, since PNEC value was retrieved from NORMAN factsheet, 2014[3]

[b] Calculated using the equilibrium partitioning method. The following values were used: $K_{sed\text{-}water}$= 1525.8 m^3m^{-3} (calculated with eq. D of section 3.3.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 3050 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 61000 L/kg (from PubChem[11]), Foc_{sed}= 0.05 kg kg-1 (default value). Conversion from wet weight to dry weight was done with eq. B (see section 3.3.2).

[c] ADI value (from Schwab et al. 2005[12]) used in equation Fas TL_{hh} (see section 3.3.5)

8. Risk Quotient (PEC/PNEC)

RQ	Human consumption (Eq. G)[a]	MEC[b]

RQ_{fw}	**6.045**	**13.93**
RQ_{sed}	**6.045**	**13.93**
$RQ_{biota,sec\ pois}$	N.R.	N.R.
$RQ_{biota,\ hh}$	N.R.	N.R.
$RQ_{dw,\ hh}$	0.10	0.22

[a] Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from PT.

[b] MEC_{95} (SE – NORMAN DB)

9. References

[1] Drugbank (2014): http://www.drugbank.ca/drugs/DB01211

[2] Dulio V, von der Ohe PC. (2013) NORMAN Prioritisation framework for emerging substances. N° W604002510 NORMAN Association - Working Group on Prioritisation of Emerging Substances,. Available at http://www.norman-network.net/sites/default/files/files/Publications/NORMAN_prioritisation_Manual_15%20April2013_final%20for%20website-f.pdf

[3] NORMAN factsheet on Ciprofloxacin, version of 31.08.2014 (available on CIRCA BC).

[4] NORMAN database at http://www.norman-network.net/?q=node/24

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[11] Pubchem website: https://pubchem.ncbi.nlm.nih.gov//compound/2764?from=summary#section=Top

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[13] Technical Guidance for Deriving Environmental Quality Standards – Guidance Document No. 27 (2011) Common Implementation Strategy of the Water Framework Directive (2000/60/EC). Available at http://ec.europa.eu/environment/water/water-dangersub/lib_pri_substances.htm

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[15] National human consumption data of antibiotics directly provided to the JRC by PT.

[16] Instituto Nacional de Estatística. http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main;

[17]Ministry of Foreign Affairs of the republic of Latvia. http://www.am.gov.lv/en/?id=4659)

[18]Hellenic Statistical Authority (EL.STAT.). http://www.statistics.gr/portal/page/portal/ESYE

[19] Statistics Denmark. Available at http://www.dst.dk/en/Statistik/emner/befolkning-og-befolkningsfremskrivning.aspx

[20] National human consumption data of antibiotics directly provided to the JRC by LV and DK.

[21] Hughes SR, Kay P, Brown LE. (2013) Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ Sci Technol. 47(2):661-77.

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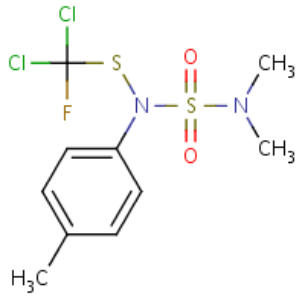
Tolylfluanid (CAS N. 731-27-1)

1. Substance identity

EC name	
EC number	211-986-9
CAS number	731-27-1
Molecular formula	C10H13Cl2FN2O2S2
Molecular weight	347.3
Structure	
SMILES	N(C)(C)S(=O)(=O)N(c1ccc(cc1)C)SC(F)(Cl)Cl

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (Pa)	2 x 10^{-4}	Biocide Assessment Report, 2009[1]
Water solubility (mg/L)	0.90	EFSA Conclusion, 2005[2]
logK_{ow}	3.9	EFSA Conclusion, 2005[2]

3. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	2200	EFSA Conclusion, 2005[2]
Biodegradability	NRB	EFSA Conclusion, 2005[2]
Bioaccumulation (BCF)	74	EFSA Conclusion, 2005[2]
BMF	1	Default value, TG n. 27 - CIS WFD[3]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	2000 (year 2000)	From previous prioritisation exercise
Uses	Biocide	
Spatial usage (by MS)	Not known	
Banned uses	Fungicide - PPP	Commission Directive 2010/20/EU[4]

ERC code	ERC8b	
Fraction of tonnage to region	0.1	
PEC_{fw} (mg/L)	0.00097	ECETOC
PEC_{sed} (mg/kg dw)	0.217	ECETOC
PEC_{biota} (mg/kg)	0.072	Calculation based on Equation L (Section 3.4.3)

4.1.1 ECETOC simulation with lower tonnages

Authorisations for plant protection products containing the active substance tolylfluanid were withdrawn by 30 November 2010. No authorisations for plant protection products containing tolylfluanid are granted or renewed from 1 December 2010[4].

However, the available tonnage of 2000 relates to the year 2000, which is prior to the banning of the substance as PPP.

At the WG Chem meeting 16-17/10/2014 it was suggested to perform a simulation on the PEC calculated with ECETOC using reduced tonnage values of tolylfluanid that could be closer to the actual tonnage after the banning, i.e. related to the use as biocide only. Since no tonnage value specific for this particular use was available, it was decided to perform the simulation considering a 20%, and 30% or 50% decrease in tonnage values. The results of the simulations are compared with the pre-banning tonnage scenario in the following Table.

Tonnes/year	2000	1600	1400	1000
Decrease respective to pre-banning tonnage	-	20%	30%	50%
PEC_{fw} (mg/L)	9.7E-04	7.8E-04	6.8E-04	4.9E-04
RQ_{fw}	3.66	2.94	2.56	1.85
Position in the ranking (higher RQ)	22 (RQ_{fw})	22 (RQ_{fw})	22 (RQ_{fw})	22 (RQ_{fw})

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
5 (FR, NL, FI, SE, IT)	NORMAN DB, 2014[5]	$MEC_{site, whole}$: 0.01 µg/L	-
	WATERBASE, 2014[6]	all values < LOQ	
	IPCheM[7]	all values < LOQ	
	SE pesticide monitoring programme[8]	all values < LOQ	
	IT monitoring programme[9]	MEC_{95}: 0.048 µg/L	

5. P, B, T, C, M, R, ED properties

Tolylfluanid is neither genotoxic nor carcinogenic[1]. There were no classification-relevant effects on reproductive or developmental toxicity[1]. The substance is not readily biodegradable (P).It shows a low potential for bioaccumulation[1].

6. Hazard assessment

6.1 Ecotoxicology data

Since the substance will be de-selected from the Watch List, because of sufficient monitoring data, ecotoxicity data are not reported at this stage.

6.2 Mammalian toxicology data

Since the substance will be de-selected from the Watch List, because of sufficient monitoring data, mammalian toxicity data are not reported at this stage.

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Daphnia magna*, 21 d, NOEC	0.00265 mg/L	10[a]	0.000265 mg/L[b]
$PNEC_{sed}$	*Chironomus riparius*, 28 d, EC_{15}	5.75 mg/L	100[c]	0.058 mg/L[d]
$PNEC_{biota,sec\ pois}$	Rat, 2-generation study, conversion factor 20, NOAEL	12 mg/kg bw/day	30[e]	8 mg/kg bw/day[f]
$PNEC_{biota,\ hh}$	ADI	0.1 mg/kg bw/day	-	6.087 mg/kg food[g]
$PNEC_{dw,\ hh}$	ADI	0.1 mg/kg bw/day	-	0.350 mg/L[h]

[a] AF of 10 because three long term values were available from the main trophic levels.
[b] PNEC value retrieved from the Biocide Assessment Report, 2009[1]
[c] AF of 100 because one long term value was available.
[d] Due to the fast degradation of the substance in sediment, no studies with the active substance on sediment dwelling organisms were considered necessary, and no sediment risk assessment was carried out (EFSA, 2005[2], Biocide AR, 2009[1]). Thus, the endpoint value used in this report is referred to its metabolite.
[e] AF of 30 selected according to the duration of the test (see TG n. 27 - CIS WFD[3])
[f] The following steps were followed for $PNEC_{biota,sec\ pois}$ calculation: a) conversion of NOAEL (12 mg/kg bw/day) value, retrieved from EFSA Conclusion 2005[2], into NOEC (240 mg/kg) by using the conversion factor of 20 ((taken from TG n. 27- CIS WFD, which depends both on species tested and age/study); b) Application of appropriate AF_{oral} (30) to the NOEC value.
[g] ADI value retrieved from EFSA Conclusion 2005[2], used for PNEC calculation according to Equation E (see section 3.3.4)
[h] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	**3.66**
RQ_{sed}	0.017
$RQ_{biota,sec\ pois}$	0.009
$RQ_{biota,\ hh}$	0.01
$RQ_{dw,\ hh}$	0.003

8. References

[1] Directive 98/8/EC concerning the placing biocidal products on the market Inclusion of active substances in Annex I or IA to Directive 98/8/EC - Assessment Report Tolylfluanid Product-type 8 (Wood preservatives) – March 2009. Available at http://dissemination.echa.europa.eu/Biocides/factsheet?id=0055-08

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[6] WATERBASE Database http://www.eea.europa.eu/data-and-maps/data/waterbase-rivers-6

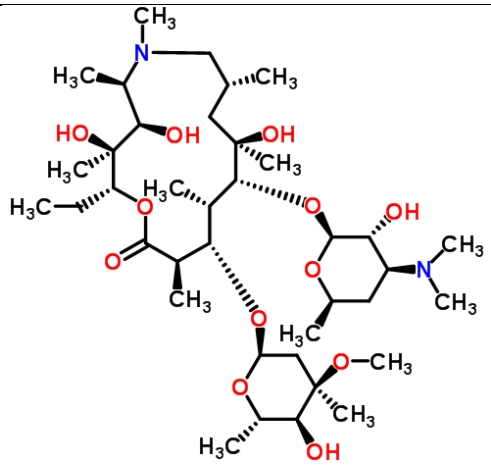
[7] IPCheM database at http://ipchem.jrc.ec.europa.eu/

[8]Swedish National Screening Programme Pesticides (data provided directly to the JRC)

[9] Italian Monitoring Programme (data provided directly to the JRC)

Azithromycin (CAS N. 83905-01-5)

1. Substance identity

Chemical name (IUPAC)	(2R,3S,4R,5R,8R,10R,11R,13S,14R)-11-[(2S,3R,4S,6R)-4-dimethylamino-3-hydroxy-6-methyloxan-2-yl]oxy-2-ethyl-3,4,10-trihydroxy-13-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one
EC number	-
CAS number	83905-01-5
Chemical class	Aazalide, a subclass of macrolide antibiotics
Molecular formula	C38H72N2O12
Molecular weight	748.98
Structure	
SMILES	CC[C@@H]1[C@@]([C@@H]([C@H](N(C[C@@H](C[C@@]([C@@H]([C@H]([C@@H]([C@H](C(=O)O1)C)O[C@H]2C[C@@]([C@H]([C@@H](O2)C)O)(C)OC)C)O[C@H]3[C@@H]([C@H](C[C@H](O3)C)N(C)C)O)(C)O)C)C)O)(C)O

2. Reason for proposal as candidate for the Watch list and suspected environmental risk

Azithromycin is a semi-synthetic macrolide antibiotic of the azalide class. Like other macrolide antibiotics, azithromycin inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome.[1]

Azithromycin has been classified as Category 2 according to the NORMAN Prioritisation Methodology[2], with a frequency of exceedance of 13% and an extent of exceedance of 1611-fold of the lowest PNEC[3], considering monitoring data from 2002-2011 in the NORMAN database[4].

3. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure (mm Hg)	2.65E-24	PubChem, 2014[5]
Water solubility (mg/L)	2.37	PubChem, 2014[5]
logK_{ow}	4.02	PubChem, 2014[5]

4. Environmental fate

Endpoint	Value	Source
Sorption potential (K_{oc})	3100	PubChem, 2014[5]
Biodegradability	NRB	NORMAN, 2014[3]
Bioaccumulation (BCF)	200 (estimated)	PubChem, 2014[5]
BMF	1	Default value, TG n. 27 - CIS WFD [6]

5. Environmental exposure assessment

	Description	Source
Tonnes/year	-	
Uses	Pharmaceutical Intermediate use (no tonnage available)	ECHA, 2014[7]
Spatial usage (by MS)	Wide dispersive use (diffuse sources, present in urban wastewater)	NORMAN, 2014[3]
Banned uses	-	
ERC code	ERC6a (intermediate use), ERC8a (suitable for pharmaceutical use)	
Fraction of tonnage to region	-	

5.1 Predicted Environmental Concentration

	Human consumption (Eq. G)[a]	MEC[b]
PEC_{fw} (mg/L)	0.000128	0.000583
PEC_{sed} (mg/kg dw)	0.0200[c]	0.0913[c]
PEC_{biota} (mg/kg)	0.026[d]	0.117[d]

[a]Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from PT.

[b] MEC_{95} (NORMAN: PT)

[c] PEC_{sed} calculated with the Equilibrium Partitioning Method, where $K_{sed\text{-}water}$= 78.3 m^3m^{-3} (calculated with eq. K of section 3.4.2), RHO_{sed}= 1300 kg m-3 (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 155 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 3100 L/kg (from PubChem[5]), Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. I (see section 3.4.2).

[d] Calculation with Equation L (Section 3.4.3)

5.1.1 PEC calculation considering different uses or sales data from MS

Uses	Calculation tool/ equation	Country	PEC_{fw} (µg/L)
Human use	PEC_b[a]	France	0.046
Human use	PEC_b[a]	Portugal	**0.130 (rounded)**
Human use	PEC_b[a]	Latvia	0.043
Human use	PEC_b[a]	Greece	0.114
Human use	PEC_b[a]	Denmark	0.037
Veterinary use	No sales data	-	-

[a] PEC_b equation was retrieved from Besse et al, 2008[8]

PEC_b equation: $PEC_{fw} = (consumption \times F_{excreta}) / (WWinhab \times hab \times dilution \times 365)$

where *WWinhab* is the volume of wastewater per person per day (default value of 200 [L/(hab*day]), *hab* are the number of inhabitants in the respective country (retrieved from PT[10], LV[11], EL[12] and DK[13] official sources). $F_{excreta}$ is the excretion factor of the active substance retrieved from Besse et al, 2008[8], *dilution* is the dilution factor (default value of 10), *consumption* is the quantity (mg/year) of active ingredient consumed by the population during 1 year. Consumption data were taken from Besse et al, 2008[8] for FR, from Iatrou et al, 2014[14] for EL, and directly provided to the JRC for PT[9], DK and LV[15].

5.2 Measured Environmental Concentration

From an analysis of pharmaceutical datasets in river systems worldwide collected from the literature, Hughes et al. (2013)[16] report that azithromycin has a mean detection frequency worldwide of 41% (from all the records for that particular substance) and median and maximum concentrations worldwide of 0.19 and 1.5 µg/L, respectively. The frequency of quantification of azithromycin in the NORMAN database is 15%[3].

n. of MS	Source of monitoring data	MEC values	RBSP
3 (NL, PT, SE)	NORMAN DB, 2014[4]	$MEC_{95,\ whole}$: (NL[a]) $MEC_{95,\ dissolved}$: 0.583 µg/L[b] (PT)	-
	SE Screening Programme Pharmaceuticals[17]	MEC_{95}: 0.030 µg/L	

[a] All values<LOQ

[b] Outliers from monitoring data were removed.

6. P, B, T, C, M, R, ED properties

Azithromycin does not cause gene mutations in microbial or mammalian cells, or chromosomal aberrations in cultured human lymphocytes or in mouse bone marrow *in vivo*[5]. A BCF value of 200 L/kg was reported in PubChem[5].

P: Very persistent (DT_{50} water > 3 years) - NORMAN, 2014[3]

7. Hazard assessment

7.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	Fish, 96 h, EC_{50}	84 mg/L	Mattson, 2010[18]
Aquatic invertebrates	*Daphnia magna* , 48h, LC_{50}	120 mg/L	Mattson, 2010[18]
Aquatic invertebrates	***Ceriodaphnia dubia*, 7 d, NOEC**	**0.0044 mg/L**	Mattson, 2010[18]
Algae	*Pseudokirchneriella subcapitata,* 96 h, biomass, EC_{50}	0.019 mg/L	Harada et al. 2008[19]
Algae	*Pseudokirchneriella subcapitata,* 96 h, NOEC	0.0052 mg/L	Harada et al. 2008[19]

[a] The references were taken from the NORMAN factsheet on azithromycin (see reference 3), and the studies were considered reliable.

7.2 Mammalian toxicology data

No study found

7.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Ceriodaphnia dubia*, 7 d, NOEC	0.0044 mg/L	50	9.00E-05 mg/L [a]
$PNEC_{sed}$	-	-	-	0.014 mg/kg dw [b]
$PNEC_{biota,sec\ pois}$	-	-	-	No info
$PNEC_{biota,\ hh}$	ADI	0.0017 mg/kg day	-	0.103 mg/kg food [c]
$PNEC_{dw,\ hh}$	ADI	0.0017 mg/kg day	-	0.006 mg/L [d]

[a] An AF of 50 was selected based on availability of two long-term values from the main trophic levels.

[b] Equilibrium partitioning method used with the following values: $K_{sed\text{-}water}$= 78.3 m^3m^{-3} (calculated with eq. D of section 3.3.2), RHO_{sed}= 1300 kg m^{-3} (default value), $Fsolid_{sed}$= 0.2 (default value), RHO_{solid}= 2500 kg m^{-3} (default value), Kp_{sed}= 155 L/kg (calculated, K_{oc} x Foc_{sed}), K_{oc}= 3100 L/kg (from PubChem[5]), Foc_{sed}= 0.05 kg kg^{-1} (default value). Conversion from wet weight to dry weight was done with eq. B (section 3.3.2).

[c] ADI value retrieved from Leung 2013 (see reference 20) used in equation E as TL. See section 3.3.4 for calculation

[d] ADI value used in equation F as TL_{hh}. See section 3.3.5 for calculation

8. Risk Quotient (PEC/PNEC)

RQ	Human consumption (Eq. G)[a]	MEC[b]
RQ_{fw}	**1.422**	**6.48**
RQ_{sed}	**1.422**	**6.48**
$RQ_{biota,sec\ pois}$	No info	No info
$RQ_{biota,\ hh}$	0.25	1.13
$RQ_{dw,\ hh}$	0.02	0.10

[a]Besse et al., 2008[14] (Equation G) for PEC equation, human consumption data from PT.

[b] MEC_{95} (NORMAN: PT)

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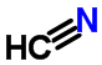
Cyanide-Free (CAS N. 57-12-5)

1. Substance identity

EC name	
EC number	
CAS number	**57-12-5**
Molecular formula	**HCN, CN^-**
Molecular weight	**27.03**
Structure	
SMILES	**C#N**

2. Physico-chemical Properties

Endpoint	Value	Source
Vapour Pressure	620 mmHg at 20°C (as HCN)	WFD – UK TAG Report, 2012[1]
Water solubility (mg/L)	1,000,000 at 25°C (as HCN)	WFD – UK TAG Report, 2012[1]
logK_{ow}	0.35–1.07 (as HCN)	WFD – UK TAG Report, 2012[1]

3. Environmental fate

Endpoint	Value	Source
Biodegradability	Biodegradation is an important transformation process for cyanide in natural surface waters and is dependent on such factors as cyanide concentrations, pH, temperature, availability of nutrients and acclimation of microbes.	WFD – UK TAG Report, 2012[1]
Bioaccumulation (BCF)	Experimental BCF values for rainbow trout range from 1.69–4.12.	WFD – UK TAG Report, 2012[1]

4. Environmental exposure assessment

4.1 Predicted Environmental Concentration

	Description	Source
Tonnes/year	-	
Uses	Cyanides are used extensively in industry and are also emitted from car exhaust fumes. They also occur ubiquitously in the environment and are found in a range of aquatic organisms such as arthropods, macrophytes, fungi and bacteria.	WFD – UK TAG Report, 2012[1]
Spatial usage (by MS):	Widespread use	
Banned uses	**-**	
ERC code	-	
Fraction of tonnage to region	-	
PEC_{fw} (mg/L)	-	
PEC_{sed} (mg/kg dw)	-	
PEC_{biota} (mg/kg)	-	

4.2 Measured Environmental Concentration

n. of MS	Source of monitoring data	MEC values	RBSP
14 (CZ, SI, EL, FR, DE, AT, ES, UK, IE, NL, PL, RO, SK, IT) Reported as cyanide in the databases	NORMAN DB, 2014[2]	$MEC_{95, whole}$: 1.07 µg/L $MEC_{95, dissolved}$: 5 µg/L	10 MS (RBSP EQS ECOSTAT – UBA report)[5] EQS set for cyanide ion and total (WRc, 2012)[6]
	WATERBASE, 2014[3]	$MEC_{95, whole}$: 20 µg/L $MEC_{95, dissolved}$: 20 µg/L	
	IPCheM[4]	MEC_{95}: 14 µg/L	

5. P, B, T, C, M, R, ED properties

Volatilisation and biodegradation are important transformation processes for cyanide in ambient waters. Hydrogen cyanide can be biodegraded by acclimated microbial cultures, but is usually toxic to unacclimated microbial systems at high concentrations (WFD- UK TAG Report, 2012[1]).

6. Hazard assessment

6.1 Ecotoxicology data

Trophic level	Endpoint	Value	Reference
Fish	Rainbow trout, 20 d, LOEC	0.005 mg/L	WFD- UK TAG Report (2012)[1]
Fish	***Lepomis macrochirus*,**	**0.0052 mg/L**	WFD- UK TAG Report

	289 d, total inhibiotin of spawning, LOEC		(2012)[1]
Fish	*Salvelinus fontinalis*, egg production, NOEC	0.0057 mg/L	WFD- UK TAG Report (2012)[1]
Aquatic Invertebrates	*Moinodaphnia macleayi*, 5 d, reproduction, NOEC	0.0096 mg/L	WFD- UK TAG Report (2012)[1]
Aquatic Invertebrates	*Gammarus pseudolimnaeus,* 98 d, growth, NOEC	0.004 mg/L	WFD- UK TAG Report (2012)[1]
Aquatic Invertebrates	*Hydra viridissima*, 6 d, population growth, NOEC	0.110 mg/L	WFD- UK TAG Report (2012)[1]
Algae	*Pseudokirchneriella subcapitata*, 72 h,growth rate and biomass, NOEC	0.010 mg/L	WFD- UK TAG Report (2012)[1]

6.2 Mammalian toxicology data

No information retrieved

6.3 PNEC derivation

PNEC	Endpoint	Endpoint value	AF	PNEC value
$PNEC_{fw}$	*Lepomis macrochirus*, 289 d, LOEC	0.0052 mg/L	20	2.6E-04 (mg/L)[a]
$PNEC_{sed}$	-	-	-	-
$PNEC_{biota,sec\ pois}$	-	-	-	-
$PNEC_{biota,\ hh}$	-	-	-	-
$PNEC_{dw,\ hh}$	-	-	-	0.05 (mg/L)[b]

N.R. Not required based on Koc and BCF values not reaching the trigger values required for sediment and biota assessment

[a] Value retrieved from WFD- UK TAG Report (2012)[1] . A more recent freshwater AA-EQS derivation of 5E-04 mg/l needs also to be considered.

[b] EU Drinking Water QS[7], refered to cyanide.

7. Risk Quotient (PEC/PNEC)

RQ	Value
RQ_{fw}	-
RQ_{sed}	-
$RQ_{biota,sec\ pois}$	-
$RQ_{biota,\ hh}$	-
$RQ_{dw,\ hh}$	-

8. References

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EUR 27142 EN – Joint Research Centre – Institute for Environment and Sustainability

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Seasonal and Diurnal Variation of Organic Ultraviolet Filters from Personal Care Products Used Along the Japanese Coast

Kenshi Sankoda · Kotaro Murata · Mai Tanihata · Kengo Suzuki · Kei Nomiyama · Ryota Shinohara

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Abstract This study aimed to investigate the behavior of organic ultraviolet (UV) filters released by recreational activities along the Japanese coastline. Seasonal variations of organic UV filters in seawater were investigated at four different recreational beaches (Mogushi, Wakamiya, Tsurugahama, and Otachimisaki beaches) in both summer (July through August) and winter (December). Moreover, short time scale diurnal changes were monitored at Otachimisaki beach in summer. Of the four sunscreen agents tested in this study, two agents—2-ethylhexyl-4-methoxycinnamate (EHMC) and 2-ethylhexyl salicylate (EHS)—were detected in all samples, whereas octyldimethyl-*p*-aminobenzonic acid and 3-(4-methylbenzylidene)-camphor were lower than detection limits. In particular, EHMC, one of the most popular organic UV filters, was dominant. The highest concentration of EHMC was observed at 1,080 ng L^{-1}, a level that exceeds those of previous studies. Both EHMC and EHS concentrations showed significant ($p < 0.05$) seasonal variations with advancing summer suggesting direct input from recreational activities. The subsequent examination showed short time scale diurnal changes of organic UV filters on the beach. The results showed that diurnal changes in EHMC concentrations were correlated to the number of bathers. EHMC concentrations increased during the afternoon and decreased during the night, although complete attenuation during the night did not occur. EHMC persists along the coast due to low mobility and may persist the next day. This is the first study to show the natural attenuation behavior of organic UV filters along recreational beaches.

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Organic ultraviolet (UV) filters are designed to absorb harmful UV light and are used in sunscreen products to protect the skin from UV radiation. These filters are also present in other personal care products such as cosmetics, hair sprays, body lotions, and shampoos (Li et al. 2007). Recent increasing interest in the adverse impact on human health by excessive sunlight exposure has resulted in the widespread use of organic UV filters (Baron et al. 2013).

The heavy consumption of organic UV filters has increased concern regarding environmental contamination by these chemicals. Recent in vitro and in vivo assays have shown that some organic UV filters potentially disrupt the endocrine system of living organisms. For example, the estrogenic effects of organic UV filters have been shown in MCF-7 assays, in rats (Schlumpf et al. 2001) and fish (Inui et al. 2003; Kunz et al. 2006). Kunz and Fent (2006a) found that the estrogenic effects of a mixture of eight UV filters on hERα of yeasts assays were stronger than a single UV filter suggesting the possible synergistic effects of these chemicals. However, organic UV filter contaminants have not only estrogenic effects but also potential antiestrogen, androgen, and antiandrogen effects. Kunz and Fent (2006b) also reported that most of these chemicals cause multiple hormonal effects. Furthermore, it was suggested that organic UV filters in seawater may have toxic effects such as coral

bleaching (Danovaro et al. 2008). The above-mentioned studies emphasize the importance of understanding the fate of organic UV filters in the aquatic environment.

Recent studies showed the persistence of several organic UV filters in the environment. Organic UV filters, including sunscreen agents and UV stabilizers, have been detected in aquatic environments such as rivers, lake waters (Fent et al. 2010; Giokas et al. 2005; Kameda et al. 2011), sediments (Nakata et al. 2009; Zhang et al. 2011), aquatic organisms (Bachelot et al. 2012; Fent et al. 2010; Gago-Ferrero et al. 2013; Kameda et al. 2011; Nakata et al. 2009, 2010, 2012), tap water, and well water (Díaz-Cruz et al. 2012). The emission sources of organic UV filters are mainly classified into indirect and direct sources (Gago-Ferrero et al. 2011). Previous studies have shown that wastewater-treatment plants (WWTPs) are the main indirect source (Balmer et al. 2005; Fent et al. 2010; Kameda et al. 2011; Plagellat et al. 2006; Zhang et al. 2011).

In this study, we focused on the direct emission sources. Humans engaging in recreational activities, such as ocean bathing, is one of the direct emission sources for organic UV filters (Cuderman and Heath 2007). Direct emissions should not be ignored because they contribute to the direct introduction of organic UV filters into the aquatic environment without any previous treatment. Although recreational activities are important sources of organic UV filters and could lead to widespread marine pollution, the available information on the direct emission of organic UV filters is limited. For example, along Norwegian coastal regions, concentrations of organic UV filters in water were greater in July than in May (Langford and Thomas 2008). Giokas et al. (2005) compared the concentrations of organic UV filters in seawater before (12:00 p.m.) and at the peak (3:00 p.m.) of recreational bathing activity and reported increased concentrations at the peak, thereby showing a direct link. However, to the best of our knowledge, short time scale diurnal changes in the concentrations of organic UV filters have not been previously reported. Thus, it is not known whether organic UV filters are attenuated or persist within coastal waters during night hours when recreational pressure is at a minimum. Elucidating the diurnal variations in the concentrations of organic UV filters in coastal water environments contributes to understanding their behavior and fate, thus further contributing to reasonable risk assessments.

This study identified and quantified organic UV filters released during recreational bathing activities along swimming beaches in Japan. In addition, transient diurnal variations in organic UV filter concentrations were determined. Additional sunlight exposure experiments were also performed to rationalize the environmental fate of organic UV filters. This is the first study to demonstrate the natural attenuation behavior of organic UV filters along recreational beaches.

Materials and Methods

Reagents and Instruments

The four tested organic UV filters are listed in Table 1. Hexane, acetone, and chrysene-d_{12} were purchased from Kanto Chemical (Tokyo, Japan). Octyl-dimethyl-*p*-aminobenzoic acid (OD-PABA) (≥97 %), EHMC (E isomer) (≥95 %), and sodium azide were purchased from Wako (Osaka, Japan). EHS (≥98 %) and 3-(4-methylbenzylidene)-camphor (4-MBC) (≥99 %) were supplied by Tokyo Chemical Industry (Tokyo, Japan) and Alfa Aesar (Massachusetts, USA), respectively. Phenanthrene-d_{10} was purchased from Cambridge Isotope Laboratories (Massachusetts, USA). Artificial seawater salt for sunlight exposure experiments was purchased from Marinetech (Tokyo, Japan). EPI suite version 4.1 (United States Environmental Protection Agency 2011) was used for deriving the log K_{ow} of the four organic UV filters (Table 1). Structural information for each organic UV filter was taken from the Chemical Abstracts Service (CAS) registry numbers.

Organic UV filter analyses were performed with a HP 6890 gas chromatograph (Agilent, California, USA) equipped with a JMS-700 mass spectrometer (JEOL, Tokyo, Japan) (gas chromatograph/mass spectrometer (GC/MS)). A fused silica capillary column DB-5MS (inside diameter 0.25 mm; film thickness 0.25 μm; column length 60 m; Agilent) was used for separation with high-purity helium as carrier gas.

Sampling

The seasonal variation in organic UV filter concentration in seawater was investigated at four recreational beaches in the Kumamoto prefecture in southwestern Japan (Fig. 1). Samples were collected from the Mogushi, Wakamiya, Tsurugahama, and Otachimisaki beaches. All studied sites were open to the public for swimming during summer (generally June through August). The numbers of bathers present at the sites during the sampling times were estimated and used for the normalization of UV filter concentration (Fig. 1). In 2010, seawater samples were collected in both summer (July through August) and winter (December).

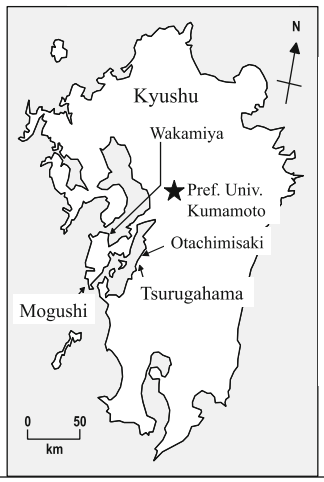
The diurnal variations in organic UV filter concentrations in seawater were investigated at Otachimisaki beach on July 26 to 27, 2011. During the sampling campaign, surface seawater was collected every 1 or 2 h from 9:00 a.m. to 8:00 a.m. the next day. The number of bathers was counted during each sampling period. Sampling times, the number of bathers, and temperatures are listed in Table 2.

Immediately after sample collection, saturated sodium azide solution was added to the samples to a concentration

Table 1 Name and physicochemical properties of tested organic UV filters

Name	Abbreviation	CAS no.	Formula	Molecular weight	Log Kow[a]	Recovery rate (%)	LOQ (ng L^{-1})	LOD (ng L^{-1})
Octyl-dimethyl-*p*-aminobenzonic acid	OD-PABA	21245-02-3	$C_{17}H_{27}NO_2$	277.4	5.8	86 ± 1.8	0.32	0.096
2-Ethyl-hexyl-4-methoxycinnamate	EHMC	5466-77-3	$C_{18}H_{26}O_3$	290.4	5.8	90 ± 3.7	0.27	0.082
2-Ethylhexyl salicylate	EHS	118-60-5	$C_{15}H_{22}O_3$	250.33	6	120 ± 3.3	0.33	0.099
3-(4-Methylbenzylidene)-camphor	4-MBC	38102-62-4	$C_{18}H_{22}O$	254.37	5.9	89 ± 0.7	0.5	0.15

[a] Calculated by EPI suite version 4.1



Site	Date	Time	Estimated bathers (min–max)	Temperature (℃)	
				Air	Water
Mogushi	Jul. 27, 2010	15:30	100–150	28	28
	Dec. 1, 2010	13:00	0	21	21
Wakamiya	Jul. 27, 2010	11:30	25–50	32	29
	Dec. 1, 2010	10:30	0	18	15
Tsurugahama	Aug. 15, 2010	11:30	100–150	32	22
	Dec. 19, 2010	10:00	0	14	16
Otachimisaki	Aug. 15, 2010	12:00	200–300	29	27
	Dec. 19, 2010	11:00	0	14	17

Fig. 1 Sampling site, date, time, estimated number of bathers, and recorded temperatures in seasonal variation tests

Table 2 Sampling time, number of bathers, and temperatures recorded at Otachimisaki beach during the 2011 sampling

Date	Time	Tourists	Temperature (°C)	
			Air	Water
July 26, 2011	9:00 a.m.	26	28	28
	10:00 a.m.	38	30	28
	11:00 a.m.	72	29	28
	12:00 a.m.	104	30	28
	1:00 p.m.	129	30	27
	2:00 p.m.	148	29	28
	3:00 p.m.	210	29	28
	4:00 p.m.	69	27	28
	5:00 p.m.	15	26	28
	6:00 p.m.	4	24	27
	7:00 p.m.	0	24	26
	8:00 p.m.	0	24	26
	10:00 p.m.	0	25	27
July 27, 2011	12:00 a.m.	0	26	27
	2:00 a.m.	0	24	26
	4:00 a.m.	0	24	25
	6:00 a.m.	0	24	26
	8:00 a.m.	3	27	26

of 0.1 % to prevent the biodegradation of the organic UV filters. The collected samples were stored at 4 °C before the analysis.

Extraction, Concentration, Measurement, and Data Processing

Hexane (50 mL) was added to seawater (200 mL) in a separatory funnel and shaken for 20 min. After extraction, the organic layer was collected and filtered using a filter paper. The extraction procedure was repeated by the addition of more hexane (50 mL). The combined extracts from seawater were concentrated to approximately 500 µL with a rotary evaporator and then further reduced to 50 µL under a gentle stream of nitrogen. Phenanthrene-d_{10} and chrysene-d_{12} were spiked into the sample to give the final test solutions for GC/MS analysis. Z isomer of EHMC in samples were also quantified based on an assumption that Z EHMC shows same chromatographic response as E EHMC (Bachelot et al. 2012), and the results displayed them as one chemical. All samples were analyzed in triplicates ($n = 3$).

The final extract was injected (2 μL) to the GC/MS in the splitless mode. The GC column temperature program was first maintained at 60 °C for 1 min and then heated to 300 °C at 10 °C min^{-1} and maintained for 10 min. The injection port, GC/MS interface, and MS ion source were kept at 280 °C. We used the electron impact ionization method and the MS ion source with an ionization voltage of 70 eV. The mass spectrometer was operated at the selective ion monitoring mode with a resolution of 3,000.

Welch's *t* test was used to analyze the seasonal variations in the detected organic UV filters in summer and winter. The level of significance was defined at $p < 0.05$. Statistical analyses were performed using GraPhpad Prism version 5 (GraphPad Software, California, USA).

Sunlight Exposure Experiments for EHS

EHS was selected for additional sunlight exposure experiments because the diurnal variation of this compound would be more rationalized when its photodegradability was taken into consideration (see "Discussion" section). The experiments were performed on July 28, 2014, along a balcony situated within the Prefectural University of Kumamoto (mapped in Fig. 1). EHS was dissolved in artificial seawater, including 1 % acetone, at a concentration of 1 mg L^{-1}. The test solutions (10 mL) were prepared in capped 50-mL borosilicate glass tubes ($n = 4$) and kept in the balcony. UV intensity, measured twice, 30 and 150 min afterward using a UV-340 (Lutron Electronics, Pennsylvania, USA), was 900 μW cm^{-2}. An aliquot (1 mL) was taken at each sampling period (0, 30, 60, 90, 120, and 150 min) and then extracted with hexane (1 mL) under ultrasonic irradiation (30 min). A portion of the extract (500 μL) was spiked with phenanthrene-d_{10} as internal standard followed by injection to the GC/MS. The rate constant for photodegradation (k) and half-life of EHS dissolved in artificial seawater was determined by the following first-order kinetic equation,

$$\ln \frac{C_t}{C_0} = -kt, \quad (1)$$

where C_t and C_0 are the concentrations of EHS at time t and time zero, respectively. The half-lifetime of EHS was calculated using Eq. (2) as follows:

$$\text{Half - life time (h)} = \frac{\ln 2}{k}. \quad (2)$$

Quality Assurance and Control

All glassware was thoroughly rinsed with acetone and hexane before use. Sample values were calibrated against blank values. Samples were pretreated at night to avoid photodegradation of the organic UV filters.

Spike-recovery experiments were run using standard references of the organic UV filters dissolved in acetone. Organic UV filters (4 ng) were fortified to ultrapure water (200 mL; $n = 3$). They were treated as described previously, and the rates were calculated. The mean recovery rates (mean ± SD) of the organic UV filters in seawater ranged from 86 ± 1.8 % (OD-PABA) to 120 ± 3.3 % (EHS). The GC/MS limit of quantification (LOQ) and limit of detection (LOD) were calculated on the basis of the detection levels from the repeated blank tests ($n = 5$). LOQ and LOD were defined as 3 and 10 times the SD in the blank samples, respectively. LOQ in seawater ranged from 0.27 to 0.5 ng L^{-1}. Recovery rates, LOQ, and LOD for all of the organic UV filters are listed in Table 1.

Results

Profile and Seasonal Variations of Organic UV Filters Along Recreational Beaches

Table 3 lists mean concentrations of organic UV filters at recreational beaches located in southwestern Japan. Two of four organic UV filters, EHMC and EHS, were detected with particular dominance of EHMC. In summer, the highest concentration of the total organic UV filters was detected in the sample from Mogushi ([mean] 1,084.3 ng L^{-1}) followed by Otachimisaki (1,033.1 ng L^{-1}), Tsurugahama (720.9 ng L^{-1}), and Wakamiya (220.0 ng L^{-1}). In contrast, the spatial variation in organic UV filter concentrations was clearly normalized by the number of bathers (4.1 to 8.7 ng L^{-1} $person^{-1}$). The coastal water concentrations of EHMC reached 1,080 ± 390 ng L^{-1} along Mogushi beach followed by 1,010 ± 174 ng L^{-1} along Otachimisaki beach, 700 ± 110 ng L^{-1} along Tsurugahama beach, and 210 ± 50 ng L^{-1} along Wakamiya beach. EHMC is one of the most widely used sunscreen agents and has been detected in environmental samples in many previous studies (Goksøyr et al. 2009; Kameda et al. 2011; Langford and Thomas 2008). In this study, the concentrations of EHMC were greater than those reported along coastal recreational areas in Greece [7.4 and 10.7 ng L^{-1} (Giokas et al. 2005)] and Norway during July [39–390 ng L^{-1} (Langford and Thomas 2008)].

In contrast to EHMC and EHS, OD-PABA and 4-MBC were not detected in any seawater samples. OD-PABA and 4-MBC are infrequently found in the environment in Japan (Kameda et al. 2011; Nakata et al. 2009). The use of 4-MBC as sunscreen agent is not allowed within Japan (Ministry of Health, Labour and Welfare 2000). In addition, 70 cosmetic products, such as sunscreen and foundation, purchased in the Japanese market did not contain any detectable OD-PABA (Ikarashi et al. 2007). In contrast, these chemicals have been detected in European countries in concentrations of up to several hundred

Table 3 Organic UV filter concentrations in seawater from beaches in Japan showing mean (ng L^{-1}) and normalized values by estimated number of bathers (ng L^{-1} $person^{-1}$)

TCH>Beach		EHMC		EHS		Total	Normalized total
		Average	SD	Average	SD		
Mogushi	Summer	1,080*	390	4.3*	0.8	1,084.3	8.7
	Winter	18	2.0	2.2	0.1	20.2	NC
Wakamiya	Summer	210*	50	10*	3	220	5.9
	Winter	11	0.90	2.0	0.04	13.0	NC
Tsurugahama	Summer	700**	110	20.9*	2.9	720.9	5.8
	Winter	20	1.9	3.8	0.6	23.8	NC
Otachimisaki	Summer	1,010**	174	23.1*	5.3	1,033.1	4.1
	Winter	14	6.1	3.4	0.8	17.4	NC

SD standard deviation, *NC* not calculated

* Significant seasonal variations between summer (* $p < 0.05$) and winter (** $p < 0.01$). Normalized totals were calculated by dividing the total concentrations in summer by the median estimated number of bathers listed in Fig. 1

nanograms per liter (Amine et al. 2012; Fent et al. 2010; Langford and Thomas 2008). Moreover, 4-MBC has been detected in fish from Swiss rivers at a maximum concentration of 1,800 ng g^{-1} lipid weight (Buser et al. 2006). The organic UV filter profile in seawater appeared to clearly reflect the difference of local market features.

In all cases, greater concentrations of organic UV filters were detected during summer compared with winter. Both EHMC and EHS showed significant (Welch's t test, $p < 0.05$) seasonal differences at all sites (Table 3). In particular, EHMC showed the clearest seasonal trends. Compared with winter, the concentration of EHMC in seawater was 19–72 times greater during the summer. These seasonal variations in the coastal environment are in agreement with previous studies in European countries (Bachelot et al. 2012; Langford and Thomas 2008) and support the notion that the concentration of organic UV filters increases with recreational activities.

Diurnal Variation of Organic UV Filters in Seawater

To confirm the temporal changes in organic UV filters, seawater was collected at hourly intervals from Otachimisaki beach coastal waters during the peak summer period with a special focus on EHMC and EHS, which showed increasing concentrations with advancing summer. The number of bathers increased from morning to afternoon and peaked at 3:00 p.m. (Table 2). No bathers were observed swimming after 7:00 p.m. until 6:00 a.m. the next day.

The number of bathers and concentrations of EHMC and EHS in seawater as a function of time within a 24-h period are shown in Fig. 2. In general, the concentrations of EHMC in seawater showed a positive correlation with the number of recreational bathers. EHMC was detected at 9:00 a.m. at a concentration of 76 ng L^{-1}. The highest concentration of EHMC at 220 ng L^{-1} was observed at 3:00 p.m. when the number of bathers also peaked. In contrast, the concentration of EHMC at 12:00 a.m. was 52 ± 33 ng L^{-1} suggesting natural attenuation during night hours by wave and ocean flow. However, no clear

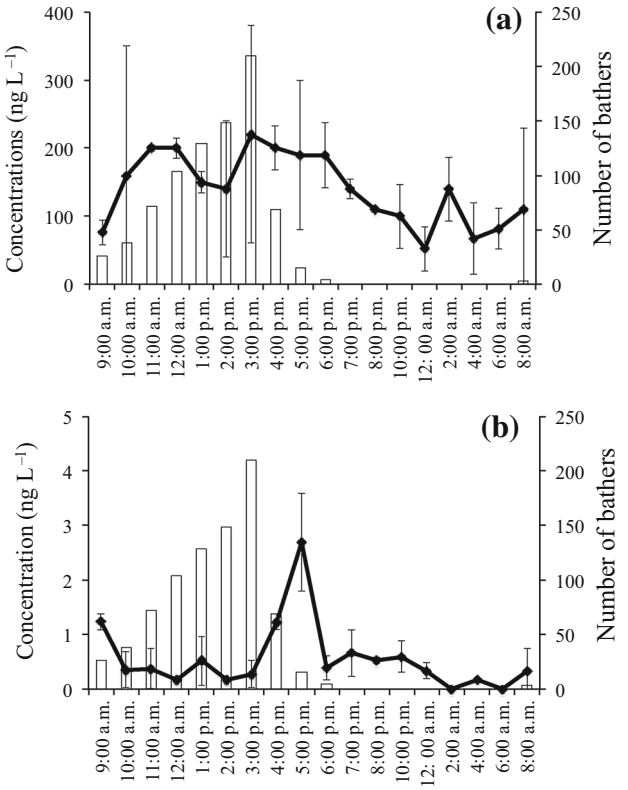


Fig. 2 Number of bathers at the beach (*bar*) and diurnal changes of two organic UV filters—**a** EHMC and **b** EHS—in seawater collected from Otachimisaki beach during summer 2011 (*line*). *Error bars* represent SD ($n = 3$). Samples that showed concentrations lower than the LOQ were treated as the half the value of LOQ for visualization

relationship between concentrations of EHS and the number of bathers was observed. Concentrations of EHS during daylight hours did not increase with the number of bathers. The concentration of EHS reached the highest concentration (2.7 ± 0.9 ng L^{-1}) at 5:00 p.m. when most bathers had already left.

Discussion

Seawater samples were collected during summer and winter from recreational beaches in southwestern Japan. The concentrations of EHMC in this study (summer 210–1,080 ng L^{-1}) were greater than those recorded during previous studies (Giokas et al. 2005; Langford and Thomas 2008). In contrast, no detectable concentrations of OD-PABA and 4-MBC were observed.

The sources of the detected organic UV filters can be discussed in relation to the seasonal variation in concentrations. EHMC and EHS showed significant seasonal variations with greater values in summer than those in winter (Table 3). Compared with the summer, concentrations of EHMC decreased >90 % in winter. These results are consistent with the report by Bachelot et al. (2012) and suggest a direct correlation between organic UV filters and recreational bathing activities. In contrast, effluent from WWTPs is another possible source for organic UV filters in seawater. Because relatively high concentrations of organic UV filters have been detected in effluent from WWTPs (Kameda et al. 2011), their concentrations in the receiving water would increase during summer by high-volume use of sunscreens. However, considering that the sampling sites are situated along popular recreational bathing beaches, the influence of effluent might be small. Moreover, Kameda et al. (2011) investigated the occurrences of organic UV filters in rivers in Japan and reported the dominance of benzotriazole UV stabilizer UV-328 in rivers that receive industrial and domestic wastewater. This suggests that the presence of UV-328 is a useful index of industrial or domestic wastewater input. We analyzed residual levels of UV-328 in all seawater samples. However, compared with the previous study, UV-328 was not detected in any of the analyzed samples (data not shown) suggesting that the effluent is not a contributor in this case. The absence of OD-PABA and 4-MBC also supports the direct emission of EHMC and EHS by recreational bathing activity. The profile of UV filters detected clearly reflects the products available in Japan, and this is evidence of localized pollution along coastal waters because OD-PABA and 4-MBC are not used as sunscreen ingredients in Japan.

The concentrations of total organic UV filters in the samples collected during 2010 differed depending on the location. Such spatial variation could be attributed to the differences in number of bathers at each site because the normalized values were in line with each other (Table 3). From these results, recreational activities are considered to be an important direct emission source for sunscreen agents EHMC and EHS in the coastal environment of Japan, whereas OD-PABA and 4-MBC are not present in significant concentrations.

Moreover, a subsequent examination at the Otachimisaki beach site showed transient diurnal variation of EHMC and EHS. The concentrations of organic UV filters in seawater collected from Otachimisaki during 2011 were lower than those collected in 2010. This may reflect the differences in the number of bathers between the two sampling campaigns.

EHMC Versus EHS

For diurnal variations, we should separately discuss EHMC and EHS. The concentrations of EHMC in seawater were dependent on the number of bathers. The EHMC concentration increased with increased number of bathers with a peak at 3 p.m. During the night hours (7:00 p.m. to 4:00 a.m.), the concentration of EHMC decreased with the lowest concentration of 52 ng L^{-1} and highest concentration of 140 ng L^{-1} being observed at 12: 00 a.m. and 7:00 p.m., respectively. The attenuation of EHMC during night hours could be attributed to the dilution effects by wave and ocean flow. Although the concentrations of EHMC decreased during night hours, complete attenuation was not present even though bathers were absent. Concentrations of EHMC during night hours were still greater than background levels observed during winter 2010 (Table 3). In general, adsorption processes to a solid phase play an important role in the fate of hydrophobic contaminants existing in aquatic systems (Nguyen et al. 2014; Schwarzenbach et al. 2005; Wijekoon et al. 2013). The adsorption of EHMC to sediments and/or suspended solids is expected to result in its persistence against dilution effects. In addition, repeated ocean wave action would continuously agitate these EHMC-associated solids in coastal sediment. Therefore, EHMC should be detected at a constant level in samples taken during night hours.

Diurnal Variation of EHS

Another point that requires discussion is the diurnal variation of EHS. Concentrations of EHS were not clearly dependent on the increasing numbers of bathers. One possible reason could be the low amount of EHS discharged during this sampling campaign. Concentrations of organic UV filters in seawater were lower during summer 2011 compared with during summer 2010. It is assumed that the low concentrations of EHS made obvious causative effects of diurnal variations difficult to detect.

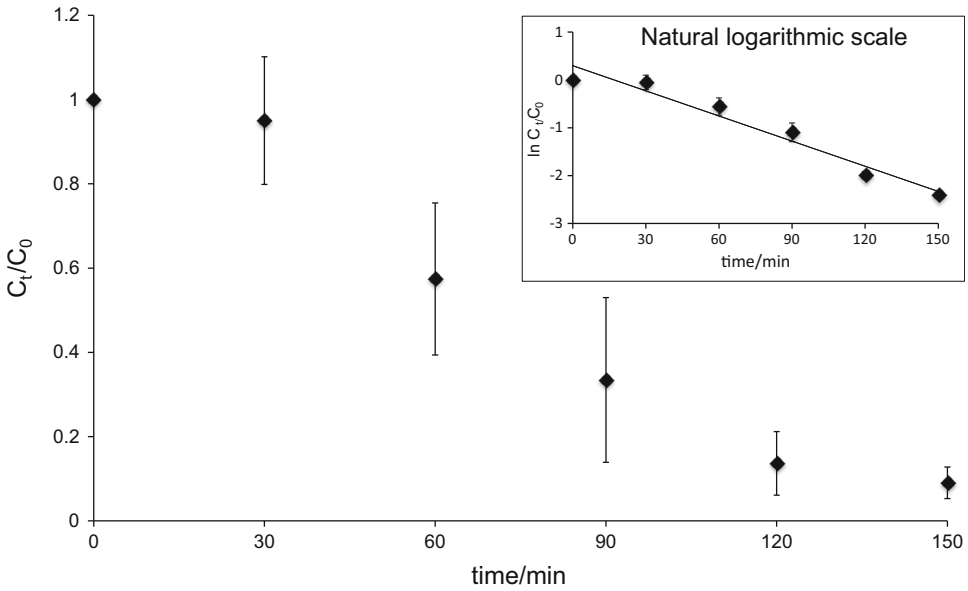


Fig. 3 Degradation of EHS by sunlight exposure in artificial seawater (1 mg L^{-1}). The results at 30 and 60 min are based on triplicates because of outliers

Another possible reason that would explain the observed diurnal variation of EHS is the rapid photodegradation of this UV filter at the ocean surface. To confirm this hypothesis, we performed additional sunlight exposure experiments for EHS. EHS was dissolved in artificial seawater (including 1 % acetone) at a concentration of 1 mg L^{-1}. Under this condition, >90 % of EHS was degraded within 150 min of irradiation with an estimated half-life of 39 min (Fig. 3). The rapid degradation implies that EHS in the aquatic environment is labile against sunlight and would explain why the concentration of EHS peaked at 5:00 p.m.; the increase of EHS during daylight hours was possibly mitigated by photodegradation. Biodegradation processes affecting EHS under natural conditions may be ruled out considering the short half-life.

Conclusion

We showed that recreational bathing activities are an important direct emission source of EHMC and EHS along the coastline in Japan. The most abundant contaminant, EHMC, was detected at relatively high concentrations that exceeded those of previous studies. The concentration of EHMC in seawater showed a positive correlation with the number of bathers. Moreover, during night hours, EHMC may persist due to low mobility, and a certain proportion of it would persist the next day. This result is important to consider when evaluating the exposure time and load of organic UV filters to which aquatic organisms could potentially be exposed. To gain insight into the environmental fate of organic UV filters released by direct sources, accumulation to sediments and partitioning between the dissolved phase and particulate phase requires further research.

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RESEARCH ARTICLE

The occurrence of UV filters in natural and drinking water in São Paulo State (Brazil)

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Abstract Ultraviolet (UV) filters are widely used in the formulation of personal care products (PCPs) to prevent damage to the skin, lips, and hair caused by excessive UV radiation. Therefore, large amounts of these substances are released daily into the aquatic environment through either recreational activities or the release of domestic sewage. The concern regarding the presence of such substances in the environment and the exposure of aquatic organisms is based on their potential for bioaccumulation and their potential as endocrine disruptors. Although there are several reports regarding the occurrence and fate of UV filters in the aquatic environment, these compounds are still overlooked in tropical areas. In this study, we investigated the occurrence of the organic UV filters benzophenone-3 (BP-3), ethylhexyl salicylate (ES), ethylhexyl methoxycinnamate (EHMC), and octocrylene (OC) in six water treatment plants in various cities in Southeast Brazil over a period of 6 months to 1 year. All of the UV filters studied were detected at some time during the sampling period; however, only EHMC and BP-3 were found in quantifiable concentrations, ranging from 55 to 101 and 18 to 115 ng L^{-1}, respectively. Seasonal variation of BP-3 was most clearly noticed in the water treatment plant in Araraquara, São Paulo, where sampling was performed for 12 months. BP-3 was not quantifiable in winter but was quantifiable in summer. The levels of BP-3 were in the same range in raw, treated and chlorinated water, indicating that the compound was not removed by the water treatment process.

Keywords UV filters · Endocrine disruptors · Brazil · River water · Drinking water · Occurrence

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Introduction

Because of concern regarding the depletion of the ozone layer and the consequential increase of UV radiation in the stratosphere that results in a higher skin cancer risk, UV filters have become an important constituent in everyday product formulations (e.g., skin moisturizing cream, body lotion, spray and hair dye, shampoo, and lipstick; Brausch and Rand 2011). UV filters have other applications, such as stabilizers in plastics, paints, textiles, and in other materials, to prevent the photodegradation of polymers and pigments (Ozáez et al. 2013). These products can act as an environmental source of UV filters, primarily from degradation in landfills (Fent et al. 2008).

UV filters are colourless or yellow substances that absorb almost no visible light but that significantly absorb light in the UVA-UVB range. A common feature of all UV filters is the presence of an aromatic ring with side chains that have various degrees of unsaturation (Díaz-Cruz and Barcelo 2009). The majority of UV filters are lipophilic; however, the presence of phenol groups in their structures can give them some mobility in the aquatic environment (Negreira et al. 2009; Richardson 2009).

UV filters are classified as inorganic (physical) or organic (chemical) according to their chemical nature. The inorganic UV filters (e.g., titanium dioxide and zinc oxide), basically act

by reflecting, scattering, and absorbing UV light, whereas the organic UV filters (e.g., benzophenone, octocrylene) act mainly by absorbing UV light (Cuderman and Heath 2007).

Some of these UV filters protect from UVB radiation, others offer protection against wavelengths in the UVA range, and few are efficient against both UVA and UVB. Thus, to ensure an efficient sun protection factor (SPF), high concentrations of UV filters have been used in personal care products (PCPs) and, in general, a combination of UV filters is used. Consequently, consumers can be exposed to high concentrations of several UV filters simultaneously (Fent et al. 2010; Manová et al. 2013). To protect the public health, the UV filters used in PCPs are regulated in some countries, mainly in the European Union and North America.

However, no worldwide environmental regulatory rules exist. In Brazil, this input to the environment is probably excessive. Considering that Brazil is a tropical country and has a high incidence of solar radiation, there is high consumption of products that contain UV filters. According to the Brazilian Association of Toiletries, Perfumes & Cosmetics, Brazil is the third largest consumer of cosmetics in the world, trailing only the USA and Japan (ABIHPEC 2014), and it still has an unreliable sanitary sewer system. According to the Brazilian Institute of Geography and Statistics, only 28.5 % of Brazilian cities collect and treat sewage, which leads to the hypothesis that the environmental levels of these compounds in Brazil can be higher than those found in other countries.

Although several papers in the literature report on the estrogenic activity of UV filters (Fent et al. 2008; Kunz and Fent 2006; Schlumpf et al. 2004), as well as on their occurrence and environmental fate (especially regarding water resources; Liu et al. 2011; Magi et al. 2012; Zhang and Lee 2012) and tap water (Basaglia et al. 2011; Díaz-Cruz et al. 2012; Zhang and Lee 2013), papers about Brazil could not be found. Only, an extraction method development for the determination of PCPs from drinking-water treatment sludge including benzophenone-3 (BP-3; Cerqueira et al. 2014). Therefore, this study aims to provide information about the occurrence and fate of the UV filters BP-3, ES, EHMC, and OC in Brazil, which are widely used in cosmetics. The study is focused on the occurrence and concentrations of these UV filters in aqueous samples from water treatment plants (WTP). The WTPs selected for study are located in the central region of São Paulo. Sao Paulo State has the better basic sanitary conditions in Brazil, collecting 87 % and treating 53 % of the sewage (SNIS 2013).

Materials and methods

Chemicals and reagents

The compounds selected for this study were four UV filters used with high frequency in sun creams largely sold in Brazil. In Brazil, there is not available information about retail marketing for cosmetics, including sunscreens. Thus, we conducted an informal survey based on the more traditional cosmetics brands sold in Brazil, by multinational and Brazilian companies. This survey included several types of sunscreen formulation and protection factors (n=52). Among the substances found most frequently in the formulation, as described in the products labels, those with the greatest estrogenic potential, according to the literature, were selected to be included in this study (BP-3, ES, EHMC, and OC).

The UV filters (BP-3, ES, EHMC, and OC), the surrogate benzophenone-d10 (BP-d10) and the internal standard, and benzyl cinnamate (BC), all of high purity (>97 %), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Names, abbreviations, chemical structures, and some physicochemical data for the UV filters are shown in Table 1. High-purity solvents (methanol [MeOH] and ethyl acetate [AcOEt] were of HPLC grade) were obtained from Mallinckrodt Baker Inc. (Paris, KY, USA). Hydrochloric acid (HCl, 37 % m/v) was purchased from the JT Baker Chemical Co. (Phillipsburg, NJ, USA) to adjust the pH of the water samples.

Standard solutions of the individual UV filters were prepared at a concentration of 1,000 mg L^{-1} in AcOEt and then diluted (with the same solvent) to achieve a stock solution mixture at 5 mg L^{-1} for each compound. All the standard solutions were stored at −20 °C. Working solutions of different concentrations were prepared daily by appropriate dilution (v/v) of the stock solution in AcOEt.

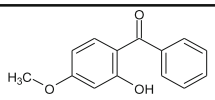
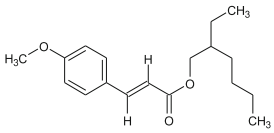
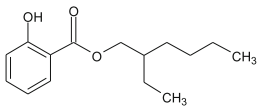
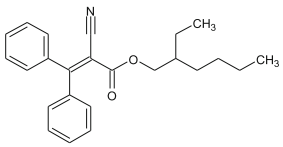
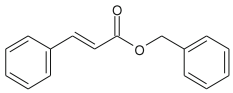
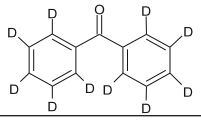
Cleaning of glassware

Considering that UV filters are ingredients of PCPs (shampoo, lipstick, soap, and sunscreen), laboratory contamination appeared to be imminent. To avoid contamination from this source, nitrile gloves were worn during all procedures during the breakup of the glassware and materials to be used, sampling, sample pre-treatment, and analysis. Glassware cleaning consisted of the following steps: a running tap water rinse, immersion in neutral Extran 5 % detergent (Merck, Darmstadt, Germany) overnight, a tap water rinse followed by a distilled water rinse and finally, an acetone rinse. The clean glassware was air dried for a minute or two, then transferred to an oven at >100 °C. Volumetric glassware was air dried. All glassware was rinsed with AcOEt immediately prior to use.

Sampling sites description

The area selected for study was the Unit of Water Resources Management number 13 (UWRM-13), located in southeastern Brazil, in the central region of São Paulo State (Fig. 1). São Paulo is divided into 22 Units of Water Resources

Table 1 Analytes, abbreviations, chemical structures, molecular weights, Log K_{ow} and pK_a values for the UV filters included in this work

Analyte (abbreviations)	Chemical structure	Molecular weight (g mol^{-1})	Log K_{ow}	pKa
Benzophenone-3 (BP-3)		228.24	3.79	7.56
Ethylhexyl methoxycinnamate (EHMC)		290.40	5.80	-
Ethylhexyl salicylate (ES)		250.33	5.97	8.13
Octocrylene (OC)		361.49	6.88	-
Benzyl cinnamate[a] (BC)		238.28	4.06	-
Benzophenone -d_{10}[b] (BP-d_{10})		192.28	3.18	-

[a]internal standard; [b]surrogate

Management (UWRM). UWRM-13, also called Hydrographic Basin Tietê-Jacaré, is composed of 34 municipalities that contain approximately 3.5 % of the São Paulo State population, about 1.5 million inhabitants (CETESB 2012) and was selected as the study area. The municipalities included in this study were Araraquara, Bueno de Andrada (Araraquara district), Bauru, Jau, São Carlos, and Trabiju. In the municipalities of Araraquara, Bauru, Jau, and São Carlos, the water treatment steps are coagulation, flocculation, sedimentation, filtration, chlorination, and fluoridation. In Bueno de Andrada and Trabiju, the water source is groundwater and the treatment consists only of disinfection by chlorination and fluorination (which occurs directly in the piping).

Sampling and sample preparation

Water samples were collected from October 2012 to February 2014, in the Water Treatment Plants (WTP)

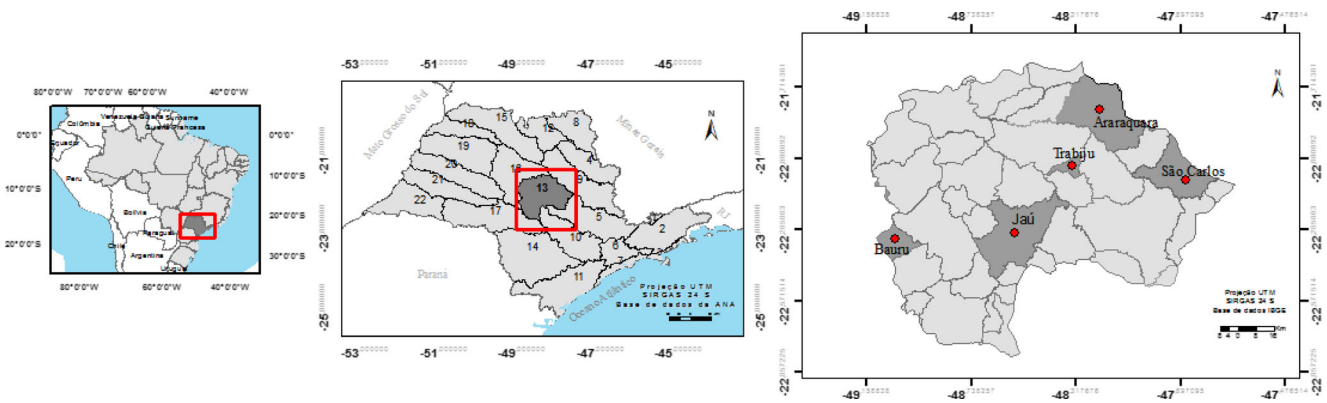


Fig. 1 Water sampling cities. São Paulo State at Brazil map and the Unit of Water Resources Management (UWRM), on the São Paulo State map, showing the cities where water samples were collected

Table 2 Concentrations of UV filters in natural, treated, and chlorinated water (ng L^{-1})

	Analyte	River water	Treated water	Chlorinated water	River water	Treated water	Chlorinated water	River water	Treated water	Chlorinated water
Araraquara		March/2013			April/2013			May/2013		
	BP-10[a]	102(5)	100(2)	110(10)	76(9)	83(3)	84(8)	89(5)	86(2)	98(11)
	ES	<LOQ	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	19(10)	18(9)	18(9)	23(3)	25(4)	21(8)	n.d.	n.d.	n.d.
	EHMC	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		June/2013			July/2013			August/2013		
	BP-10[a]	59(6)	63(5)	71(2)	80(5)	92(1)	85(4)	89(9)	90(11)	97(10)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
	EHMC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	59(67)	<LOQ
		September/2013			October/2013			November/2013		
	BP-10[a]	111(6)	109(11)	109(12)	68(11)	82(7)	85(4)	77(8)	73(8)	86(5)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	22(1)	19(2)	20(4)	33(2)	28(15)	25(7)	38(2)	29(9)	31(4)
	EHMC	n.d.	n.d.	75(11)	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		December/2013			January/2014			February/2014		
	BP-10[a]	58(8)	61(12)	69(8)	59(3)	63(15)	65(5)	106(9)	106(16)	141(5)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	26(4)	23(3)	23(10)	36(5)	32(4)	28(3)	30(6)	25(13)	23(7)
	EHMC	<LOQ	<LOQ	57(64)	65(19)	55(11)	70(58)	55(10)	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bauru		April/2013			May/2013			June/2013		
	BP-10[a]	107(15)	105(5)	131(3)	48(6)	81(17)	115(23)	59(6)	63(5)	71(2)
	ES	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	28(15)	33(14)	115(1)	20(36)	26(27)	72(54)	n.d.	n.d.	n.d.
	EHMC	<LOQ	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	<LOQ	<LOQ	87(57)	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.
		July/2013			August/2013			September/2013		
	BP-10[a]	45(12)	57(3)	73(8)	93(6)	100(18)	111(9)	123(16)	106(9)	105(3)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EHMC	n.d.	n.d.	n.d.	669(5)[b]	707(6)[b]	309(6)[b]	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	188(2)[b]	211(4)[b]	199(4)[b]	n.d.	n.d.	n.d.
São Carlos		April/2013			May/2013			June/2013		
	BP-10[a]	110(8)	121(7)	93(3)	93(9)	85(5)	81(11)	80(2)	82(10)	82(4)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	n.d.	n.d.	n.d.	<LOQ	<LOQ	n.d.	44(10)	60(31)	n.d.
	EHMC	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	101(10)	<LOQ	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		July/2013			August/2013			September/2013		
	BP-10[a]	86(11)	84(5)	80(8)	94(10)	94(15)	92(40)	105(6)	104(9)	114(6)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	<LOQ	n.d.
	BP-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EHMC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2 (continued)

	Analyte	River water	Treated water	Chlorinated water	River water	Treated water	Chlorinated water	River water	Treated water	Chlorinated water
Jau		March/2013			April/2013			May/2013		
	BP-10[a]	96(5)	100(3)	105(6)	79(5)	79(11)	82(5)	65(23)	73(10)	74(17)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	18(8)	18(1)	n.d.	24(8)	26(6)	28(15)	n.d.	n.d.	n.d.
	EHMC	<LOQ	n.d.	n.d.	<LOQ	<LOQ	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		June/2013			July/2013			August/2013		
	BP-10[a]	60(1)	59(7)	66(4)	83(6)	84(1)	92(6)	115(6)	123(6)	135(13)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	21(20)	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
	EHMC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	755(8)[b]	629(16)[b]	292(3)[b]
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	208(12)[b]	176(10)[b]	183(24)[b]

BP-10 was used as a surrogate. Average recoveries and relative standard deviations (RDS) for $n=3$

n.d. not detected, *<LOQ* below the limit of quantification

[a] BP-10 used as surrogate at 200 ng L^{-1}

[b] Quantification disregarded (contamination was suspected)

at the municipalities included in this study at three points: the entrance of the WTP, before any treatment (*river water*), after treatment without chlorination (*treated water*), and after chlorination (*chlorinated water*). Sampling in Bauru, Bueno de Andrada, Jau, São Carlos, and Trabiju was performed over a period of 6 months, while in Araraquara, the sampling was performed over a period of 12 months to evaluate the influence of seasonality on the occurrence of the UV filters.

All samples were collected inside the water treatment plant, except in Bauru, where there was no collection point for one of the sample types (river water or raw water) inside the station, so it had to be directly collected from the river. In Trabiju and Bueno de Andrada, the water is collected underground and is disinfected directly in the pipe, so only two types of water samples were analyzed: groundwater and chlorinated water.

The samples were collected in the morning between 07:00 and 10:30 in amber Pyrex glass bottles with screw caps of 4 L capacity. After collection, the samples were transported to the laboratory under refrigeration at 4 °C (ice packs) and protected from light. In the laboratory prior to extraction, the samples were filtered through a glass fibre filter with a pore size of 0.6 μm (Macherey-Nagel GF3), acidified to pH 3, spiked to 200 ng L^{-1} with a surrogate (BP-d10) and immediately extracted, and analyzed according to the previously developed and validated solid-phase extraction (SPE) method and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS; Silva et al. 2013; Silva et al. 2015).

SPE extraction and GC-MS/MS analysis

The SPE method employed was a previously published method by Silva et al. (2013). The extraction of the sample analytes was performed using 200 mg/6 mL, Strata X SPE cartridges obtained from Phenomenex (Torrance, USA). The SPE cartridges were first conditioned with 5 mL of AcOEt, 5 mL of MeOH and 5 mL of deionized water. Sample volumes of 500 mL at pH 3 were passed through the cartridge. The cartridge was then dried under total vacuum for 5 min and 500 μL of MeOH was added. The analytes were eluted with 3×2 mL of AcOEt. Then, 500 μL of an internal standard solution of 100 μg L^{-1} BC was added to the eluate. The eluate was evaporated to 1 mL under a gentle flow of nitrogen gas prior to analysis by GC-MS/MS.

GC-MS/MS analysis was performed on a Varian CP-3800 gas chromatograph (Varian, Walnut Creek, CA, USA) that was equipped with a Saturn 2000 ion trap mass spectrometer . The chromatography was performed under the following conditions: helium was the carrier gas (maintained at a constant flow rate of 1.2 mL min^{-1}); an injector temperature of 300 °C, operating in splitless injection mode with a sampling time of 1 min. The separation of the analytes was performed with a fused-silica ZB-5MSi capillary column (30 m length × 0.25 mm i.d. and 0.25 mm film thickness; Phenomenex, Torrance, CA, USA). The temperature program was as follows: 60 °C for 1 min; ramped at 25 °C min^{-1} to 160 °C, which was maintained for 1 min; and ramped at 10 °C min^{-1} to 300 °C, which was maintained for 2 min. The total run time was 22.0 min. The transfer line, trap, and manifold temperatures were maintained at 300, 50, and 220 °C, respectively.

Results and discussion

Quality parameters of the method

The analytical method used to determine UV filters in water, in this work, was based on SPE and GC-MS/MS. The detailed study of multivariate optimization procedure for this method was published previously (Silva et al. 2013), as well as, the validation parameters, including matrix effect (Silva et al. 2015).

For all samples (6 months to 1 year) for all cities (Araraquara, Bauru, Jau, São Carlos, Trabiju and Bueno de Andrada), the recovery of the surrogate (BP-d10) ranged 70–120 %, and the relative standard deviations (RSDs) was below 20 %, as shown in Tables 2 and 3. Therefore, the method satisfied the requirements for precision and accuracy for quantification.

Sampling aspects

The most favorable Brazilian scenario (south-eastern Brazil) regarding basic sanitation, where the sewage collection far exceeds the national average, was used as the study area. However, note that the treatment is performed in only 48 % of collector municipalities. This absence of sewage treatment in more than half of the collector municipalities in the region of the country that has the best sanitary conditions clearly shows the seriousness of the national environmental situation. The poor collection and treatment of sewage in Brazil implies serious negative consequences to public health and the environment. In this context, the quantitative determination of UV filters in the raw water and the drinking water under the "best" conditions (south-east) is of importance.

The state of São Paulo in the Southeast Brazil is divided into 22 UWRM. UWRM-13 was selected as the study area

Table 3 Concentrations of UV filters in ground and chlorinated water (ng L^{-1})

	Analyte	Ground water	Chlorinated water	Ground water	Chlorinated water	Ground water	Chlorinated water
Bueno de Andrada		March/2013		April/2013		May/2013	
	BP-10[a]	133(7)	143(11)	107(10)	99(9)	108(5)	112(10)
	ES	n.d.	<LOQ	n.d.	n.d.	<LOQ	n.d.
	BP-3	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.
	EHMC	<LOQ	n.d.	<LOQ	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		June/2013		July/2013		August/2013	
	BP-10[a]	71(8)	72(23)	105(13)	122(3)	130(40)	80(30)
	ES	n.d.	<LOQ	<LOQ	n.d.	n.d.	n.d.
	BP-3	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.
	EHMC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trabiju		March/2013		April/2013		May/2013	
	BP-10[a]	105(21)	113(3)	88(8)	88(9)	59(14)	66(14)
	ES	n.d.	105(258)	n.d.	n.d.	n.d.	n.d.
	BP-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EHMC	137(148)	<LOQ	n.d.	n.d.	n.d.	n.d.
	OC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		June/2013		July/2013		August/2013	
	BP-10[a]	60(5)	61(6)	93(3)	85(2)	115(15)	112(6)
	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP-3	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.
	EHMC	n.d.	n.d.	n.d.	n.d.	724(4)[b]	273(2)[b]
	OC	n.d.	n.d.	n.d.	n.d.	175(22)[b]	174(19)[b]

BP-10 was used as a surrogate. Average recoveries and relative standard deviations (RDS) for $n=3$

n.d. not detected, *<LOQ* below the limit of quantification

[a] BP-10 used as surrogate at 200 ng L^{-1}

[b] Quantification disregarded (contamination was suspected)

because it is a populous region in which the sewage collection is nearly 100 %, although in some municipalities, the sewage collected is not treated. For example, Bauru, the most populous municipality in this basin, treats only 10 % of the sewage collected (CETESB 2012).

Study of the occurrence of UV filters in environmental samples

The concentrations of the UV filters in samples from the six WTPs in the UWRM-13 are presented in Tables 2 and 3. All UV filters were detected in the samples from the WTPs, however, quantifiable concentrations were found only for BP-3 and EHMC. The concentrations of BP-3 and EHMC ranged 18–115 and 55–101 ng L^{-1}, respectively. Although the other analytes were present in some samples, they were not present at levels that could be reliably quantified.

Both BP-3 and EHMC, among other UV filters, have been found throughout the world. They have been detected in water, soil, sediment, sludge, and biota. BP-3 and EHMC have been detected in river and tap water worldwide (Table 4). The maximum level of BP-3 detected in river water was 114 ng L^{-1} in Slovenia (Cuderman and Heath 2007), according to a review by Kim and Choi (2014). EHMC has been found in slightly higher concentrations; Kameda and collaborators found concentrations up to 1,040 ng L^{-1} in highly polluted rivers in Japan (Kameda et al. 2011). It was reported by Díaz-Cruz et al. (2012) that concentration of BP-3 and EHMC were up to 295 and 256 ng L^{-1}, respectively, in the public water supply. These literature values are comparable to the values obtained for river water and drinking water in this study.

BP-3, EHMC, and OC are the UV filters most used in PCPs. BP-3 is the most polar, which is possibly why it occurs with the highest frequency in the samples studied. BP-3 was quantified in Araraquara during almost every sample period (March 2013 to February 2014) except only May, June, July, and August 2013. BP-3 was quantified in Bauru during April and May 2013; in São Carlos, only in June 2013; and in Jau during March, April, and June 2013. EHMC was quantified in Araraquara only in September 2013, January 2014, and February 2014; and it was quantified in São Carlos in June 2013. The other two UV filters (ES and OC) were below quantifiable levels, although they were detected. Figure 2 shows (1) a chromatogram of the SPE products and the GC-MS/MS analysis for the UV filters at the limit of quantification and (2) a chromatogram of the SPE products and the GC-MS/MS analysis for the UV filters in river water from the Araraquara WTP in January 2014.

The high concentrations of EHMC and OC in August in Bauru, Jau, and Trabiju might be explained by contamination during the collection and/or extraction of the sample. Although these compounds were detected (below the LOQ) in previous months, these values appear to be discordant with others from the same sampling sites Even if their coefficients of variation are excellent, the values are suspect because these analytes were detected in Bauru, Jau, and Trabiju at similar concentrations. Because the samples from the three municipalities were collected on the same day and also processed together, the possibility of contamination cannot be discarded. Contamination was also not confirmed because an entire cleaning procedure of glassware and materials was conducted,

Table 4 Summary of the measured concentrations of UV filters in river water and tap water (ng L^{-1})

Sample	Maximum concentration of BP-3 (ng L^{-1})	Maximum concentration of EHMC (ng L^{-1})	Country	Reference
River water	44	101	Brazil	This article
Drinking water	105	75	Brazil	This article
River water	114	88	Slovenia	Cuderman and Heath (2007)
River water	30	21	Spain	Rodil and Moeder (2008)
River water	52	–	Spain	Negreira et al. (2009)
River water	<LOQ	<LOQ	Australia	Liu et al. (2011)
River water	12	1040	Japan	Kameda et al. (2011)
River water	n.d.	n.d.	Singapore	Zhang and Lee (2012)
Tap water	295	256	Spain	Diaz-Cruz et al. (2012)
River water	69	10	Italy	Magi et al. (2012)
River water and groundwater	38	–	Spain	Gago-Ferrero et al. (2013)
River water	15	–	Taiwan	Wu et al. (2013)
Tap water	n.d.	n.d.	Singapore	Zhang and Lee (2013)
Groundwater	19	–	Spain	Jurado et al. (2014)

<*LOQ* below the limit of quantification, *n.d.* not detected, – not studied

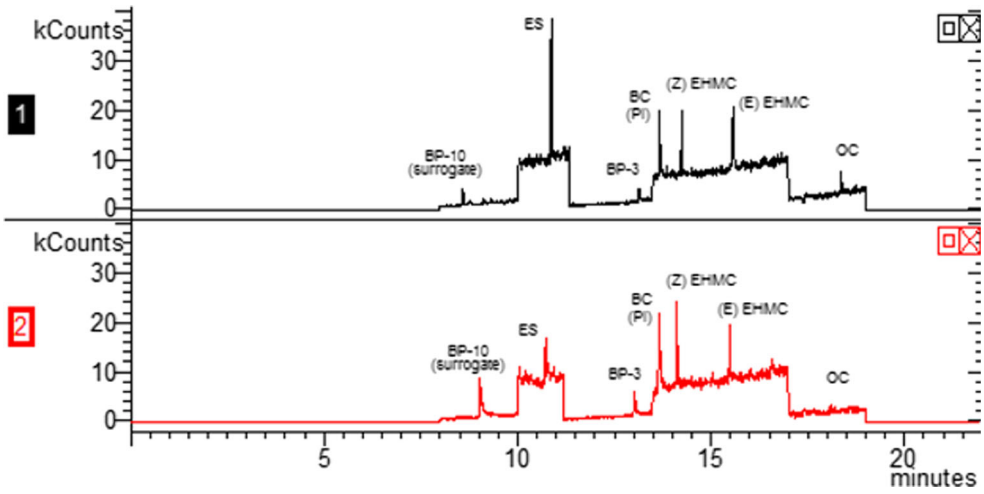


Fig. 2 SPE and GC-MS/MS chromatogram in MRM mode of the analysis of *1* WTP (treated water—Araraquara) spiked at 10, 100, 10, 50, and 50 ng L^{-1} to BP10, ES, BP3, EHMC, and OC, respectively. The internal standard BC was spiked at 50 μg L^{-1}. *2* WTP (river water—Araraquara—January) the surrogate BP-10 and the internal standard BC was spiked at 100 μg L^{-1}. EHMCs are present as both (*E*) and (*Z*) isomer

in addition to care with the use of gloves during the procedure for separation of the glassware for the collection until the moment of injections.

In Araraquara, the sampling was performed over one year period (March 2013 to February 2014), so in that municipality, the seasonal occurrence of BP-3 was evident, as shown in Fig. 3. It should be noted that during winter time (May–August) UV filters were not quantifiable probably because of the lower consumption of sunscreens in the winter season.

Figure 3 indicates a lower concentration of BP-3 in chlorinated water samples compared to treated and river samples, probably because of the chlorination of the aromatic ring of the BP-3 (Negreira et al. 2008). However, the concentration in chlorinated water is only slightly lower than in treated and river water. It is suggested that the water treatment procedure used was not sufficient to eliminate these substances from the drinking water. This finding is disturbing because BP-3 has often been reported as an endocrine disruptor (Fent et al. 2008), inducing hormonal activity in vitro and in vivo. Benzophenones have been associated with the induction of vitellogenin, alterations in the gonads, a reduction in fertility and reproduction, and feminization of sexual characteristics in male fish (Díaz-Cruz and Barcelo 2009).

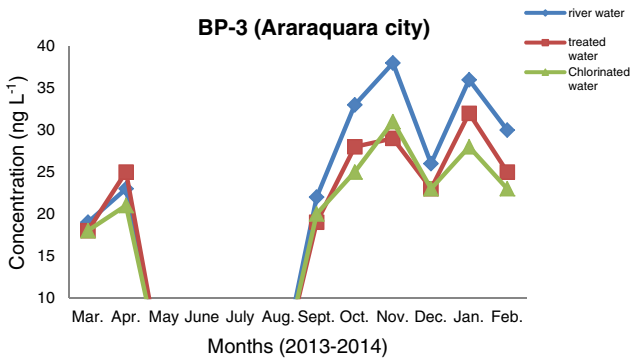


Fig. 3 Average monthly concentration ($n=3$) of BP-3 in river water (◆), treated water (●), and chlorinated water (▲) in the Araraquara WTP

Despite the low concentration found in this work for natural water analysis, to achieve a more complete environmental risk analysis discussion is mandatory to include UV filters determination in other environmental compartments as sediment and water particulate matter. On the other hand, in the literature, conclusive studies regarding the temporal effects of environmentally relevant concentrations are absent. However, even at very low concentrations, long-term exposure can present deleterious effects (Díaz-Cruz and Barcelo 2009). Additionally, the propensity for rapid accumulation of these substances and their potential combined effects should not be neglected because they are introduced into the environment daily in a mixture with other compounds. All these issues clearly indicate the need for further studies to evaluate the environmental occurrence and fate of UV filters, as well as toxicological and ecotoxicological studies (which are currently lacking) that can support the worldwide environmental regulation of these compounds.

Conclusions

UV filters were found in low concentrations in raw water and in the public water supply from treatment plants in the UWRM-13. All of UV filters studied were detected at some point during the study, both in the raw water and the drinking water, but only BP-3 (18–115 ng L^{-1}) and EHMC (55–101 ng L^{-1}) were found in quantifiable concentrations.

The concentrations of BP-3 increased during the summer, most likely due to direct and indirect environmental input. Although the concentrations found were low (in nanograms per liter level), it does not indicate that these substances are not liable to cause environmental or human damage because safe environmental or human exposure levels for these compounds have not yet been determined. Additionally, this is the first report of these compounds in Brazil, but only water samples were analyzed. The physicochemical properties of some of

these compounds indicate a high potential for accumulation in particulate matter, sediments and even in the biota.

The occurrence and quantification of UV filters in drinking water raises the question about the safe levels of these substances, indicating the need for improved water treatment processes for their removal. Thus, our results contribute to a discussion about the improvement of the water and sewage treatment in Brazil, as well as the development of global environmental legislation regarding emerging contaminants.

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:10 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 3 of 9
Attachments: 13 Diaz Cruz Analytical and Bioanalytical Chemistry.docx; 14 Plagellat UV filters in Swiss wastewater.docx; 15 Amine UV filters, ethylhexyl methoxycinnamate.pdf; 16 Sharifan UV filters environmental threat.pdf; 17 Global Harmonized System Guide Oct 05.pdf; 18 Ref for FDA Voluntary Cosmetic Registration Program available at https.docx; 19 Klimova Skin absorption and human exposure.pdf; 20 21 and 23 Absorption References.docx; 22 Janjua J Invest Dermatol.docx; 24 Gago-Ferrero ABC-Review biota UV F_publicado.pdf; 25 Danovaro et al Sunscreen Cause Coral Bleaching.pdf; 26 Schlumpf Milk Transfer UV filters 2010.pdf; 27 Alonso Toxic Heritage.pdf; 28 Gago-Ferrero UV filter bioaccumulation in fish from Oberian river basin SoTE.pdf; 29 Vione The role of direct photolysis.pdf; 30 Benvokenuti Avobenzone breakdown by OCM.docx; 31 to 38 Contact Photocontact Dermatitis articles.docx

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

Analytical and Bioanalytical Chemistry
March 2012, Volume 402, Issue 7, pp 2325–2333| Cite as
Analysis of UV filters in tap water and other clean waters in Spain

M. Silvia Díaz-Cruz, Pablo Gago-Ferrero, Marta Llorca, Damià Barceló

Abstract
The present paper describes the development of a method for the simultaneous determination of five hormonally active UV filters namely benzophenone-3 (BP3), 3-(4-methylbenzylidene) camphor (4MBC), 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA), 2-ethylhexyl 4-methoxycinnamate (EHMC) and octocrylene (OC) by means of solid-phase extraction and gas chromatography–electron impact ionization–mass spectrometry. Under optimized conditions, this methodology achieved low method limits of detection (needed for clean waters, especially drinking water analysis), between 0.02 and 8.42 ng/L, and quantitative recovery rates higher than 73% in all cases. Inter- and intraday precision for all compounds were lower than 7% and 11%, respectively. The optimized methodology was applied to perform the first survey of UV absorbing compounds in tap water from the metropolitan area and the city of Barcelona (Catalonia, Spain). In addition, other types of clean water matrices (mineral bottled water, well water and tap water treated with an ion-exchange resin) were investigated as well. Results evidenced that all the UV filters investigated were detected in the water samples analyzed. The compounds most frequently found were EHMC and OC. Maximum concentrations reached in tap water were 290 (BP3), 35 (4MBC), 110 (OD-PABA), 260 (EHMC), and 170 ng/L (OC). This study constitutes the first evidence of the presence of UV filter residues in tap water in Europe.

Chemosphere. 2006 Feb;62(6):915-25. Epub 2005 Jul 5.

Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland.

Plagellat C[1], Kupper T, Furrer R, de Alencastro LF, Grandjean D, Tarradellas J.

Abstract

Many substances related to human activities end up in wastewater and accumulate in sewage sludge. The present study focuses on the analysis of widely used UV filters 3-(4-methylbenzylidene) camphor (4-MBC), octyl-methoxycinnamate (OMC), octocrylene (OC) and octyl-triazone (OT) in sewage sludge originating from a monitoring network in Switzerland. Mean concentrations in stabilised sludge from 14 wastewater treatment plants were 1780, 110, 4840 and 5510 microg/kg dry matter for 4-MBC, OMC, OC and OT, respectively. Specific loads in sewage sludge show that UV filters originate mainly from private households, but surface runoff and industries may be considered as additional sources. This indicates that besides use for sunscreens and cosmetics UV filters might occur in plastics and other materials and be released to the environment by volatilization or leaching. Differences between the modeled per capita loads of UV filters in sewage sludge and the observed specific loads in sewage sludge are probably due to erroneous figures of production volumes, degradation and sorption during wastewater treatment as well as degradation processes during transport in the sewer or sludge treatment. Thus, further research is needed to elucidate the fate of UV filters after application and release into the environment. Other compounds used as UV filters should be included in future studies.

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UV filters, ethylhexyl methoxycinnamate, octocrylene and ethylhexyl dimethyl PABA from untreated wastewater in sediment from eastern Mediterranean river transition and coastal zones

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ABSTRACT

UVF may occur in the aquatic environment through two principal sources: direct inputs from recreational activities and indirect wastewater- and river-borne inputs. The aim of this study was to obtain a first overview of levels of three UVF (EHMC, OC and OD-PABA) in coastal areas subjected to river inputs, untreated wastewater discharges and dumpsite leachates. We selected three eastern Mediterranean rivers that have been impacted for decades by untreated wastewater release and collected sediment in the coastal zone during the hot and humid seasons. Western Mediterranean sites receiving treated wastewaters were analyzed for comparison. The results gave an overview of sediment contamination under these two contrasted situations representative of Mediterranean coastal areas without bathing activities. The analysis of the three UVF revealed the ubiquity and high point source contamination by EHMC and OC in transition and coastal zones, with levels as high as 128 ng g^{-1} d.w. OD-PABA was also frequently detected, but at lower concentrations (<LOD-17 ng g^{-1} d.w.). A temporal trend was observed, with a higher sediment concentration in the dry period (August and October). Based on these results, we conclude that there is background contamination from river input that could be exacerbated by the direct contribution in coastal bathing zones.

1. Introduction

Organic sun-blocking substances absorb UV light and are used as UV filters (UVF) or UV light stabilizers to prevent photo-deterioration of human skin and plastic products. Some of these compounds, such as ethylhexyl methoxycinnamate (EHMC) or octocrylene (OC), are often used as both UVF and UV light stabilizers in cosmetics, polymer-based products and paints. One-hundred thirty one ingredients are listed as UVF in the International Cosmetic Ingredient Dictionary and Handbook. Moeder et al. (2010) reported that EHMC and OC accounted for 81% and 35%, respectively, of the market of sun-screen formulations in 2001. New information on the global consumption of these substances as UVF and UV light stabilizer remains very sparse. The environmental concern with respect to these substances is their potential to cause hormonal activities (estrogenic, antiestrogenic, androgenic, and antiandrogenic) that have been documented *in vitro* as well as *in vivo* (Christen et al., 2011; Kunz and Fent, 2006). For example, Zucchi et al. (2010) showed that EHMC induced a decrease of both spermatocytes in testes and previtellogenic oocytes in ovaries of zebra fish. Other observations on these substances showed that EHMC and benzophenone-3 (BP-3) were involved in coral bleaching by promoting viral infections (Danovaro et al., 2008).

UVF are widespread in the aquatic environment. Several have been detected in different countries at concentrations ranging from 0.1 to 19 μg L^{-1} in raw wastewater and from <0.01 to 2.7 μg L^{-1} in treated wastewater (Balmer et al., 2005; Li et al., 2007; Rodil et al., 2009), suggesting some removal. Table 1 illustrates the measured concentrations of EHMC, OC and ethylhexyl dimethyl PABA (OD-PABA) in raw and treated wastewaters and in other environmental samples. As a receptor compartment, surface water also contains UVF, including EHMC, OC and OD-PABA. The major sources of these substances appear to be sewage treatment plant effluents and recreational activities. Indeed, studies conducted in swimming pools, game pools and bathing water showed EHMC concentrations in the 3 to 10.7 ng L^{-1} range (Giokas et al., 2004, 2005; Lambropoulou et al., 2002). These concentrations increased from 12:00 h to 15:00 h, i.e. the period when there is increased bathing (Giokas et al., 2005). OD-PABA showed the highest concentration of UVF measured in game pools and in shower wastes, reaching 6.2 μg L^{-1}

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Table 1
Environmental levels of ethylhexyl methoxycinnamate (EHMC), octocrylene (OC) and ethylhexyl dimethyl PABA (OD-PABA). Aqueous and solid matrix concentrations are expressed in ng L^{-1} and ng g^{-1} dry weight (d.w.), respectively, nd: not detected.

Matrix		Location	EHMC	OC	OD-PABA	References
WWTP	Influent	Switzerland	500–19,000	100–6100		Balmer et al. (2005)
		Germany	1732	5322	nd	Rodil et al. (2009)
	Effluent	Switzerland	<10–100	<10–270		Balmer et al. (2005)
		Germany	nd	179	nd	Rodil et al. (2009)
		Japan	12	nd	nd	Kameda et al. (2011)
	Sewage sludge	Switzerland	10–390	320–18,740		Plagellat et al. (2006)
Surface water	Streams	Japan	21–260	6–14	2	Kameda et al. (2011)
	Lakes	Japan	18	1	5	Kameda et al. (2011)
	Rivers (highly polluted)	Japan	125–1040	nd	nd	Kameda et al. (2011)
	Rivers (moderately polluted)	Japan	12–91	1	1–2	Kameda et al. (2011)
Recreational water	Swimming pools	Greece			nd	Lambropoulou et al. (2002)
		Greece	4.5			Giokas et al. (2004)
	Game pools	Greece			2100	Lambropoulou et al. (2002)
		Greece	3.8–4.4			Lambropoulou et al. (2002)
		Greece	3.0			Giokas et al. (2004)
	Shower wastes	Greece			5300–6200	Lambropoulou et al. (2002)
		Greece	4.1			Giokas et al. (2004)
	Bathing water (12 h PM)	Greece	7.4			Giokas et al. (2005)
	Bathing water (15 h PM)	Greece	10.7			Giokas et al. (2005)
	Recreational lakes	Switzerland	<2–26	<2		Poiger et al. (2004)
		Switzerland	<2–19	<2–27		Poiger et al. (2004)
		Germany	3009	4381	nd	Rodil et al. (2009)
		Germany	<LOD-20	10–250	2	Rodil and Moeder (2008a)
Sea water	Ionian Sea	Greece			nd	Lambropoulou et al. (2002)
	Ionian Sea	Greece	nd			Giokas et al. (2004)
	Pacific Ocean (microlayer)	Polynesia	13–92			Goksoyr et al. (2009)
Surface sediments	Havel and Spree rivers	Germany	nd-4			Ricking et al. (2003)
	Rivers (highly polluted)	Japan	2.2–9.6	2.7–50.0	nd	Kameda et al. (2011)
	Rivers (moderately polluted)	Japan	3.8–30.0	0.4–8.1	nd	Kameda et al. (2011)
	Ebro river	Spain	nd-42	nd-2400	nd-5.2	Gago-Ferrero et al. (2011)
	Lakes (recreational)	Germany	14–34	61–93		Rodil and Moeder (2008b)
	Lakes	Japan	2.0–8.0	1.0–12.0	nd	Kameda et al. (2011)
	Streams	Japan	3.0–101.0	3.0–635.0	nd	Kameda et al. (2011)

(Lambropoulou et al., 2002). Furthermore, in lakes receiving inputs from recreational activities in Germany, the levels of these three UVF vary seasonally between <LOD (16) and 33 ng L^{-1} for EHMC, between 10 and 250 ng L^{-1} for OC, and between <LOD (0.2) and 5 ng L^{-1} for OD-PABA (Rodil and Moeder, 2008a). Even in very remote environments such as the Pacific Ocean (Polynesia), EHMC, BP-3 and 3-benzylidene-camphor (3-BC) was detected in the surface microlayer (Goksoyr et al., 2009).

Due to their high lipophilicity (log K_{ow} = 3–7) and stability in the environment (Brausch and Rand, 2011), UVF can be highly submitted to sorption by sediment. Indeed, Kameda et al. (2011) found total concentrations of 18 UVF ranging from 2.0 to 3422 ng g^{-1} d.w. in river surface sediment. They also found widespread EHMC and OC adsorption onto sediments, while OD-PABA was not observed in sediment despite its presence in surface water. Rodil and Moeder (2008b) reported levels of EHMC from 14 to 34 ng g^{-1} d.w. and of OC from 61 to 93 ng g^{-1} d.w., while OD-PABA was not detected in lake sediments in Germany. More recently, Gago-Ferrero et al. (2011) revealed the widespread occurrence of OC, reaching concentrations of up to 2400 ng g^{-1} d.w in river sediments in Spain. They also found, for the first time, OD-PABA at concentrations of up to 5.2 ng g^{-1} d.w., and EHMC not exceeding 42 ng g^{-1} - d.w. Nakata et al. (2009) detected four benzotriazole UV stabilizers in marine sediments collected from the Ariake Sea in Japan. Total concentrations ranged from 7.9 to 720 ng g^{-1} d.w in coastal and river sediments around the Ariake Sea.

Despite the information that has been reported on the occurrence of UVF in the aquatic environment and on their potential impact on aquatic species, several questions arise concerning their occurrence and fate in transition and coastal areas. In these zones, contamination of the aquatic environment with UVF may occur through two principal sources already identified in rivers and lakes: direct inputs from recreational activities (Lambropoulou et al., 2002) and indirect wastewater- and river-borne inputs (Giokas et al., 2004; Kameda et al., 2011). Direct inputs are subjected to seasonal variations of unknown amplitude in terms of fluxes and concentrations. Indirect inputs are dependent on urban and industrial pressures. It is important to develop a strategy in order to differentiate the behavior of UVF under different release patterns. This could be done through a selection of several sites exempt of direct inputs that could interfere with the main input from wastewater.

The aim of this study was to obtain a first overview of the levels of three UVF, their spatial and temporal trends, in coastal areas subjected to river inputs, untreated wastewater discharges and dumpsite leachates but not subjected to direct inputs from bathing activities. A comparison with a zone receiving treated wastewaters was performed.

2. Materials and methods

2.1. Reagents and standards

EHMC (CAS 5466-77-3, 99.5%) and OD-PABA (CAS 21245-02-3, 98.5%) – Eusolex® were supplied by Merck (Germany). OC (CAS 6197-30-4, 97%) was provided by Sigma–Aldrich (USA). Chrysene-d12 (99.9%) and PCB 30 (96.5%), used as internal standards, were supplied by SUPELCO (USA) and Dr. Ehrenstorfer GmbH (Germany), respectively. Hydrochloric acid was obtained from Riedel-de-Haën (Seelze, Germany). All solvents used for extraction and analysis were of pesticide quality, or HPLC ultrapure grade and were supplied by SDS, Carlo Erba. Copper granules (diameter: 0.2 and 0.6 mm) were supplied by Sigma–Aldrich (St. Quentin Fallavier, France). Anhydrous sodium sulfate was dried at 110 °C for

8 h prior to use. Standard mixtures were prepared in ethanol and used to spike sediments for quality control. All solutions were stored in the dark at −20 °C prior to use.

2.2. Sampling sites in the eastern Mediterranean

Three sampling campaigns were conducted at 37 sampling sites in August 2010, October 2010 (dry period) and January 2011 (wet period). Thirty one sites were located in the upstream (U), transition (T) and coastal (C) zones of three Lebanese rivers: Abu Ali river (AA), El-Bared river (EB) and El-Kebir river (EK). Six sites were positioned in sectors on the El-Mina coastline (M), corresponding to specific activities: one commercial harbor (CH), one fishing harbor (FH) and four sewage outfalls, numbered from 1 to 4 (SO1, SO2, SO3 and SO4) from north to south along M (Table 2 and Fig. 1).

A Mediterranean climate prevails, with a moderately warm dry summer (30 ± 3 °C) and a moderately cold, windy, and wet winter (13 ± 4 °C). Around 80–90% of the precipitation occurs between November and March, resulting in a torrential flow regime in the three rivers. Low flows occur in dry periods between July and October. The three selected rivers are mainly subjected to urban and agricultural pressure. The population levels in the river catchments are 600,000, 198,000 and 400,000 inhabitants, respectively, in AA, EB and EK (Table 2). AA is also impacted by the industrial activity around the city of Tripoli (Lebanon) situated in the coastal site (Massoud et al., 2006a,b). The coastal marine environment of AA is subjected to river inputs, untreated wastewater discharges and Tripoli dumpsite leachates. All activities at the studied watershed generated an average annual discharge rate estimated in 2001 at 262 million m^3, 282 million m^3, 190 million m^3 for AA, EB and EK, respectively (SOER, 2001). No data on more recent discharge rates were available. El-Mina is a coastal city of 53,000 inhabitants, where six specific sectors of activity were chosen, depending on their proximity to potential sources of organic and chemical contamination (harbor, dumping site, untreated wastewater discharge, etc.).

2.3. Sampling sites in the western Mediterranean

Six western Mediterranean sites were also sampled. Three sites were located in the transition zone of the Lez river (France), a 315,000 inhabitant watershed: one site was directly positioned on the Lez river (LR) under 250,000 inhabitants wastewater treatment plant (WWTP) effluent, two sites were positioned on two coastal lagoons (CL). CL Arnel received direct discharge from a WWTP of 8500 inhabitants. The CL Mejean received indirect discharge through its connection with the LR. LR and CL sediments were collected in April 2004 and October 2005 for a previous study (David et al., 2010), just before the stop of WWTPs discharges. The three remaining sites, sampled in 2010, were located in the coastal zone, in the vicinity of a submarine outfall (MO) build in 2005 for the new 450,000 inhabitant WWTP. The MO sites were about 350 m north (MO1), east (MO2) and west (MO3) of the outfall.

2.4. Sediment collection and treatment

Five hundred grams of sediment (0–20 cm depth) was collected, in accordance with EPA methods (2001), with a stainless steel grab sampler. After collection, sediments were homogenized, air dried, sieved and the fraction below 2 mm was collected in aluminum boxes, frozen under −20 °C, freeze-dried and stored in the dark and dry environment until analysis.

At the sampling site, water salinity and dissolved oxygen (DO) were measured. Salinity measurements were based on the standard method 4500-Cl-B (argent metric method) and DO was measured with a membrane electrode (Multi-Parameter Instrument: WTW, Multi 340 i) according to the standard 4500-O-G method. Total organic carbon (TOC) was determined with a HighTOC II analyzer (Hanau, Germany) in sediment collected in October.

2.5. Extraction and purification

5 ± 0.1 g of homogenated samples were extracted via microwave-assisted extraction in a Multiwave 3000 (Anton Paar) with 30 mL of an acetone/heptane mixture (1:1, v:v) after addition of the surrogate standard (Chrysene-d12). The extraction temperature was increased to 115 °C within 15 min. After extraction, the liner was rinsed with three portions of 5 mL acetone/heptane mixture. In the extract, 1–2 g of activated copper granules were added for 1–2 h to remove sulfur. Copper granules were activated by the Standard Operating Procedure N° 5-192-05 and the EPA method 3660B. Extracts were then filtered (0.2 μm GF-C) through 10 g of anhydrous sodium sulphate, rotary evaporated to dryness and redissolved in 1.0 mL of heptane containing the internal standard (PCB 30). The presence of UVF in sediment was confirmed by extraction and analysis of duplicates. Chrysene-d12 had a similar

Table 2
Sampling locations in the eastern and western Mediterranean sites.

River/inhabitants	Zone	Distance[a] (km)	Altitude (m)	Description
East Mediterranean (Lebanon)				
Abu Ali (AA)/600,000	C (n = 3)	0	0	Major urban complex: Tripoli (350,000 inhab.)
	T (n = 3)	0	0	Sewage outfalls
	U (n = 5)	0.2–9.5	2–33	Dump site leachate discharge and sewage outfalls
El-Bared (EB)/198,000	C (n = 3)	0	0	Major urban complex: Camp (50,000 inhab.)
	T (n = 3)	0	0	Sewage outfalls
	U (n = 3)	0.1–3.1	1.5–40	Urban areas and agricultural areas
El-Kebir (EK)/400,000	C (n = 3)	0	0	Syrian and Lebanese sites
	T (n = 3)	0	0	Sewage outfalls
	U (n = 5)	0.2–6.5	2–45	Residential and agricultural area (poultry farms)
El-Mina (M)/53,000	CH (n = 1)	0		Commercial harbor
	FH (n = 1)	1.8		Fishing harbor
	SO (n = 4)	3.6–10.5		Sewage outfalls
West Mediterranean (France)				
Lez river/315,000	LR (n = 1)			Downstream WWTP
Coastal lagoons	CL (n = 2)			Arnel (area: 475 ha, average depth: 0.4 m)
				Mejean (area: 550 ha, average depth: 0.7 m)
Marine outfall/400,000	MO (n = 3)			

n: sampling sites; C, T and U zones: rivers coastal, transition and upstream zones; CH: commercial harbors; FH: fishing harbors; SO: sewage outfalls along the El-Mina coastline; LR, CL and MO: Lez river, coastal lagoons and WWTP marine outfall.

[a] Km from river's mouth and km from CH in the El-Mina coast.

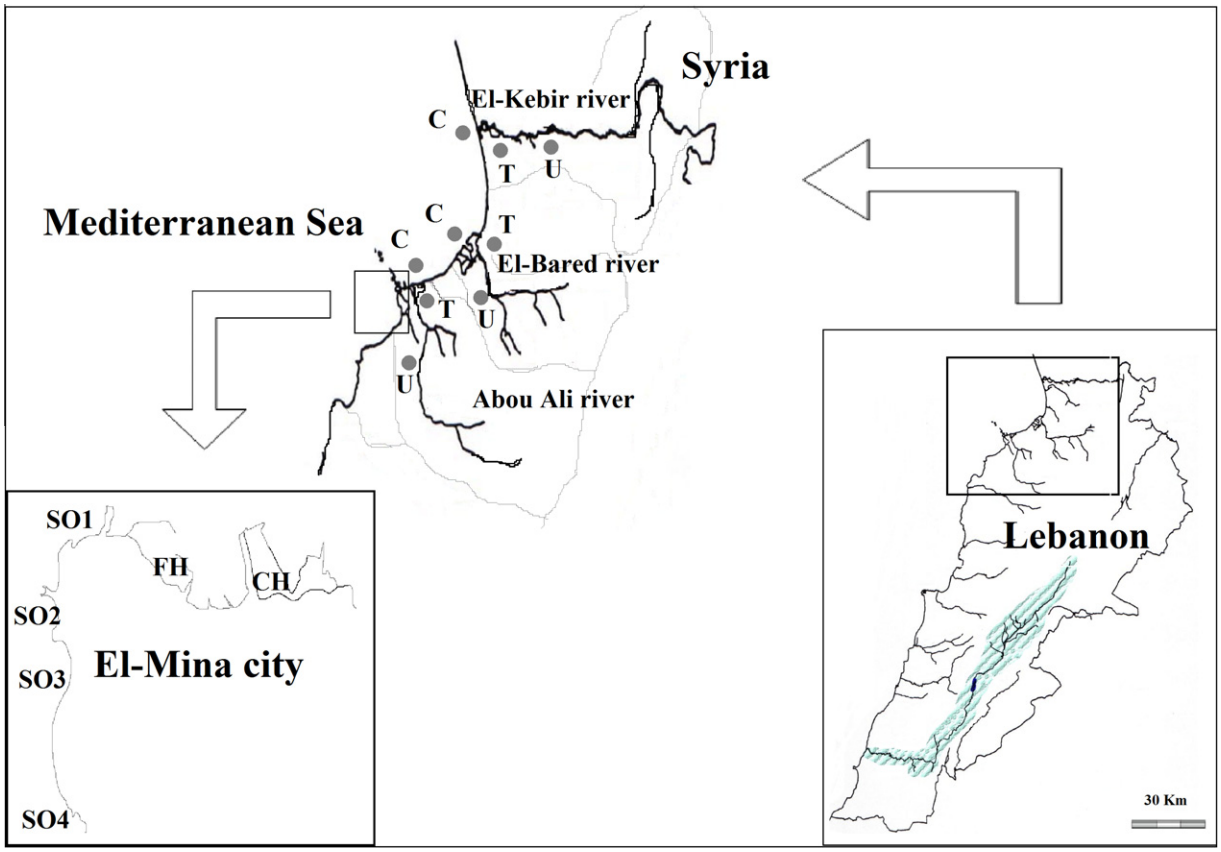

Fig. 1. The locations of the sampling zones in the eastern Mediterranean. U, T, C refer respectively to the upstream, transition and coastal zones along the three rivers. CH, FH, SO refer respectively to the commercial harbor, the fishing harbor and sewage outfalls along El Mina city coastline.

behavior to the different UVF during extraction and purification. This molecule was preferred to benzophenone-d10, the labeled UVF available from suppliers.

2.6. GC-MSn analysis

Analysis was carried out on a GC Ultra trace 3000 (Thermo) connected to an ion trap mass spectrometer (MSn) detector Polaris Q (Thermo). An SGE-BPX5® capillary column (30 m, 0.25 mm I/D, 0.25 μm film thickness) was used. One micro liter was injected with an autosampler (AI 3 000) in splitless mode at 250 °C. Helium was used as carrier gas at a flow rate of 1.7 mL min^{-1}. The GC temperature program was as follows: 80 °C, 0.5 min isothermal, 20 °C min^{-1} to 150 °C, then at 5 °C min^{-1} to 200 °C, 20 °C min^{-1} to 220 °C, finally at 5 °C min^{-1} to 300 °C followed by an isothermal hold of 10 min. The ion trap source and the GC–MS interface temperatures were set at 220 °C and 300 °C, respectively. Data were acquired in the selected ion monitoring (SIM) for standards PCB 30 and chrysene-d12, and in the MS–MS mode for Z- and E-EHMC, OC, and OD-PABA. The collision energies, Qz, parent and quantification ions, and retention times are presented in Table 3. Peak detection and integration were carried out using Xcalibur software (Thermo). Z-EHMC was assumed to give the same response factor as E-EHMC. The results are expressed as the sum of both Z- and E-EHMC.

2.7. Quantification and quality control

Spiked samples were analyzed as described above together with a blank sample. A procedural blank was performed with every set of six samples. The linearity (n = 7) of the analytical procedure was verified by spiking samples with the three UVF over a concentration range of 1 to 50 ng g^{-1} d.w. (Table 4). Since there were no reference sediments for UVF analysis, recovery studies were conducted with surface sediments spiked with known amounts of the analytes (six replicates of three spiking levels: 5, 25 and 50 ng g^{-1} d.w.). The three spiking levels (low, medium and high) were chosen referring to the values found in literature and representing the levels that are found in sediments. The average recoveries, assessed by the subtraction concentration observed in non-spiked samples from those spiked at 25 ng g^{-1} d.w. (medium spike level), ranged from 97% to 115%, (Table 4). The extraction recoveries were confirmed from the low spike level (5 ng g^{-1} d.w.). The recoveries of surrogate standard Chrysene-d12, ranged from 86% to 107%. The accuracy, calculated as the percentage deviation of the mean observed concentrations from the nominal concentration, was under 16%. The precision was evaluated by analyzing spiked samples (three replicates) during three different days. The RSD values obtained were under 13%. The limits of detection (LOD) and the limits of quantification (LOQ) were determined as the concentrations which would give three and ten times, respectively, the standard deviation of the peak height for six replicates of the blank sample. LOD and LOQ were respectively 1.5 ng g^{-1} d.w. and 5 ng g^{-1} d.w. for EHMC and OD-PABA, and 2.0 ng g^{-1} d.w. and 6 ng g^{-1} d.w. for OC (Table 4).

2.8. Statistical analysis

The software package SPSS (Statistical Package for Social Sciences) version 19.0 was employed for statistical analysis. Assumption tests of normality and equal variance were performed before ANOVA. The Kolmogorov–Smirnov test and Levine's test were

Table 3
Analytical parameters used for quantification of EHMC, OC and OD-PABA in GC–MS/MS mode.

Compound	Parent ion	Excitation amplitude (V)	Quantification ions	Retention time (min)
EHMC	178	1.3	121 + 132 + 161	Z-19.83 E-21.99
OC	248	1.45	220 + 165	24.35
OD-PABA	165	1.3	91 + 118 + 148	21.69

Table 4
Linear correlation coefficient, recovery, accuracy, relative standard deviation (RSD, $n = 6$), limit of detection (LOD) and limit of quantification (LOQ) of UV filter analyses in sediment. r^2 was calculated over a concentration range of 1–50 ng g^{-1} d.w.

Compound	Correlation coefficient (r^2)	Recovery (%) 5 ng g^{-1} d.w. 25 ng g^{-1} d.w. 50 ng g^{-1} d.w.	Accuracy (%)	RSD (%)	LOD ng g^{-1} d.w.	LOQ ng g^{-1} d.w.
EHMC	0.997	111 ± 12	+11	9	1.5	5.0
		99 ± 8	−1	12		
		113 ± 11	+13	10		
OC	0.995	108 ± 12	+8	9	2.0	6.0
		97 ± 9	−3	11		
		115 ± 11	+15	10		
OD-PABA	0.995	101 ± 10	+1	10	1.5	5.0
		98 ± 9	−2	11		
		104 ± 9	+4	12		

applied to test normal distribution and homogeneity of variance, respectively. An adjustment or transformation stage was necessary if the variable distribution was not normal. One-way analysis of variance (ANOVA) followed by Tukey's HSD and Fisher's LSD multiple comparison tests (MCT) were applied, respectively, to assemble sites based on their salinity into homogeneous subsets or zones (C, T and U). Arithmetic means and relative standard deviations (RSD) were provided to express the average concentrations of UVF and physical parameters. A one-way ANOVA test was used to investigate differences between a specific sample variable (UVF concentration) from one sampling period to another (August–October and January) and from one sampling zone to another (C, T and U) for the three rivers (AA, EB and EK) and El-Mina coastline (M). When significant differences between means were detected ($P < 0.05^{*}$, $P < 0.01^{**}$, $P < 0.001^{***}$), MCT were carried out to evaluate them.

3. Results

Total UVF concentrations, calculated as the addition of EHMC, OC and OD-PABA concentrations, ranged from 12 ng g^{-1} d.w. to 304 ng g^{-1} d.w. in sediment from the 37 eastern Mediterranean sites and from <LOD to 33 ng g^{-1} d.w. in the 6 western Mediterranean sites. In the eastern Mediterranean, among the three UVF, OC showed the highest sediment concentrations, reaching 128 ± 5 ng g^{-1} d.w., followed by EHMC (45 ± 6 ng g^{-1} d.w.) and OD-PABA (from <LOD to 17 ± 3 ng g^{-1} d.w.).

3.1. UVF at the eastern Mediterranean sites

On the eastern Mediterranean coast, the river sampling zones were characterized by their salinity. The upstream, transition and coastal zones had salinity levels of 0.08, 0.26 and 32 g L^{-1}, respectively. DO did not differentiate the zones, but exhibited hypoxia (1.6 ± 0.2 mg L^{-1}) during the dry (January) and hot (August and October) season in AA. For each river, MCT showed statistical differences in UVF concentrations between the three physically different zones (C, T and U), indicating a significant spatial variation. TOC in sediment ranged from <0.10% to 3.25% and its distribution do not correlate with C, T and U zones. UVF concentration increased from the coast to the upstream zone, which was affected by Tripoli dumpsite leachates in AA. In EB and EK rivers, the highest concentrations were observed in the transition zone (Fig. 2).

There was also a significant difference in sediment concentration between sampling months for every UVF. The concentrations measured in August and October (dry season) were statistically higher than those of January (wet season) for the three different zones along the three rivers (Fig. 2).

The highest sediment concentrations were obtained in AA, regardless of the sampling month. In AA, EHMC reached 35.8 ng g^{-1} d.w. while the maximum concentration was 19.3 ng g^{-1} d.w. in EB and 17.2 ng g^{-1} d.w. in EK (Fig. 2). OD-PABA and OC were quantifiable in all rivers regardless of the sampling zone and month, whereas EHMC was not quantifiable in EK zones when sampling was performed in January. In EK river, EHMC concentrations ranged from 10 to 17 ng g^{-1} d.w. to <LOD, showing a broad range of temporal variability, whereas OC and OD-PABA persisted during the wet period.

Along the El-Mina coastline, the three UVF were detected in all sediments at the same levels for the three sampling campaigns, with no significant temporal trend. The highest concentrations were measured in the four SO (9.0 ± 1.1 and 79.0 ± 3.2 ng g^{-1} d.w. for OD-PABA and OC, respectively) in comparison to the harbors CH and FH (6.0 ± 0.2 and 51.0 ± 3.2 ng g^{-1} d.w. for OD-PABA and OC, respectively). The OD-PABA and OC concentrations at SO were in the same range as those observed in AA river. The EHMC concentrations along the El-Mina coastline, including SO, CH and FH (9.0 ± 0.7 ng g^{-1} d.w.), were similar to those noted in the coastal zone of the three rivers in August.

3.2. UVF at the western Mediterranean sites

In LR sediments, EHMC and OC were quantified at 7.9 ± 1.2 and 32.8 ± 3.3 ng g^{-1} d.w., respectively, while OD-PABA was below the detection limit. These concentrations were lower than those observed in the eastern Mediterranean transition zones. In CL and

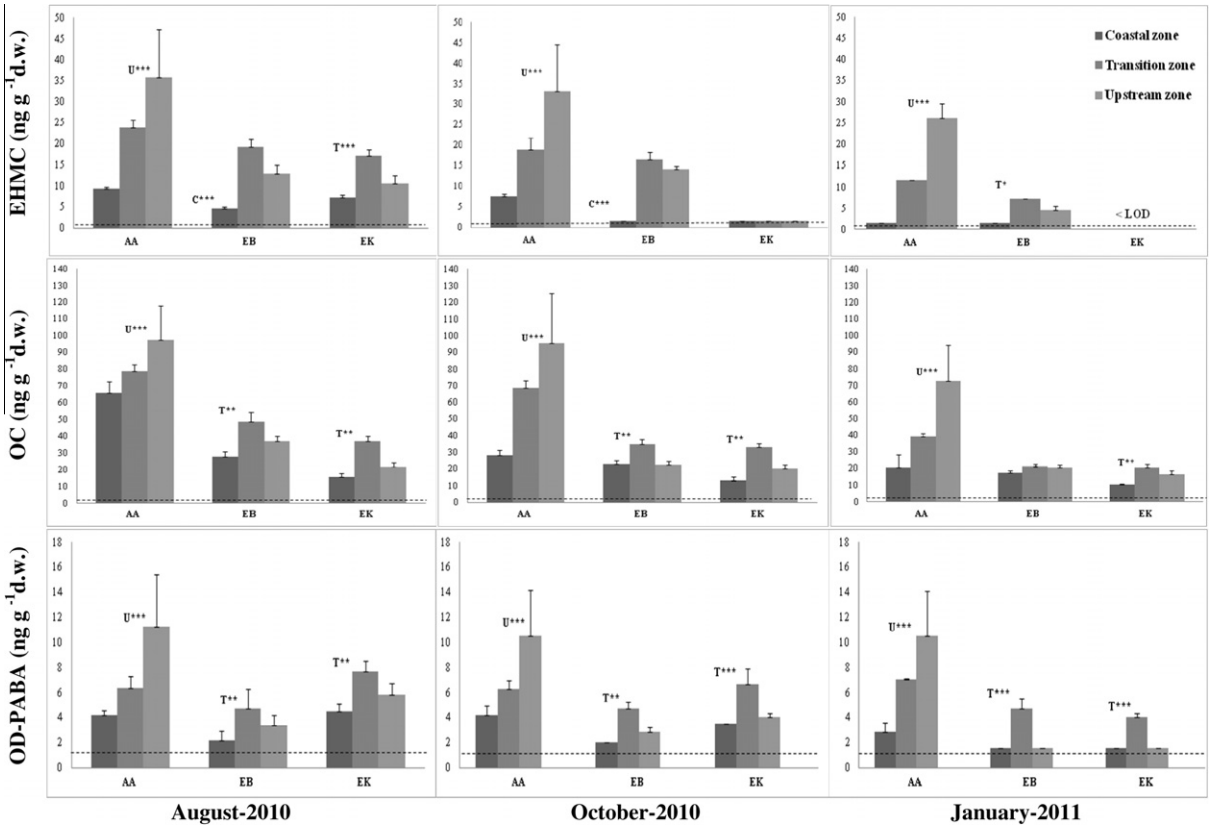

Fig. 2. EHMC, OC and OD-PABA concentrations in surface sediments from Abu Ali (AA), El Bared (EB) and El Kebir (EK) rivers of north Lebanon for the three sampling campaigns (——— LOD). C, T and U refer to the coastal, transition and upstream zones, respectively. The *P*-values indicate significant difference (Post Hoc test – Fisher LSD) between mean values of the studied variable from one sampling zone to the two remaining zones at the 95.0% confidence level. $P < 0.05^{*}$, $P < 0.01^{**}$, $P < 0.001^{***}$.

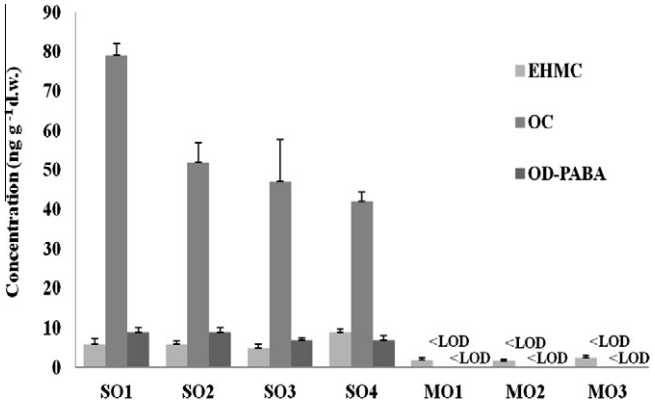

Fig. 3. EHMC, OC and OD-PABA concentrations in surface sediments receiving sewage outfall discharge in the eastern Mediterranean (SO1, SO2, SO3 and SO4) along El-Mina city coastline, and a submarine outfall in the western Mediterranean (MO1, MO2 and MO3).

MO sediments, OC and OD-PABA were under the detection limit, while EHMC was detected at 1.6 ± 0.4 ng g^{-1} d.w. and 2.5 ± 0.6 ng g^{-1} d.w., respectively. These concentrations were lower than those measured along the M coastline (Fig. 3).

4. Discussion

For lipophilic organic UVF, sediment constitutes a trapping compartment. Our results suggest that organic UVF discharged with wastewater can be locally trapped in sediment at high concentrations, especially in transition zones. The presence of such contamination could be attributed to the pressure of anthropogenic activity (urbanization, industrial production) and the lack of wastewater treatment facilities. Sediment contamination by EHMC, OC and OD-PABA in transition zones of the three studied rivers were on the same level as those reported for highly polluted rivers (3.8–30.0 and 2.7–50.0 ng g^{-1} d.w. for EHMC and OC, respectively) (Kameda et al., 2011) or recreational lakes (14–34 and 61–93 ng g^{-1} d.w. for EHMC and OC, respectively) (Rodil and Moeder, 2008b). The gradient investigated in the three studied rivers suggested that coastal zones are contaminated mainly via river input. Such evidence has been shown for other contaminants like nonylphenols (David et al., 2009), where a gradient was noted between coastal samples subjected to continental pressure and deep sea samples.

A temporal trend in sediment contamination was already described by Kim and Carlson (2007) for antibiotics. These authors observed the highest concentrations during low flow conditions as it was observed in the present work for the three Lebanese rivers: the highest UVF concentrations were measured during the dry season, in August, with a marked decrease in the wet season, in January. The consumption of UVF, that is expected to increase in August, should also contribute to this temporal variation.

The three UVF persisted in sediment of the three rivers. However, data concerning the persistence and/or degradation of UVF are very scarce, especially for sediment. In water, some authors reported that OD-PABA and EHMC were photodegradable, while BP-3, OC and 4-MBC were highly stable in ultrapure water (Ricci et al., 2003; Rodil et al., 2009). The structural changes in EHMC that occurred under direct irradiation were consistent with isomerization and polymerization, whereas OD-PABA was degraded by dealkylation (Rodil

et al., 2009) in byproducts stable for several days in water (Rodil et al., 2009). Experimental data on EHMC photolysis in water indicated the formation of cyclodimers (MacManus-Spencer et al., 2011), but there is no data on their occurrence and fate in the environment. Despite the fact that EHMC rapidly photodegrades by direct photolysis in water, EHMC in sediment might be more stable due to the limited light penetration in the sediment compartment. According to these transformation pathways, the lowest sediment concentration found in the present study was observed for OD-PABA, followed by EHMC and them OC, which is known to be photostable. A better understanding of UVF behavior in sediment should include investigation on biodegradation under both freshwater and marine conditions. Indeed, specific transformation processes might occur in coastal zones. It has been experimentally shown that, in the presence of chlorine, some UVF are transformed into halogenated byproducts (Negreira et al., 2008). In brackish environments, this halogenation reaction was reported for the pharmaceutical carbamazepine (Chiron et al., 2006).

Overall, concentrations of the three UVF at eastern Mediterranean sites that had received untreated effluents for decades were higher than those measured at western Mediterranean sites receiving treated effluents. It has been shown that conventional wastewater treatment leads to a decrease in UVF concentrations released in the environment. The removal efficiencies reported in the literature ranged from 40% to 43% for EHMC, and 36% to 38% for OC (Li et al., 2007). Sediment contamination levels therefore differ depending on whether there is or not wastewater treatment plants.

More generally, with UVF occurring in sediment up to 100 ng g^{-1} d.w., biota exposure should be evaluated. In freshwater organisms, OC has been found in fish caught downstream from WWTP at concentrations of around 600 ng g^{-1} lipids (Buser et al., 2006). EHMC has been quantified in crustaceans (*Gammarus* sp.), mollusks (*Dreissena polymorpha*) and in several fish species at levels reaching 133, 150 and 337 ng g^{-1} lipids, respectively (Fent et al., 2010). In marine ecosystems, a recent study reported the presence of EHMC and OC in mussels collected in coastal areas where bathing activities prevail at concentrations of up to 256 ng g^{-1} d.w. for EHMC and 7112 ng g^{-1} d.w. for OC (Bachelot et al., 2012). An experimental study on mussels showed that EHMC and OC, after punctual feeding exposure, were excreted within 24 h (Gomez et al., 2012). This last information indicate that a continuous exposure is needed for attain the levels of UVF measured by Bachelot et al. (2012) and suggest that the bioavailability of the compound and the metabolisation capacities of the exposed organism must be taken into account previously to any risk assessment.

5. Conclusion

Wastewater treatment highly impacts the occurrence and the levels of UVF in the aquatic environment. This study revealed the ubiquity and high point source UVF contamination in eastern Mediterranean sites that had received untreated effluents for decades compared to western Mediterranean sites receiving treated effluents. The concentrations observed in sediment helped to identify the influence of sources and the spatial and temporal trends. Nevertheless, transformation processes need to be studied in depth in this compartment. The behavior of OC, EHMC and OD-PABA in sediment should be studied and compared to the processes involved in other compartments (water and biota) for improving exposure assessment. Sediment should be considered as the target compartment for further studies.

This study indicates that there is a background UVF contamination level mainly due to river input that might be exacerbated in coastal bathing zones. In this case, specific management program should be implemented.

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ORIGINAL RESEARCH ARTICLE

UV filters are an environmental threat in the Gulf of Mexico: a case study of Texas coastal zones

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Summary UV filters are the main ingredients in many cosmetics and personal care products. A significant amount of lipophilic UV filters annually enters the surface water due to large numbers of swimmers and sunbathers. The nature of these compounds cause bioaccumulation in commercial fish, particularly in estuarine areas. Consequently, biomagnification in the food chain will occur. This study estimated the amount of four common UV filters (ethylhexyl methoxycinnamate, EHMC; octocrylene, OC; butyl methoxydibenzoylmethane, BM-DBM; and benzophenone-3, BP3), which may enter surface water in the Gulf of Mexico. Our data analysis was based on the available research data and EPA standards (age classification/human body parts). The results indicated that among the 14 counties in Texas coastal zones, Nueces, with 43 beaches, has a high potential of water contamination through UV filters; EHMC: 477 kg year^{-1}; OC: 318 kg year^{-1}; BM-DBM: 258 kg year^{-1}; and BP by 159 kg year^{-1}. Refugio County, with a minimum number of beaches, indicated the lowest potential of UV filter contamination. The sensitive estuarine areas of Galveston receive a significant amount of UV filters. This article suggests action for protecting Texas estuarine areas and controlling the number of tourists and ecotourism that occurs in sensitive areas of the Gulf of Mexico.

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1. Introduction

Ultraviolet (UV) filters are common ingredients in many cosmetics and personal care products such as sunscreens, soap, shampoos, and hair sprays (Li et al., 2007; Sharifan et al., 2016; Silvia Díaz-Cruz et al., 2008). UV filters and their transformation products, which are washed off from the skin and clothes during swimming and bathing, enter the surface water (Giokas et al., 2007; Li et al., 2007; Nakajima et al., 2009; Plagellat et al., 2006; Poiger et al., 2004; Ramos et al., 2016) and are considered to be a source of surface water contamination (Ekpeghere et al., 2016; Poiger et al., 2004; Ramos et al., 2016). UV filters are added to consumer sunscreen products at different concentrations due to sunscreen formulations (Amine et al., 2012; Kupper et al., 2006; Li et al., 2007; Plagellat et al., 2006; Silvia Díaz-Cruz et al., 2008). The water contamination by UV filters is an increasing public concern due to the secondary effects (i.e. bioaccumulation) of pharmaceuticals and personal care products (PPCPs) in receiving waters, which may reach detectable and potentially toxic concentration levels (Gago-Ferrero et al., 2013; Sharifan et al., 2016).

Furthermore, due to the lipophilic characteristics of UV filters, they can bioaccumulate and biomagnify through the food chain, and their presence is associated with estrogenic effects (Broniowska et al., 2016; Mueller et al., 2003; Vila et al., 2016). Ultimately, these filters can bioaccumulate in humans (Broniowska et al., 2016; Valle-Sistac et al., 2016). Due to a high log octanol–water partition coefficient (log-K_{ow}) of UV-filters (3.8–5.9), these compounds are associated with a high accumulation rate in fish (Broniowska et al., 2016; Ekpeghere et al., 2016; Kim and Choi, 2014).

Fish has a strong tendency to accumulate UV filters (Giokas et al., 2007; Liu et al., 2015). Reported concentrations of UV filters in fish ranged from 9 to 2400 ng g^{-1} lipid weight (Gago-Ferrero et al., 2015). For example, two fish species of perch and roach accumulated UV filters, respectively, by 2000 ng g^{-1} and 500 ng g^{-1} lipids (Li et al., 2007). Though the accumulation rate of UV-filters in fish has been studied both in the field and in laboratories (Blüthgen et al., 2014; Gago-Ferrero et al., 2013; Liu et al., 2015), the toxicokinetic mechanisms of these compounds in fish remain unclear.

In addition to accumulating in the food chain, UV filters have shown severe effects on coral reefs by bleaching corals at very low concentrations (Danovaro et al., 2008). Recently, the UV filters were detected at concentration levels greater than 3700 ng L^{-1} along the coastal areas of South Carolina in the USA (Bratkovics et al., 2015). This concentration may actively link to the life of U.S. endangered coral species such as *Acropora palmata* at the Flower Garden Banks National Marine Sanctuary in the northwestern Gulf of Mexico (Zimmer et al., 2006).

The long shoreline in South Texas (approximately 367 miles/590 km) is a center of recreational activities throughout the year. All 14 counties of this shoreline have 169 beaches for water activities (EPA, 2013). Every year, due to millions of beach visitors and swimmers, significant amounts of UV filters directly or indirectly (i.e. through mistreatment of wastewater, contamination of sand, etc.) enter the surface water in the Gulf of Mexico. However, UV filter concentration information is geographically restricted to some European and Asian countries, as well as Australia, whereas data from other regions, namely the Americas, is missing (Ramos et al., 2016). The potential release of these compounds has never been studied in the Gulf of Mexico. A major challenge for the potential risk effects of UV filters on aquatic life and the food chain is the availability of reliable analytical procedures that determine these substances in aquatic systems (Giokas et al., 2004, 2005; Rodil and Moeder, 2008). However, the empirical research (laboratory experiments and field surveys) is strictly limited due to financial and practical constraints (Arnot and Gobas, 2003; Korsman et al., 2015).

In order to fill the knowledge gap on the ecotoxicity of UV filters in the Gulf of Mexico region, this study aims to identify potentially hazardous substances in an effective and conservative manner. The objective of this study is to estimate the amount of UV filters: ethylhexyl methoxycinnamate (EHMC), octocrylene (OC), butyl methoxydibenzoylmethane (BM-DBM), and benzophenone-3 (BP3) entering the Gulf waters from Texas beaches.

2. Material and methods

2.1. Study area of Gulf of Mexico (Texas)

Based on an EPA report, the total number of beaches in the Texas shoreline, all 14 counties, contain 169 beaches (EPA, 2013), which are aquatic centers for swimmers and beachgoers. The counties of Nueces and Galveston have the highest number of beaches, 43 and 36, respectively. Since 1970, the population of this region increased more than 50% by 2003 based on available statistical data (Lynch et al., 2003). Texas coastal zones have the second largest number of beach visitors (3.8 million) and swimmers (3.07 million) in the entire USA (Lynch et al., 2003). Fig. 1 shows the geographical distribution of Texas counties along the Gulf of Mexico.

2.2. Chemicals

Currently, 14 organic UV filters are authorized in the USA (Ao et al., 2015; Rodil et al., 2009). Four commonly-used UV filters, which are authorized in the USA were studied herein (Santos et al., 2012; Scalia and Mezzena, 2009). The chemical structures of these four compounds, BP3, EHMC, OC and BM-DBM, are described in Table 1, which are presenting the typical structure of chemical UV filters with an aromatic moiety and a side-chain indicating different degrees of unsaturation (Silvia Díaz-Cruz et al., 2008).

2.3. Concentration of UV filters

The average content of each UV filter in cosmetic products (Table 1) was calculated as a weighted average from the composition of individual products via Eq. (1), which was developed by Poiger et al. (2004). The data of UV filter content in sunscreen products used in this study were extracted from a study by Poiger et al. (2004).

$$c_{j,\mathrm{av}} = \frac{\sum n_j c_{j,i}}{\sum n_j}. \quad (1)$$

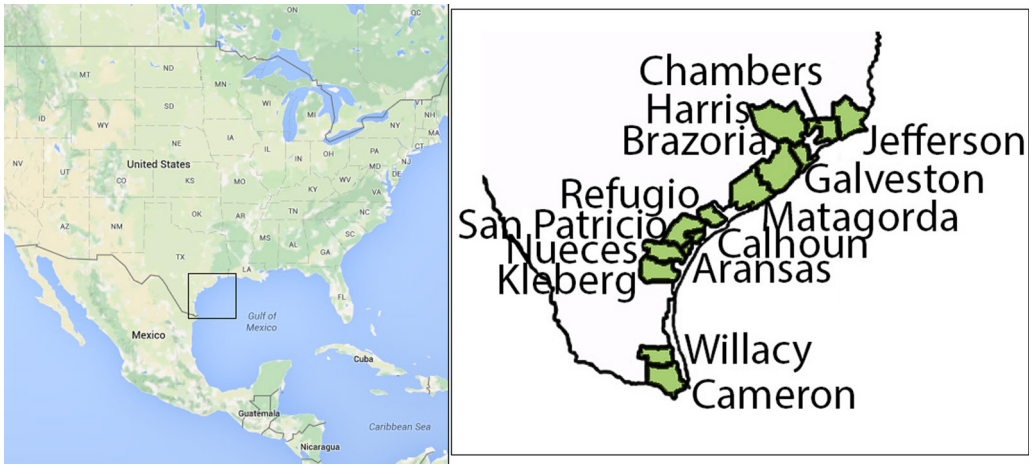


Figure 1 The map of the beaches in 14 counties along the Gulf coast of Texas.
Source: EPA (EPA, 2013), Google.

In this equation $c_{j,\mathrm{av}}$ is the average content of UV filter j in the products used during the survey, n_i is the number of people using product i, and $c_{j,i}$ is the concentration of UV filter j in product i.

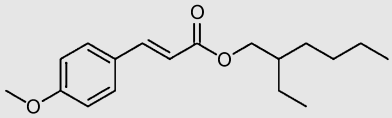
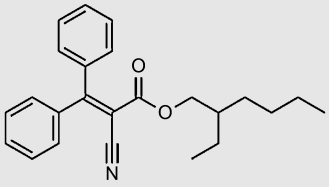
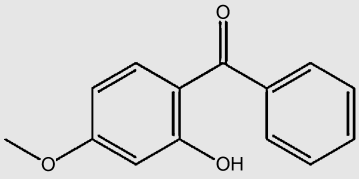
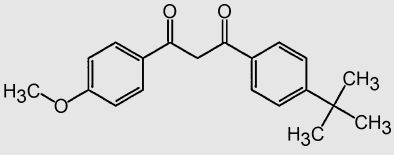
2.4. Surface area of the body

In this study, the surface area of the body parts for different standard age groupings as recommended by the EPA (Table 2) was applied to estimate how much each individual body part could contribute to the release of UV filters into the surface water. The age classes between 1 and 21 are combined by gender. Both genders of male and female are classified from age 21 to 80 (EPA, 2011).

2.5. Release estimation

The direct input of UV filters to the surface water is a function of the fraction of UV filters released from the skin (wash-off rate) during swimming and sunbathing at the beach. The

Table 1 Chemical characteristics, structures and corresponding abbreviations of the surveyed UV filters.

INCI name[a]	Abbreviation	Solubility ([mg L^{-1}] at 25°C)[b]	Average UV filter content in sunscreen products [mg g^{-1}]	Log K_{ow}	Structure
Ethylhexyl methoxycinnamate	EHMC	0.15	2.4	5.8	
Octocrylene	OC	0.02	1.6	6.88	
Benzophenone-3	BP3	68.56	0.8	3.79	
Butyl methoxydibenzoylmethane	BM-DBM	2.2	1.3	4.51	

[a] International Nomenclature of Cosmetic Ingredients.
[b] *Source*: https://pubchem.ncbi.nlm.nih.gov.

Table 2 Surface area of each individual body part [m^2] for different age classifications.

Age class	Face	Trunk	Arms	Hands	Legs	Feet
1–<2	0.029	0.188	0.069	0.03	0.122	0.033
2–<3	0.017	0.25	0.088	0.028	0.154	0.038
3–<6	0.02	0.313	0.106	0.037	0.195	0.049
6–<11	0.022	0.428	0.151	0.051	0.311	0.073
11–<16	0.024	0.63	0.227	0.072	0.483	0.105
16–<21	0.025	0.759	0.269	0.083	0.543	0.112
>21 male	0.045	0.827	0.314	0.107	0.682	0.137
>21 female	0.038	0.654	0.237	0.089	0.598	0.122

lipophilic and hydrophilic UV filters account for 50 and 100% fraction rate (wash-off rate from the skin), respectively. Such a high fraction rate was studied on swimmers on beaches in Galveston County, Texas (Wright et al., 2001). In order to estimate the amount of release in each beach – because of a lack of demographical data on age classification of visitors to beaches – the average surface body of each adult swimmer, 1.94 m^2 recommended by the EPA (>21 years), was applied in this study to account for the worst case of water contamination with all adult swimmers (EPA, 2011). The empirical formula used for this estimation is shown in Eq. (2), which was developed in the previous study by author (Sharifan et al., 2016).

$$c_{j,\mathrm{rel}} = c_{j,\mathrm{av}} \times \alpha \times \beta \times S \times A. \quad (2)$$

The index $c_{j,\mathrm{rel}}$ indicates an estimation of the UV filters released from skin surface area for an adult swimmer (average of male and female), α is the amount of sunscreen cm^{-2} of skin, β is the application rate (dimensionless), S represents the surface area of the body and A represents the percentage of body which was covered by sunscreen products. Table 3 presents the experimental values of these parameters used by researchers in recent studies. In this study, A = 87%, α = 2 and β = 1.5 have been assumed (Poiger et al., 2004; Sharifan et al., 2016; Wright et al., 2001).

The number of swimmers on each beach was estimated by evenly distributing the total number of swimmers in Texas. The potential release rate of UV filters to the surface water in the Gulf of Mexico (index $c_{j,\mathrm{rel,p}}$) was calculated based on experimental Eq. (3), which was developed in this study.

$$c_{j,\mathrm{rel,p}} = c_{j,\mathrm{rel}} \times \emptyset \times P. \quad (3)$$

In this equation, $\emptyset$ indicates the fraction of UV filter (50%) and index P shows the number of swimmers or bathers who are visiting the beach during the swimming season (Sharifan et al., 2016). The annual number of swimmers in Texas was estimated to be approximately 3.07 million (Lynch et al., 2003).

3. Results and discussion

3.1. Release of UV filters from the body parts

The body surface area data (Table 2) and Eq. (2) that was used to calculate the potential release of UV filters from the surface area of swimmers is presented in Fig. 2, highlighting the impact of the different body parts on UV filter release. Evaluation of the data achieved from Eq. (3) showed that the release of UV filters significantly increased proportionately with the age of the groups. An adult male has the greatest majority of UV filter release from their skin due to higher surface area. Adult females show a significant rate of release except for the amount of EHMC, which is greater for the age group of 16–21. This difference can be explained by a larger surface area for teenagers. EHMC has the highest potential of release among the three other UV filters, which can be explained by a higher content in sunscreen products compared to other UV filter ingredients. OC showed greater value than BM-DBM, and BP3 indicated a lower value than BM-DBM in all average surface body parts in all ages. OC has higher K_{ow} (6.8) and has the highest content amount after EHMC compound. The trunk of the body has the largest surface area and shows the maximum potential for release. The hands and feet, respectively, have the lowest potential for release in the water. Trunk surface of the body for an adult can release a considerable concentration of UV filters that for EHMC could be the maximum level of approximately 200 mg per square meter of skin. Both hands and feet for a child (between 1 and 11 years) may release the minimum amount of UV filters due to smaller surface areas of the body.

Table 3 Experimental parameters for the application of sunscreens, which may vary in different studies.

References	Experimental parameters		
	α (amount of sunscreen [mg] cm^{-2} of skin)	β (application rate)	A (percentage of body [%])
Giokas et al. (2007); Poiger et al. (2004)	2, 3, 8	–	–
Poiger et al. (2004)	1	1.5	87
Neale et al. (2002)	1.5	1.03	80
Wright et al. (2001)	0.5, 1.5, 2	–	–

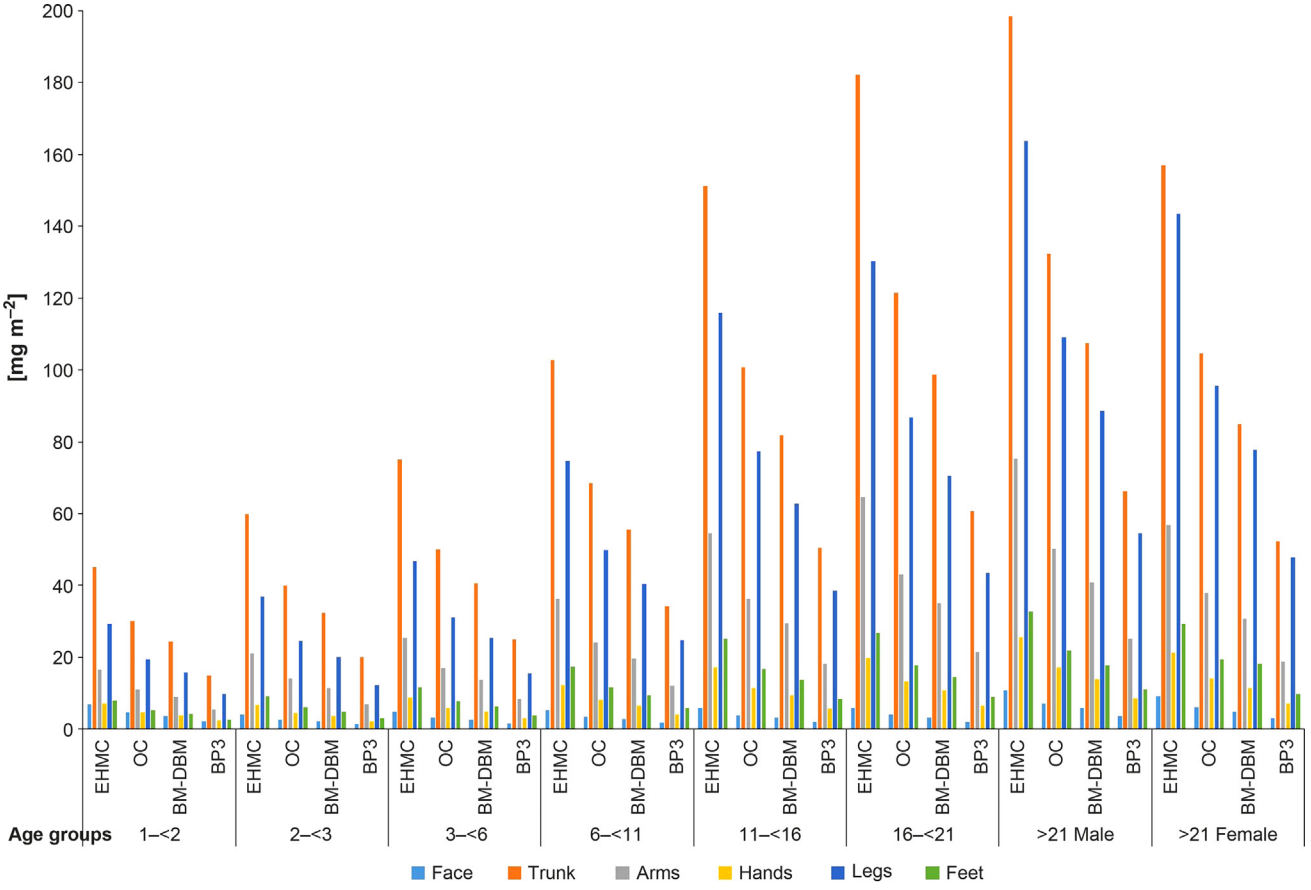


Figure 2 Results for UV filter release per body parts for different age classifications.

With respect to solubility, BP3 has the highest solubility in water (68.5 mg L^{-1}) among the other UV filters studied; therefore, it may have more potential for release in the surface water. However, BP3 may indicate a lower lipophilicity and bioaccumulation rate.

Fig. 3 shows that all UV filters have a higher potential release from the body surface of an adult male, and the age grouping of 16–21 has the second largest potential to release UV filters in surface water. This potential is due to the greater size of the body compared to other age classifications. Though there was no statistical data on the number of male and female visitors to the Gulf of Mexico in Texas, if women have a more than 50% higher tendency than men to apply sunscreens (Wright et al., 2001) on their body, the rate of release may significantly increase. However, regardless of a tendency to apply sunscreens, this analysis indicated that an average surface body of an adult female, after a male body, has a relatively high potential to release UV filters.

Due to the higher solubility of BP3 in the water, it may wash off faster than any other UV agents and may require reapplying the sunscreens. BP3 is approximately 4500 times more soluble in water than EHMC; and, at the same concentration levels, BP3 may enter the water at significantly larger amounts compared to EHMC. A study on beachgoers in Texas shows that half of beach visitors are more likely to stay in the sun longer when applying sunscreen, and approximately 70% of them believe the sunscreen will last at least 3 h without reapplying (Wright et al., 2001). Therefore, preference to stay longer periods of time on the beach may strengthen the hypothesis of higher wash-off rate from skin, as long as exposure times to the sunlight increase.

3.2. Release of UV filters to the surface water

The amount of UV filters that directly enters the Gulf further depends on the amount of UV filters released from the skin during swimming/bathing. The results of this analysis were used to estimate the average input of UV filters discharged to surface water in Texas coastal zones including 14 counties. Estuarine areas of the Texas coastline are productive aquatic systems for providing recreational and commercial marine species such as crabs, shrimp, and fish (Cai et al., 2007; Sager, 2002). For example, the Galveston Bay area has the second greatest number of beaches after Nueces, which is in the vicinity of the second largest populated region in Texas. This area has been contaminated by organic and inorganic aromatic compounds through anthropogenic sources (Glenn and James Lester, 2010; Liu et al., 2016). Therefore, an evaluation of the number of swimmers and potential release of organic UV filters from their body to the surface water is critical for the protection of the marine ecosystem of the Gulf and estuarine areas of the Texas region.

The results of this study (Table 4) estimated the Nueces with 43 beaches has the highest amount of sunscreen release to the water by 477 kg of EHMC, 318 kg of OC, 258 kg of BM-DBM and 159 kg of BP3. The county of Refugio with minimum

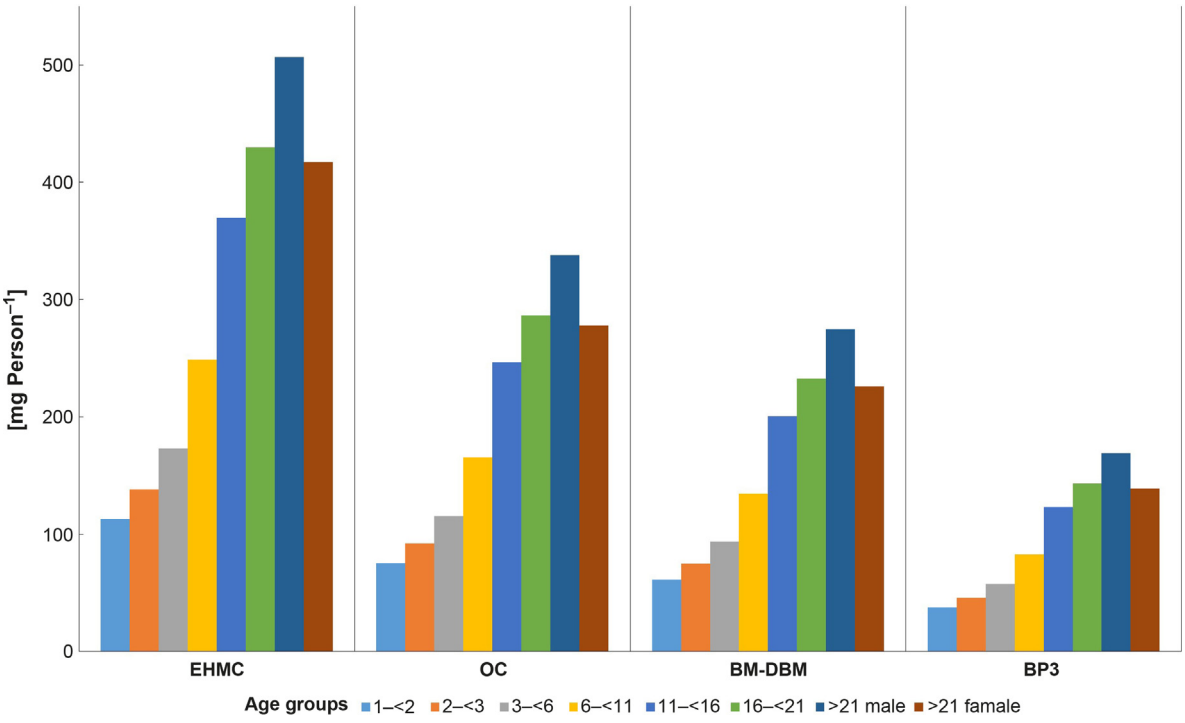


Figure 3 Results of releasing UV filters from the whole surface of a human body at different ages to the receiving water.

beach areas indicated the lowest potential of contamination with sunscreen products at the rate of approximately EHMC = 11 kg, OC = 7.5 kg, BM-DBM = 6 kg and BP3 = 4 kg. The quantity of sunscreen released during the swimming season could be far higher than what was estimated in this study because the number of beachgoers in Texas was greater than three million per year, which was not considered in this analysis. In addition, due to water activities (e.g. swimming, surfing, etc.) in the upstream of discharging rivers to the Gulf, a significant amount of UV filters may enter the water through a variety of streams, which increases the concentrations of UV filters in the Gulf.

Table 4 Estimated input of UV filters to surface water from swimmers in each county on the Texas coastline.

County	No. of beaches[a]	Beach percentage[b]	Swimmers (million)[c]	Estimated input of UV filters to surface water from swimmers [kg][d]			
				EHMC	OC	BM-DBM	BP3
Aransas	9	0.05	0.16	99.8	66.5	54.1	33.3
Brazoria	10	0.06	0.18	110.9	73.9	60.1	37.0
Calhoun	18	0.11	0.33	199.6	133.1	108.1	66.5
Cameron	12	0.07	0.22	133.1	88.7	72.1	44.4
Chambers	2	0.01	0.04	22.2	14.8	12.0	7.4
Galveston	36	0.21	0.66	399.2	266.1	216.2	133.1
Harris	8	0.05	0.15	88.7	59.1	48.0	29.6
Jefferson	2	0.01	0.04	22.2	14.8	12.0	7.4
Kleberg	7	0.04	0.13	77.6	51.7	42.0	25.9
Matagorda	12	0.07	0.22	133.1	88.7	72.1	44.4
Nueces	43	0.25	0.78	476.8	317.8	258.3	158.9
Refugio	1	0.01	0.02	11.1	7.4	6.0	3.7
San Patricio	6	0.04	0.11	66.5	44.4	36.0	22.2
Willacy	3	0.02	0.05	33.3	22.2	18.0	11.1
Total	169	1.00	3.08	1873.8	1249.2	1015.0	624.6

[a] Data from EPA report on Texas Beaches (EPA, 2013).
[b] Percentage of the beaches based on the EPA report on Texas Beaches.
[c] Calculated based on total population of swimmers in Texas reported by Lynch et al. (2003) and assumption of even distributions between beaches.
[d] Extrapolated from calculated data of potential release of UV filters from an adult (average of male and female) from the EPA report (EPA, 2015) and estimation of swimmers in a year.

However, the concentration of a variety of pyrogenic polyaromatic compounds (PAH) in the Gulf of Mexico has been increasing during the last decades (Ruiz-Fernández et al., 2016). The high concentration of UV filters released from the human body along with significant amounts of other PAH may have accumulative effects in estuarine areas of the Gulf coast of Texas. Due to chemical/microbiological stability, low water solubility, lipophilic properties, and vapor pressure, these aromatic-based compounds may accumulate highly in both aquatic and terrestrial estuary areas (Adhikari et al., 2016; Park et al., 2001). As an interpretation, the combination of both UV filter compounds and residues of petroleum pollution to the marine ecosystem may transform the oil into compounds with less volatility and longer residence time in the sediment. The carcinogenic, toxic, mutagenic and persistent nature of PAHs (Adhikari et al., 2016; Singleton et al., 2016) and the high tendency of UV filters to bioaccumulate (Giokas et al., 2007; Liu et al., 2015) may adversly affect the large fishing industry (e.g. mutagenic and estrogenic) in the Gulf of Mexico (Klimová et al., 2015; Ozáez et al., 2016), and make them a critical group of organic pollutants that need to be monitored thoroughly.

4. Conclusion

This study was a scientific approach based on the analysis of the available research data and EPA standards (age classification/body parts of human). Through this method, the release of UV filters from the surface body was estimated and the release of UV filters in susceptible coastal areas in Texas was predicted to provide bioaccumulation data. This information can be used to determine the hazard risks to aquatic wildlife of the region, which is linked to the food web.

Texas coastal zones consist of several sensitive estuarine that may be significantly affected by cumulative effects of UV filter release and contamination by PAHs. Rather than direct release through wash off from the skin, a considerable amount of UV filters may be released through showering or rubbing off with towels or clothes. This number may increase more during laundering or showering by using other personal care products containing UV filters (i.e. shampoos, cosmetics, etc.) and indirectly be discharged to the surface bodies through wastewater. Further studies are needed to investigate the ecotoxicological effects of the UV filters in aquatic organisms, particularly the cumulative effects of PAH compounds in estuarine areas of Texas coastal zones. Therefore, several bay areas in Texas can be considered as a sensitive ecosystem which are exposed to a significant amount of UV filters. Research on this issue may affect the environmental policies for the protection of the reservoirs such as zoning of marine areas as well as make markets reconsider the sunscreen formulation for a safe combination of ingredients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oceano.2016.07.002.

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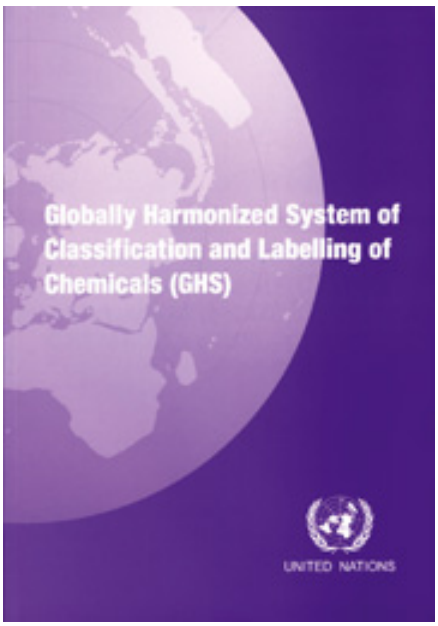
A Guide to *The Globally Harmonized System of Classification and Labeling of Chemicals* (GHS)



Acronyms/Abbreviations

The following list presents some acronyms and abbreviations used in this document. The **6.0 - Glossary** contains a more complete list.

ANSI: American National Standards Institute

APEC: Asia-Pacific Economic Cooperation

ASTM: American Society of Testing and Materials

CA: Competent Authority

CAS: Chemical Abstract Service

CBI: Confidential Business Information

CFR: Code of Federal Regulations

CG/HCCS: Coordinating Group for the Harmonization of Chemical Classification Systems

CPSC: Consumer Product Safety Commission

DOT: Department of Transportation

EINECS: European Inventory of Existing Commercial Chemical Substances

EPA: Environmental Protection Agency

EU: European Union

FIFRA: Federal Insecticide, Fungicide and Rodenticide Act

GHS: Globally Harmonized System of Classification and Labelling of Chemicals

HCS: Hazard Communication Standard

IARC: International Agency for the Research on Cancer

IFCS: International Forum on Chemical Safety

ILO: International Labor Organization

IOMC: Inter-organization Program on the Sound Management of Chemicals

ISO: International Standards Organization

IUPAC: International Union of Pure and Applied Chemistry

LD_{50} : Lethal dose 50

mg/kg: Milligram per kilogram

MSDS: Material Safety Data Sheet

NAFTA: North American Free Trade Agreement

OSHA: Occupational Safety and Health Administration

OECD: The Organization for Economic Cooperation and Development

QSARs: Quantitative Structure-Activity Relationships

SDS: Safety Data Sheet

SME: Small and medium sized enterprises

TFHCL: Task Force on the Harmonization of Classification and Labeling

TSCA: Toxic Substances Control Act

UN: United Nations

UNCED: United Nations Conference on Environment and Development

UNCETDG: United Nations Committee of Experts on the Transport of Dangerous Goods

UNCETDG/GHS: United Nations Committee of Experts on the Transport of Dangerous Goods and on the Globally Harmonized System of Classification and Labelling of Chemicals

UNITAR: United Nations Institute for Training and Research

WG: work group

WHMIS: Workplace Hazardous Materials Information System

WSSD: World Summit on Sustainable Development

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1.0 BACKGROUND

The purpose of this document is to describe the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS), why it was developed, and how it relates to the sound management of chemicals. The full official text of the system is available on the web at: http://www.unece.org/trans/danger/publi/ghs/ghs_rev00/00files_e.html

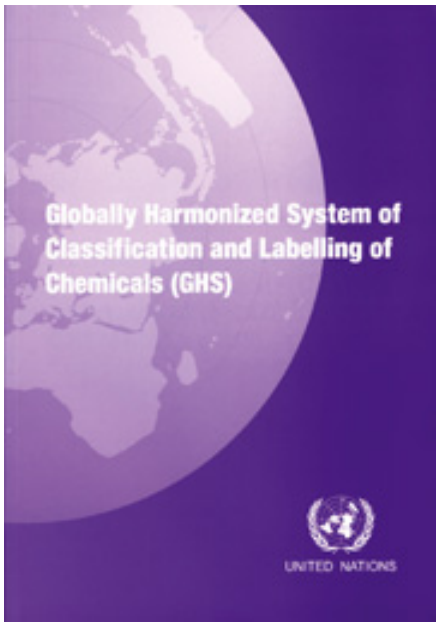
1.1 What is the GHS?

The GHS is an acronym for *The Globally Harmonized System of Classification and Labeling of Chemicals*. The GHS is a system for standardizing and harmonizing the classification and labeling of chemicals. It is a logical and comprehensive approach to:

- Defining health, physical and environmental hazards of chemicals;
- Creating classification processes that use available data on chemicals for comparison with the defined hazard criteria; and
- Communicating hazard information, as well as protective measures, on labels and Safety Data Sheets (SDS).

Many countries already have regulatory systems in place for these types of requirements. These systems may be similar in content and approach, but their differences are significant enough to require multiple classifications, labels and safety data sheets for the same product when marketed in different countries, or even in the same country when parts of the life cycle are covered by different regulatory authorities. This leads to inconsistent protection for those potentially exposed to the chemicals, as well as creating extensive regulatory burdens on companies producing chemicals For example, in the United.States (U.S.) there are requirements for classification and labeling of chemicals for the Consumer Product Safety Commission, the Department of Transportation, the Environmental Protection Agency, and the Occupational Safety and Health Administration.

Figure 1.1 GHS Document ("Purple Book")



The GHS itself is not a regulation or a standard. The GHS Document (referred to as "The Purple Book", shown in Figure 1.1) establishes agreed hazard classification and communication provisions with explanatory information on how to apply the system. The elements in the GHS supply a mechanism to meet the basic requirement of any hazard communication system, which is to decide if the chemical product produced and/or supplied is hazardous and to prepare a label and/or Safety Data Sheet as appropriate. Regulatory authorities in countries adopting the GHS will thus take the agreed criteria and provisions, and implement them through their own regulatory process and procedures rather than simply incorporating the text of the GHS into their national requirements.

The GHS Document thus provides countries with the regulatory building blocks to develop or modify existing national programs that address classification of hazards and transmittal of information about those hazards and associated protective measures. This helps to ensure the safe use of chemicals as they move through the product life cycle from "cradle to grave."

1.2 Why was the GHS developed?

The production and use of chemicals is fundamental to all economies. The global chemical business is more than a $1.7 trillion per year enterprise. In the U.S., chemicals are more than a $450 billion business and exports are greater than $80 billion per year.

Chemicals directly or indirectly affect our lives and are essential to our food, our health, and our lifestyle. The widespread use of chemicals has resulted in the development of sector-specific regulations (transport, production, workplace, agriculture, trade, and consumer products). Having readily available information on the hazardous properties of chemicals, and recommended control measures, allows the production, transport, use and disposal of chemicals to be managed safely. Thus, human health and the environment are protected.

The sound management of chemicals should include systems through which chemical hazards are identified and communicated to all who are potentially exposed. These groups include workers, consumers, emergency responders and the public. It is important to know what chemicals are present and/or used, their hazards to human health and the environment, and the means to control them. A number of classification and labeling systems, each addressing specific use patterns and groups of chemicals, exist at the national, regional and international levels. The existing hazard classification and labeling systems address potential exposure to chemicals in all the types of use settings listed above.

While the existing laws and regulations are similar, they are different enough to require multiple labels for the same product both within the U.S. and in international trade and to require multiple safety data sheets for the same product in international trade. Several U.S. regulatory agencies and various countries have different requirements for hazard definitions as well as for information to be included on labels or material safety data sheets.

Figure 1.2

Acute oral toxicity LD_{50} (mg/kg)

Organization/Country /Regulation or Standard	High / Hazard / Low					
	0 ----------		< 50 ----------		< 500 ----------	< 5,000 -----
ANSI/US/Z129.1	< 50 Highly Toxic		> 50 < 500 Toxic		> 500 < 2,000 Harmful	
OSHA/US/HCS	< 50 Highly Toxic		> 50 < 500 Toxic			
EPA/US/FIFRA	0 ≤ 50 Toxicity Category I		> 50 ≤ 500 Toxicity Category II		> 500 ≤ 5,000 Toxicity Category III	> 5,000 Toxicity Category IV
CPSC/US/FHSA	< 50 Highly Toxic		> 50 < 5,000 Toxic			
GHS	**≤ 5**	**> 5 ≤ 50**	**> 50 ≤ 300**	**> 300 ≤ 2,000**	**> 2000 ≤ 5000**	
DOT/US	< 5 Packing Group I	> 5 < 50 Packing Group II	> 50 < 200 (solid) Packing > 50 < 500 (liquid) Group III			
NFPA/US	≤ 5 Hazard Category 4	> 5 ≤ 50 Hazard Category 3	> 50 ≤ 500 Hazard Category 2		> 500 ≤ 2,000 Hazard Category 1	> 2,000 Hazard Category 0
NPCA/US/HMIS	≤ 1 Toxicity Rating 4	> 1 ≤ 50 Toxicity Rating 3	> 50 ≤ 500 Toxicity Rating 2		> 500 ≤ 5,000 Toxicity Rating 1	> 5,000 Toxicity Rating 0
EU	< 25 Very Toxic	> 25 < 200 Toxic	> 200 < 2,000 Harmful			
WHMIS/Canada	≤ 50 Very Toxic WHMIS Class D, Division 1, Subdivision A	> 50 ≤ 500 Toxic WHMIS Class D, Division 1, Subdivision B				
Australia/NOHSC	< 25 Very Toxic	> 25 < 200 Toxic	> 200 < 2,000 Harmful			
Mexico	< 1 Extremely Toxic	>20<50 Highly Toxic	>50 < 500 Moderately Toxic		>500 < 3000 Mildly Toxic	
Malaysia	<25 Very Toxic		200 to 500 Harmful			
Japan	< 30 Poisonous			300 to 3000 Powerful		
Korea	< 25 Very Toxic		>50 < 200 Toxic	>200<2000 Harmful		

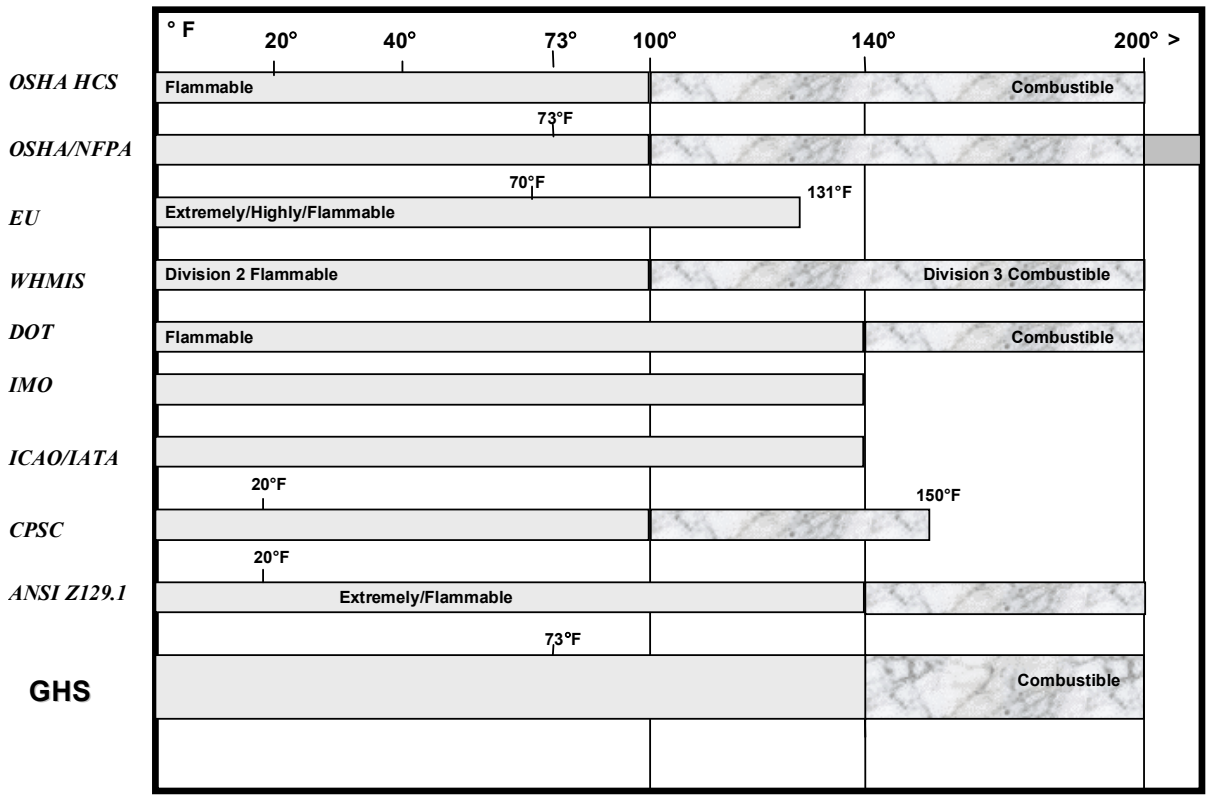
The numerical values on the hazard index scale in the table are not to scale.

For example, a product may be considered flammable or toxic by one agency or country, but not by another.

We can see by comparing a few hazards how complex it is to comply with all domestic and global regulations. Acute oral toxicity (LD_{50}) is a good example (Figure 1.2). Although most existing systems cover acute toxicity, we can see in the figure that what is considered hazardous varies considerably. These differences allow the same product to be hazardous in one country/system and not in another. At the very least, the same product has different labels and SDSs.

Figure 1.3

FLAMMABILITY



The numerical values on the hazard index scale in the table are not to scale.

Flammable liquid is another hazard that is covered by most existing systems. As shown in Figure 1.3, the coverage varies between existing systems within the U.S. and globally. This means that the same product can be non-hazardous or hazardous with different labels/SDSs. In Section 4,

Figures 4.1 through 4.7 show the diverse domestic and international labels for a fictitious product (ToxiFlam) which has both oral toxicity and flammability hazards.

These differences in hazards and SDS/labels impact both protection and trade. In the area of protection, users may see different label warnings or safety data sheet information for the same chemical. In the area of trade, the need to comply with multiple regulations regarding hazard classification and labeling is costly and time-consuming. Some multinational companies have estimated that there are over 100 diverse hazard communication regulations for their products globally. For small and medium size enterprises (SMEs) regulatory compliance is complex and costly, and it can act as a barrier to international trade in chemicals.

1.3 What was the International Mandate?

Figure 1.4
International Mandate from UNCED Agenda 21, Chapter 19

"A globally harmonized hazard classification and compatible labelling system, including material safety data sheets and easily understandable symbols, should be available, if feasible, by the year 2000."

The single most important force that drove the creation of the GHS was the international mandate (Figure 1.4) adopted in the 1992 United Nations Conference on Environment and Development (UNCED), often called the "Earth Summit". The harmonization of classification and labeling of chemicals was one of six program areas that were endorsed by the United Nations General Assembly to strengthen international efforts concerning the environmentally sound management of chemicals. It was recognized that an internationally harmonized approach to classification and labeling would provide the foundation for all countries to develop comprehensive national programs to ensure the safe use of chemicals.

1.4 How was the GHS developed?

In conjunction with its Convention and Recommendation on Safety in the Use of Chemicals at Work, the International Labor Organization (ILO) studied the tasks required to achieve harmonization. The ILO concluded that there were four major existing systems that needed to be harmonized to achieve a global approach.

No international organization covers all aspects of chemical classification and labeling. A broad scope and extensive expertise and resources were required to develop a system. In order to proceed, several decisions were needed:

(a) what systems would be considered "major" and thus the basis for harmonization, and (b) how could the work be divided to get the best expertise for different aspects. Four existing systems

Figure 1.5
Existing Systems Included in the Harmonization Process

- UN Transport Recommendations
- U.S. Requirements for Workplace, Consumer and Pesticides
- European Union Dangerous Substance and Preparations Directives
- Canadian Requirements for Workplace, Consumers and Pesticides

(Figure #1.5) were deemed to be major and the primary basis for the GHS. While not considered major, requirements of other systems were examined as appropriate, and taken into account as proposals were developed.

A Coordinating Group for the Harmonization of Chemical Classification Systems (CG/HCCS) was created under the Inter-organization Program for the Sound Management of Chemicals (IOMC) and they were charged with coordinating and managing development of the system.

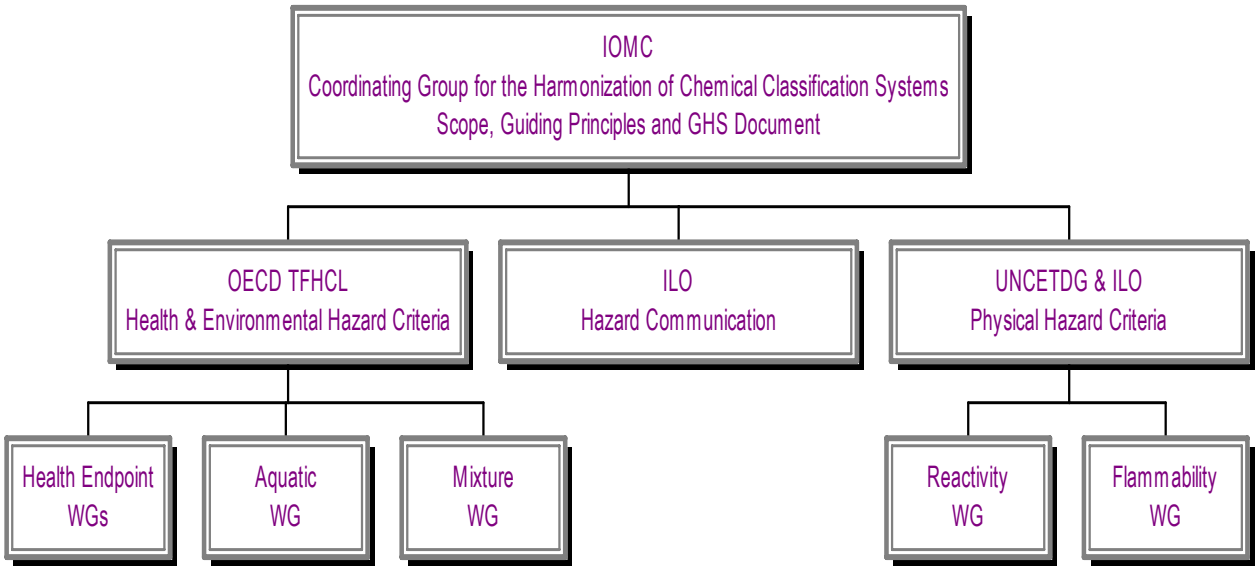
The GC/HCCS worked on a consensus basis and included representatives from major stakeholders, including national governments, industry and workers. They created a set of guiding principles (Figure 1.6). The scope and guiding principles created a common framework for the organizations that were charged with developing the different elements of the system.

Figure 1.6
Key Guiding Principles of the Harmonization Process

- Protection will not be reduced;
- Will be based on intrinsic properties (hazards) of chemicals;
- All types of chemicals will be covered;
- All systems will have to be changed;
- Involvement of all stakeholders should be ensured;
- Comprehensibility must be addressed.

In order to get the best expertise and resources, the work was divided among three technical focal points. Figure 1.7 shows how the work was assigned to the three technical focal points and the overall responsibilities of the Coordinating Group itself. The UN Committee of Experts on Transport of Dangerous Goods was selected as the lead for work on physical hazards, in cooperation with the ILO. Based on their work in the testing guidelines and other chemical issues, the Organization for Economic Cooperation and Development (OECD) was selected for health/environmental hazards and mixtures. ILO has a long history in MSDS/labels, and was selected to be the lead in hazard communication. The OECD and ILO groups also included representatives from governments, industry and workers.

Figure 1.7



1.5 How will the GHS be maintained and updated?

In October 1999, the United Nations Economic and Social Council decided (resolution 1999/65) to enlarge the mandate of the Committee of Experts on the Transport of Dangerous Goods by reconfiguring it into a Committee of Experts on the Transport of Dangerous Goods and on the Globally Harmonized System of Classification and Labeling of Chemicals (UNCETDG/GHS). At the same time, a new Sub-Committee of Experts on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS Sub-Committee) was also created.

When the IOMC completed developing the GHS, the system was presented to the UN GHS Sub-Committee, which formally adopted the system at its first session in December 2002. It was subsequently endorsed by the UNCETDG/GHS. The UN Economic and Social Council endorsed the GHS in July 2003.

The Sub-Committee of Experts on the Globally Harmonized System of Classification will:

- Act as custodian of the system, managing and giving direction to the harmonization process,
- Keep the system up-to-date, as necessary, considering the need to introduce changes or updates to ensure its continued relevance,
- Promote understanding and use of the system and encourage feedback,
- Make the system available for worldwide use,
- Make guidance available on the application of the system, and on the interpretation and use of technical criteria to support consistency of application,
- Prepare work programs and submit recommendations to the UNCETDG/GHS.

1.6 When will the GHS be implemented?

There is no international implementation schedule for the GHS. It is likely that different national systems/sectors will require different timeframes for GHS implementation. Existing systems will need to consider phase-in strategies for transition from their current requirements to the new GHS requirements.

Several international bodies have proposed implementation goals. The World Summit on Sustainable Development (WSSD) and the Intergovernmental Forum for Chemical Safety (IFCS) have encouraged countries to implement the new GHS as soon as possible with a view to having the system fully operational by 2008. The Ministers of the Asia-Pacific Economic Cooperation (APEC) have also said that as many APEC economies as possible should implement, on a voluntary basis, the GHS by 2006. Under the North American Free Trade Agreement (NAFTA), the Tri-national Occupational Safety and Health Group and the NAFTA Pesticides Technical Working Group are discussing the GHS.

Some of the major existing systems have begun discussions about GHS implementation and situational analyses comparing existing requirements to GHS requirements. Some countries are considering harmonization to the greatest extent possible between their national sectors.

1.7 What are the benefits?

The basic goal of hazard communication is to ensure that employers, employees and the public are provided with adequate, practical, reliable and comprehensible information on the hazards of chemicals, so that they can take effective preventive and protective measure for their health and safety. Thus, implementation of effective hazard communication provides benefits for **governments**, **companies**, **workers**, and **members of the public**.

The GHS has maximum value if it is accepted in all major regulatory systems for chemical hazard communication. The diversity of hazard definitions is shown in Figures 1.2 and 1.3. The array of domestic and global labels for one product is shown in Figures 4.1 to 4.7. In the USA implementation of the GHS would harmonize hazard definitions and label information among U.S. regulatory agencies (CPSC, DOT, EPA, OSHA, etc.). If the GHS is implemented globally, consistent information will be communicated on labels and SDSs.

It is anticipated that application of the GHS will:

- Enhance the protection of human health and the environment by providing an internationally comprehensible system,
- Provide a recognized framework to develop regulations for those countries without existing systems,
- Facilitate international trade in chemicals whose hazards have been identified on an international basis,
- Reduce the need for testing and evaluation against multiple classification systems.

The tangible benefits to **governments** are:

- Fewer chemical accidents and incidents,
- Lower health care costs,
- Improved protection of workers and the public from chemical hazards,
- Avoiding duplication of effort in creating national systems,
- Reduction in the costs of enforcement,
- Improved reputation on chemical issues, both domestically and internationally.

Benefits to **companies** include:

- A safer work environment and improved relations with employees,
- An increase in efficiency and reduced costs from compliance with hazard communication regulations,
- Application of expert systems resulting in maximizing expert resources and minimizing labor and costs,
- Facilitation of electronic transmission systems with international scope,
- Expanded use of training programs on health and safety,
- Reduced costs due to fewer accidents and illnesses,
- Improved corporate image and credibility.

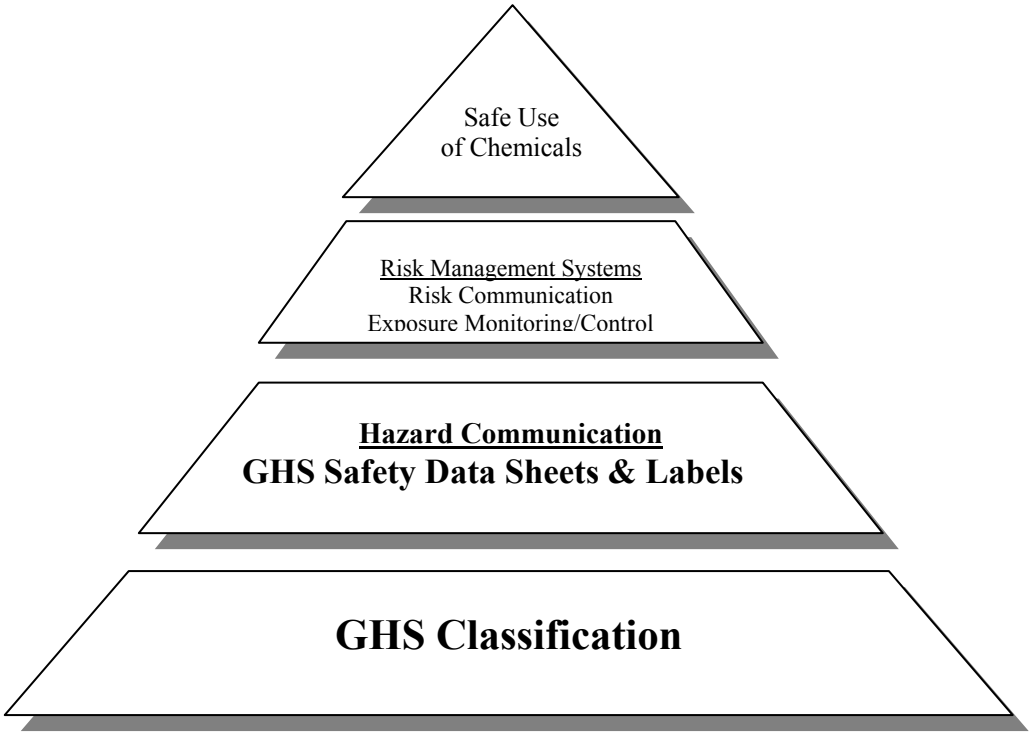
Benefits to **workers** and **members of the public** include:

- Improved safety for workers and others through consistent and simplified communications on chemical hazards and practices to follow for safe handling and use,
- Greater awareness of hazards, resulting in safer use of chemicals in the workplace and in the home.

2.0 HOW IS THE GHS TO BE APPLIED?

The GHS Classification and Communication elements are the foundation of programs to ensure the safe use of chemicals, as shown in Figure 2.1. The first two steps in any program to ensure the safe use of chemicals are to identify intrinsic hazard(s) (i.e., classification) and then to communicate that information. The design of the GHS communication elements reflect the different needs of various target audiences, such as workers and consumers. To proceed further up the pyramid, some existing national programs also include risk management systems as part of an overall program on the sound management of chemicals. The general goal of these systems is to minimize exposure, resulting in reduced risk. The systems vary in focus and include activities such as establishing exposure limits, recommending exposure monitoring methods and creating engineering controls. However, the target audiences of such systems are generally limited to workplace settings. With or without formal risk management systems, the GHS is designed to promote the safe use of chemicals.

Figure 2.1



2.1 Are all chemicals covered by the GHS?

The GHS covers all hazardous chemicals. There are no complete exemptions from the scope of the GHS for a particular type of chemical or product. The term “chemical” is used broadly to include substances, products, mixtures, preparations, or any other terms that may be used by existing systems. The goal of the GHS is to identify the intrinsic hazards of chemical substances

and mixtures and to convey hazard information about these hazards. The GHS is not intended to harmonize risk assessment procedures or risk management decisions, as described above.

"Articles" as defined in the OSHA Hazard Communication Standard (HCS) (29 CFR 1910.1200), or by similar definitions, are outside the scope of the GHS. Chemical inventory (e.g., TSCA, EINECS, etc.) and chemical control requirements in various countries are not harmonized by the GHS.

Classification in the GHS is criteria-based, not limiting coverage to a list that can become outdated. It is not anticipated that the GHS will develop or maintain an international classification authority or international classification list. Several countries currently maintain regulatory lists. GHS classification criteria can be used to reclassify chemicals on lists, if desired. Existing lists, such as those provide by organizations that evaluate cancer hazards, could be used in conjunction with the GHS to promote harmonization.

2.2 Will all hazardous chemicals require a GHS label and Safety Data Sheet?

The need for GHS labels and/or Safety Data Sheets is expected to vary by product category or stage in the chemical's lifecycle from research/production to end use. The sequence of lifecycle events is shown in Figure 2.2. For example, pharmaceuticals, food additives, cosmetics and pesticide residues in food will **not** be covered by the GHS at the point of consumption, but will be covered where workers may be exposed (workplaces), and in transport. Also, the medical use of human or veterinary pharmaceuticals is generally addressed in package inserts and is not part of existing hazard communication systems. Similarly, foods are generally not labeled under existing hazard communication systems. The exact requirements for labels and Safety Data Sheets will continue to be defined in national regulations. However, national requirements are expected to be consistent with the detailed discussion of scope provided in ***Chapter 1.1 of the GHS document***.

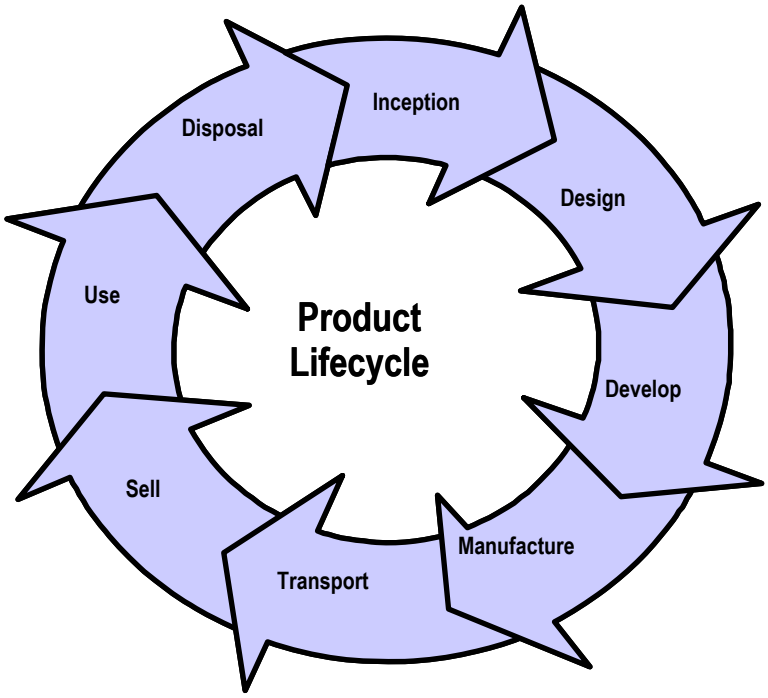


Figure 2.2

2.3 How will the GHS impact existing regulations?

The GHS is a voluntary international system that imposes no binding treaty obligations on countries. To the extent that countries adopt the GHS into their systems, the regulatory changes

would be binding for covered industries. For countries with existing systems, it is expected that the GHS components will be applied within the framework/infrastructure of existing hazard communication regulatory schemes. For example, exceptions and exemptions found in existing regulations would not be expected to change (e.g., transportation of limited quantities).

However, the specific hazard criteria, classification processes, label elements and SDS requirements within an existing regulation will need to be modified to be consistent with the harmonized elements of the GHS. It is anticipated that **ALL** existing hazard communication systems will need to be changed in order to apply the GHS. For example, in the U.S. EPA and OSHA would be expected to require hazard pictograms/symbols on labels. Canada and the EU would be expected to adopt the GHS pictograms/symbols instead of those currently in use. The transport sector is expected to adopt the changed criteria (LD_{50}/LC_{50}) for the GHS Acute Toxicity Categories 1 - 3. OSHA HCS, WHMIS and the EU would all need to change their acute toxicity criteria.

Test data already generated for the classification of chemicals under existing systems should be accepted when classifying these chemicals under the GHS, thereby avoiding duplicative testing and the unnecessary use of test animals.

2.4 What is meant by GHS Building Blocks?

The GHS classification and communication requirements can be thought of as a collection of building blocks. In regulatory schemes, coverage and communication of hazards vary by the needs of target audiences/sectors. Accordingly, the GHS was designed to contain the hazard endpoints and communication tools necessary for application to known regulatory schemes. The GHS is structured so that the appropriate elements for classification and communication, which address the target audiences, can be selected.

The full range of harmonized elements is available to everyone, and should be used if a country or organization chooses to cover a certain effect when it adopts the GHS. The full range of these elements does not have to be adopted. Countries can determine which of the building blocks will be applied in different parts of their systems (consumer, workplace, transport, pesticides, etc.). For example, some options for implementing the GHS include:

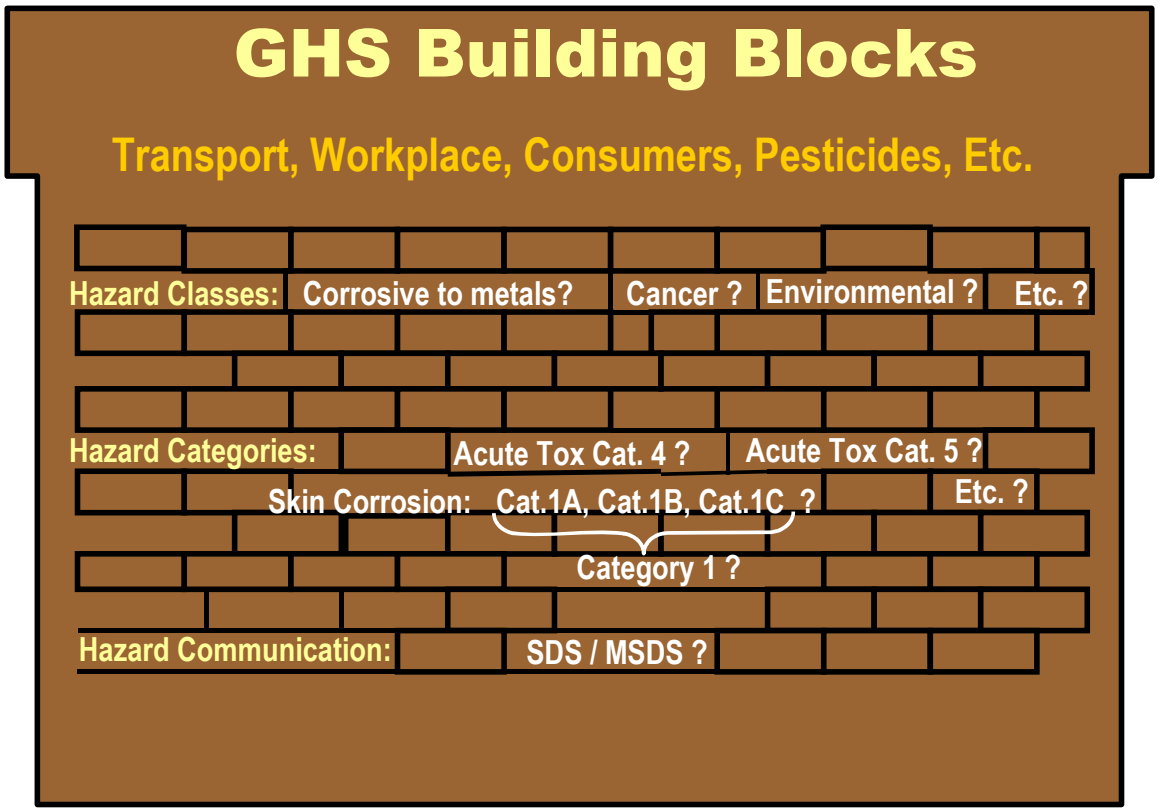
- Not using a GHS class (e.g., cancer, hazardous to the aquatic environment, etc.);
- Not using a GHS category (normally at the beginning or end of a class, e.g., Acute Toxicity Cat. 5);
- Combining categories (e.g., Acute Toxicity Cat.# 1 and Cat.# 2; Skin Corrosion Cat.1A, 1B and 1C).

2.5 How should the GHS Building Blocks by applied?

Appropriate implementation of the GHS means that the hazards covered by a Competent Authority (CA) are covered consistently with the GHS criteria and requirements. The EPA, Health Canada and OSHA are examples of Competent Authorities. Competent Authorities will decide how to apply the various elements of the GHS based on the CA needs and the needs of target audiences.

When a regulatory scheme covers something that is in the GHS, and implements the GHS, that coverage should be consistent. Once an endpoint and subclasses are selected, as needed, the GHS classification criteria, assigned label elements and SDS provisions should be followed as specified in the GHS. If a regulatory system covers carcinogenicity, for example, it should follow the harmonized classification scheme, the harmonized label elements and, where appropriate, the SDS. Figure 2.3 shows some of the hazard endpoint/subcategory and hazard communication building block choices for the transport, workplace, consumer and pesticide sectors.

Figure 2.3



To gain a better understanding of the building block approach, it is helpful to look at the specific sectors/target audiences. The needs and regulations of the various sectors vary depending on the type of chemical and use pattern. Different target audiences or sectors receive and use hazard information in different ways. The primary sectors/target audiences are transport, workplace, consumers and agriculture (pesticides). These sectors are described in more detail below.

2.5.1 Transport

For transport, it is expected that application of the GHS will be similar to application of current transport requirements.

- GHS physical, acute and environmental hazard criteria are expected to be adopted in the transport sector.
- Containers of dangerous goods will have pictograms that address acute toxicity, physical hazards, and environmental hazards.

- GHS hazard communication elements such as signal words, hazard statements and SDS are not expected to be adopted in the transport sector.

2.5.2 Workplace

In the workplace, it is expected that most of the GHS elements will be adopted, including;

- GHS physical and health hazard criteria, as appropriate;
- Labels that have the harmonized core information under the GHS (signal words, hazard statements and symbols, etc.);
- Safety Data Sheets;
- Employee training to help ensure effective communication is also anticipated;

All workplace systems may not have the jurisdiction to adopt environmental hazards.

2.5.3 Consumer

For the consumer sector, it is expected that labels will be the primary focus of GHS application.

- The appropriate GHS hazard criteria are expected to be adopted;
- These labels will include the core elements of the GHS (signal words, hazard statements and symbols, etc.), subject to some sector-specific considerations in certain systems (e.g., risk-based labeling).

2.5.4 Pesticides

For pesticides, it is expected that the GHS will be adopted.

- The appropriate GHS hazard criteria are expected to be adopted;
- Pesticide labels will include the core elements of the GHS (signal words, hazard statements and symbols, etc.), subject to some sector-specific considerations in certain systems.

2.6 How will the GHS impact countries without existing regulations?

Developing and maintaining a classification and labeling system is not a simple task. The GHS can be used as a tool for developing national regulations. It is expected that countries that do not have systems will adopt GHS as their basic scheme. The GHS provides the building blocks from which countries can construct chemical safety programs. Although the GHS will facilitate the process, many challenges exist in creating new regulations. For example:

- What is the appropriate legal framework for adopting/implementing the GHS?
- What government agencies should be involved? Are there ministries/agencies ready to implement and maintain the GHS?
- How will stakeholder cooperation and support for implementing the GHS be managed?

Work has begun in international organizations (e.g, UNITAR and ILO) under the guidance of the UN GHS Sub-Committee, to develop technical assistance for developing countries to write new regulations using the GHS elements. Guidance has been developed on how to implement a national GHS action plan. Additionally, pilot implementations have begun in a few countries. The opportunities and challenges learned from the pilot programs will be documented and are expected to facilitate future implementations.

3.0 WHAT IS CLASSIFICATION?

Classification is the starting point for hazard communication. It involves the identification of the hazard(s) of a chemical or mixture by assigning a category of hazard/danger using defined criteria. The GHS is designed to be consistent and transparent. It draws a clear distinction between classes and categories in order to allow for **"self classification"**. For many hazards a decision tree approach (e.g., eye irritation) is provided in the GHS Document. For several hazards the GHS criteria are semi-quantitative or qualitative. Expert judgment may be required to interpret these data.

Figure 3.1 shows the harmonized definition for hazard classification, which can be applied to all hazard categories in the system.

Figure 3.1

Hazard Classification

The term "hazard classification" is used to indicate that only the intrinsic hazardous properties of substances and mixtures are considered and involves the following 3 steps:

a) Identification of relevant data regarding the hazards of a substance or mixture;
b) Subsequent review of those data to ascertain the hazards associated with the substance or mixture; and
c) A decision on whether the substance or mixture will be classified as a hazardous substance or mixture and the degree of hazard, where appropriate, by comparison of the data with agreed hazard classification criteria.

The data used for classification may be obtained from tests, literature, and practical experience. The GHS health and environmental hazard criteria/definitions are test method neutral. Accordingly, tests that determine hazardous properties conducted according to internationally recognized scientific principles can be used for purposes of hazard classification.

The GHS endpoints that cover physical, health and environmental hazards are listed in Figures 3.2 and 3.3, respectively. As mentioned earlier, the GHS hazard definitions are criteria-based. The following information provides an overview of the GHS definitions and classification criteria. It is recommended that the person responsible for GHS implementation consult the GHS Document or "Purple Book" for more complete information.

3.1 What are the GHS Physical Hazards?

The GHS physical hazards criteria, developed by the ILO and UNCETDG, were largely based on the existing criteria used by the UN Model Regulation on the Transport of Dangerous Goods. Therefore, many of the criteria are already being used on a worldwide basis. However, some additions and changes were necessary since the scope of the GHS includes all target audiences. The physical hazards classification process provides specific references to approved test methods and criteria for classification. The GHS physical hazard criteria apply to mixtures. It is assumed that mixtures will be tested for physical hazards.

In general, the GHS criteria for physical hazards are quantitative or semi-quantitative with multiple hazard levels within an endpoint. This is different from several of the existing systems that currently have qualitative criteria for various physical hazards (e.g., organic peroxide criteria

under WHMIS and OSHA HCS). This could make classification under the GHS more consistent.

In developing GHS criteria for physical hazards it was necessary to define physical states. In the GHS,

- a **gas** is a substance or mixture which at 50°C has a vapor pressure greater than 300 kPa; or is completely gaseous at 20°C and a standard pressure of 101.3 kPa.
- a **liquid** is a substance or mixture that is not a gas and which has a melting point or initial melting point of 20°C or less at standard pressure of 101.3 kPa.
- a **solid** is a substance or mixture that does not meet the definitions of a liquid or a gas.

The GHS physical hazards are briefly described below. For many of the physical hazards the GHS Document contains Guidance Sections with practical information to assist in applying the criteria.

Figure 3.2

Physical Hazards

- Explosives
- Flammable Gases
- Flammable Aerosols
- Oxidizing Gases
- Gases Under Pressure
- Flammable Liquids
- Flammable Solids
- Self-Reactive Substances
- Pyrophoric Liquids
- Pyrophoric Solids
- Self-Heating Substances
- Substances which, in contact with water, emit flammable gases
- Oxidizing Liquids
- Oxidizing Solids
- Organic Peroxides
- Corrosive to Metals

3.1.1 Explosives

An explosive substance (or mixture) is a solid or liquid which is in itself capable by chemical reaction of producing gas at such a temperature and pressure and at such a speed as to cause damage to the surroundings. Pyrotechnic substances are included even when they do not evolve gases. A pyrotechnic substance (or mixture) is designed to produce an effect by heat, light, sound, gas or smoke or a combination of these as the result of non-detonative, self-sustaining, exothermic chemical reactions.

Classification as an explosive and allocation to a division is a three-step process:

- Ascertain if the material has explosive effects (Test Series 1);
- Acceptance procedure (Test Series 2 to 4);
- Assignment to one of six hazard divisions (Test Series 5 to 7).

Explosive properties are associated with certain chemical groups that can react to give very rapid increases in temperature or pressure. The GHS provides a screening procedure that is aimed at identifying the presence of such reactive groups and the potential for rapid energy release. If the screening procedure identifies the substance or mixture to be a potential explosive, the acceptance procedure has to be

Table 3.1 Explosives

Division	Characteristics
1.1	Mass explosion hazard
1.2	Projection hazard
1.3	Fire hazard or minor projection hazard
1.4	No significant hazard
1.5	Very insensitive substances with mass explosion hazard
1.6	Extremely insensitive articles with no mass explosion hazard

performed.

Substances, mixtures and articles are assigned to one of six divisions, 1.1 to 1.6, depending on the type of hazard they present. See, *UN Manual of Tests and Criteria* Part I Test Series 2 to 7. Currently, only the transport sector uses six categories for explosives.

3.1.2 Flammable Gases

Flammable gas means a gas having a flammable range in air at 20°C and a standard pressure of 101.3 kPa. Substances and mixtures of this hazard class are assigned to one of two hazard categories on the basis of the outcome of the test or calculation method (ISO 10156:1996).

3.1.3 Flammable Aerosols

Aerosols are any gas compressed, liquefied or dissolved under pressure within a non-refillable container made of metal, glass or plastic, with or without a liquid, paste or powder. The container is fitted with a release device allowing the contents to be ejected as solid or liquid particles in suspension in a gas, as a foam, paste or powder or in a liquid or gaseous state.

Aerosols should be considered for classification as either a Category 1 or Category 2 Flammable Aerosol if they contain any component classified as flammable according to the GHS criteria for flammable liquids, flammable gases, or flammable solids. Classification is based on:

- Concentration of flammable components;
- Chemical heat of combustion (mainly for transport/storage);
- Results from the foam test (foam aerosols) (mainly for worker/consumer);
- Ignition distance test (spray aerosols) (mainly for worker/consumer);
- Enclosed space test (spray aerosols) (mainly for worker/consumer).

Aerosols are considered:

- Nonflammable, if the concentration of the flammable components ≤ 1% and the heat of combustion is < 20 kJ/g.
- Extremely flammable, if the concentration of the flammable components >85% and the heat of combustion is ≥ 30 kJ/g to avoid excessive testing.

See the *UN Manual of Tests and Criteria* for the test method.

3.1.4 Oxidizing Gases

Oxidizing gas means any gas which may, generally by providing oxygen, cause or contribute to the combustion of other material more than air does. Substances and mixtures of this hazard class are assigned to a single hazard category on the basis that, generally by providing oxygen, they cause or contribute to the combustion of other material more than air does. The test method is ISO 10156:1996. Currently, several workplace hazard communication systems cover oxidizers (solids, liquids, gases) as a class of chemicals.

3.1.5 Gases under Pressure

Gases under pressure are gases that are contained in a receptacle at a pressure not less than 280 Pa at 20°C or as a refrigerated liquid. This endpoint covers four types of gases or gaseous mixtures to address the effects of sudden release of pressure or freezing which may lead to serious damage to people, property, or the environment independent of other hazards the gases may pose.

For this group of gases, the following information is required:

- vapor pressure at 50°C;
- physical state at 20°C at standard ambient pressure;
- critical temperature.

Criteria that use the physical state or compressed gases will be a different classification basis for some workplace systems.

Table 3.2 Gases under Pressure

Group	Criteria
Compressed gas	Entirely gaseous at - 50°C
Liquefied gas	Partially liquid at temperatures > - 50°C
Refrigerated liquefied gas	Partially liquid because of its low temperature
Dissolved gas	Dissolved in a liquid phase solvent

Data can be found in the literature, and calculated or determined by testing. Most pure gases are already classified in the UN Model Regulations. Gases are classified, according to their physical state when packaged, into one of four groups as shown in Table 3.2.

3.1.6 Flammable Liquids

Flammable liquid means a liquid having a flash point of not more than 93°C. Substances and mixtures of this hazard class are assigned to one of four hazard categories on the basis of the flash point and boiling point (See Table 3.3). Flash Point is determined by closed cup methods as provided in the GHS document, Chapter 2.5, paragraph 11.

Table 3.3 Flammable Liquids

Category	Criteria
1	Flash point < 23°C (73°F) and initial boiling point ≤ 35°C (95°F)
2	Flash point < 23 °C (73°F) and initial boiling point > 35°C (95°F)
3	Flash point ≥ 23 °C (73°F) and ≤ 60 °C (140°F)
4	Flash point > 60 °C (140°F) and ≤ 93 °C (200°F)

3.1.7 Flammable Solids

Flammable solids are solids that are readily combustible, or may cause or contribute to fire through friction. Readily combustible solids are powdered, granular, or pasty substances which are dangerous if they can be easily ignited by brief contact with an ignition source, such as a burning match, and if the flame spreads rapidly.

Table 3.4 Flammable Solids

Category	Criteria
1	Metal Powders: burning time ≤ 5 minutes Others: wetted zone does not stop fire & burning time < 45 seconds or burning > 2.2 mm/second
2	Metal Powders: burning time > 5 and ≤ 10 minutes Others: wetted zone stop fire for at least 4 minutes & burning time < 45 seconds or burning rate > 2.2 mm/second

Substances and mixtures of this hazard class are assigned to one of two hazard categories (Table 3.4) on the basis of the outcome of the UN Test N.1 (*UN Manual of Tests and Criteria*). The tests include burning time, burning rate and behavior of fire in a wetted zone of the test sample.

3.1.8 Self-Reactive Substances

Self-reactive substances are thermally unstable liquids or solids liable to undergo a strongly exothermic thermal decomposition even without participation of oxygen (air). This definition excludes materials classified under the GHS as explosive, organic peroxides or as oxidizing. These materials may have similar properties, but such hazards are addressed in their specific endpoints. There are exceptions to the self-reactive classification for material: (i) with heat of decomposition <300 J/g or (ii) with self-accelerating decomposition temperature (SADT) > 75°C for a 50 kg package.

Substances and mixtures of this hazard class are assigned to one of the seven 'Types', A to G, on the basis of the outcome of the UN Test Series A to H (*UN Manual of Tests and Criteria*). Currently, only the transport sector uses seven categories for self-reactive substances (Table 3.5).

Table 3.5 Self-Reactive Substances

Type	Criteria
A	Can detonate or deflagrate rapidly, as packaged.
B	Possess explosive properties and which, as packaged, neither detonates nor deflagrates rapidly, but is liable to undergo a thermal explosion in that package.
C	Possess explosive properties when the substance or mixture as packaged cannot detonate or deflagrate rapidly or undergo a thermal explosion.
D	• Detonates partially, does not deflagrate rapidly and shows no violent effect when heated under confinement; or • Does not detonate at all, deflagrates slowly and shows no violent effect when heated under confinement; or • Does not detonate or deflagrate at all and shows a medium effect when heated under confinement.
E	Neither detonates nor deflagrates at all and shows low or no effect when heated under confinement.
F	Neither detonates in the cavitated bubble state nor deflagrates at all and shows only a low or no effect when heated under confinement as well as low or no explosive power.
G	Neither detonates in the cavitated state nor deflagrates at all and shows no effect when heated under confinement nor any explosive power, provided that it is thermally stable (self-accelerating decomposition temperature is 60°C to 75°C for a 50 kg package), and, for liquid mixtures, a diluent having a boiling point not less than 150°C is used for desensitization.

Pyrophorics

3.1.9 Pyrophoric Liquids

A pyrophoric liquid is a liquid which, even in small quantities, is liable to ignite within five minutes after coming into contact with air. Substances and mixtures of this hazard class are assigned to a single hazard category on the basis of the outcome of the UN Test N.3 (*UN Manual of Tests and Criteria)*.

3.1.10 Pyrophoric Solids

A pyrophoric solid is a solid which, even in small quantities, is liable to ignite within five minutes after coming into contact with air. Substances and mixtures of this hazard class are assigned to a single hazard category on the basis of the outcome of the UN Test N.2 (*UN Manual of Tests and Criteria)*.

3.1.11 Self-Heating Substances

A self-heating substance is a solid or liquid, other than a pyrophoric substance, which, by reaction with air and without energy supply, is liable to self-heat. This endpoint differs from a pyrophoric substance in that it will ignite only when in large amounts (kilograms) and after long periods of time (hours or days). Substances and mixtures of this hazard class are assigned to one of two hazard categories on the basis of the outcome of the UN Test N.4 (*UN Manual of Tests and Criteria)*.

3.1.12 Substances which on Contact with Water Emit Flammable Gases

Substances that, in contact with water, emit flammable gases are solids or liquids which, by interaction with water, are liable to become spontaneously flammable or to give off flammable gases in dangerous quantities. Substances and mixtures of this hazard class are assigned to one of three hazard categories on the basis of test results (UN Test N.5 *UN Manual of Tests and Criteria*) which measure gas evolution and speed of evolution.

Table 3.6 Substances which on Contact with Water Emit Flammable Gases

Category	Criteria
1	≥10 L/kg/1 minute
2	≥20 L/kg/1 hour + < 10 L/kg/1 min
3	≥1 L/kg/1 hour + < 20 L/kg/1 hour
Not classified	< 1 L/kg/1 hour

3.1.13 Oxidizing Liquids

An oxidizing liquid is a liquid which, while in itself not necessarily combustible, may, generally by yielding oxygen, cause or contribute to the combustion of other material. Substances and mixtures of this hazard class are assigned to one of three hazard categories on the basis of test results (UN Test O.2 *UN Manual of Tests and Criteria*) which measure ignition or pressure rise time compared to defined mixtures.

3.1.14 Oxidizing Solids

An oxidizing solid is a solid which, while in itself not necessarily combustible, may, generally by yielding oxygen, cause or contribute to the combustion of other material. Substances and mixtures of this hazard class are assigned to one of three hazard categories on the basis of test results (UN Test O.1 *UN Manual of Tests and Criteria*) which measure mean burning time and are compared to defined mixtures. Currently, several workplace hazard communication systems cover oxidizers (solids, liquids, gases) as a class of chemicals.

3.1.15 Organic Peroxides

An organic peroxide is an organic liquid or solid which contains the bivalent -0-0- structure and may be considered a derivative of hydrogen peroxide, where one or both of the hydrogen atoms have been replaced by organic radicals. The term also includes organic peroxide formulations (mixtures). Such substances and mixtures may:

- be liable to explosive decomposition;
- burn rapidly;
- be sensitive to impact or friction;
- react dangerously with other substances.

Substances and mixtures of this hazard class are assigned to one of seven 'Types', A to G, on the basis of the outcome of the UN Test Series A to H (*UN Manual of Tests and Criteria*). Currently, only the transport sector uses seven categories for organic peroxides.

Table 3.7 Organic Peroxides

Type	Criteria
A	Can detonate or deflagrate rapidly, as packaged.
B	Possess explosive properties and which, as packaged, neither detonates nor deflagrates rapidly, but is liable to undergo a thermal explosion in that package.
C	Posses explosive properties when the substance or mixture as packaged cannot detonate or deflagrate rapidly or undergo a thermal explosion.
D	• Detonates partially, does not deflagrate rapidly and shows no violent effect when heated under confinement; or • Does not detonate at all, deflagrates slowly and shows no violent effect when heated under confinement; or • Does not detonate or deflagrate at all and shows a medium effect when heated under confinement.
E	Neither detonates nor deflagrates at all and shows low or no effect when heated under confinement.
F	Neither detonates in the cavitated bubble state nor deflagrates at all and shows only a low or no effect when heated under confinement as well as low or no explosive power.
G	Neither detonates in the cavitated state nor deflagrates at all and shows no effect when heated under confinement nor any explosive power, provided that it is thermally stable (self-accelerating decomposition temperature is 60°C to 75°C for a 50 kg package), and, for liquid mixtures, a diluent having a boiling point not less than 150°C is used for desensitization.

3.1.16 Substances Corrosive to Metal

A substance or a mixture that by chemical action will materially damage, or even destroy, metals is termed 'corrosive to metal'. These substances or mixtures are classified in a single hazard category on the basis of tests (Steel: ISO 9328 (II): 1991 - Steel type P235; Aluminum: ASTM G31-72 (1990) – non-clad types 7075-T6 or AZ5GU-T66). The GHS criteria are a corrosion rate on steel or aluminum surfaces exceeding 6.25 mm per year at a test temperature of 55°C.

The concern in this case is the protection of metal equipment or installations in case of leakage (e.g., plane, ship, tank), not material compatibility between the container/tank and the product. This hazard is not currently covered in all systems.

3.2 What are the GHS Health and Environmental Hazards?

The GHS health and environmental hazard criteria represent a harmonized approach for existing classification systems (see Figure 3.3). The work at the OECD to develop the GHS criteria included:

- A thorough analysis of existing classification systems, including the scientific basis for a system and its criteria, its rationale and an explanation of the mode of use;
- A proposal for harmonized criteria for each category. For some categories the harmonized approach was easy to develop because the existing systems had similar approaches. In cases where the approach was different, a compromise consensus proposal was developed.
- Health and environmental criteria were established for substances and mixtures.

Figure 3.3

The GHS Health and Environmental Endpoints

The following paragraphs briefly describe the GHS health and environmental endpoints. The criteria for classifying substances are presented first. Then the GHS approach to classifying mixtures is briefly discussed. It is recommended that the person responsible for GHS implementation consult the GHS Document or "Purple Book" for more complete information.

3.2.1 Acute Toxicity

Five GHS categories have been included in the GHS Acute Toxicity scheme from which the appropriate elements relevant to transport, consumer, worker and environment protection can be selected. Substances are assigned to one of the five toxicity categories on the basis of LD_{50} (oral, dermal) or LC_{50} (inhalation). The LC_{50} values are based on 4-hour tests in animals. The GHS provides guidance on converting 1-hour inhalation test results to a 4-hour equivalent. The five categories are shown in the Table 3.8 Acute Toxicity.

Table 3.8 Acute Toxicity

Acute toxicity	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Category 5
Oral (mg/kg)	≤ 5	> 5 ≤ 50	> 50 ≤ 300	> 300 ≤ 2000	Criteria: • Anticipated oral LD50 between 2000 and 5000 mg/kg; • Indication of significant effect in humans;* • Any mortality at class 4;* • Significant clinical signs at class 4;* • Indications from other studies.* *If assignment to a more hazardous class is not warranted.
Dermal (mg/kg)	≤ 50	> 50 ≤ 200	> 200 ≤ 1000	> 1000 ≤ 2000	
Gases (ppm)	≤ 100	> 100 ≤ 500	> 500 ≤ 2500	> 2500 ≤ 5000	
Vapors (mg/l)	≤ 0.5	> 0.5 ≤ 2.0	> 2.0 ≤ 10	> 10 ≤ 20	
Dust & mists (mg/l)	≤ 0.05	> 0.05 ≤ 0.5	> 0.5 ≤ 1.0	> 1.0 ≤ 5	

Category 1, the most severe toxicity category, has cut-off values currently used primarily by the transport sector for classification for packing groups. Some Competent Authorities may consider combining Acute Categories 1 and 2. Category 5 is for chemicals which are of relatively low acute toxicity but which, under certain circumstances, may pose a hazard to vulnerable populations. Criteria other than LD50/LC50 data are provided to identify substances in Category 5 unless a more hazardous class is warranted.

3.2.2 Skin Corrosion

Skin corrosion means the production of irreversible damage to the skin following the application of a test substance for up to 4 hours. Substances and mixtures in this hazard class are assigned to a single harmonized corrosion category. For Competent Authorities, such as transport packing groups, needing more than one designation for corrosivity, up to three subcategories are provided within the corrosive category. See the Skin Corrosion/Irritation Table 3.9.

Several factors should be considered in determining the corrosion potential before testing is initiated:

- Human experience showing irreversible damage to the skin;
- Structure/activity or structure property relationship to a substance or mixture already classified as corrosive;
- pH extremes of ≤ 2 and ≥ 11.5 including acid/alkali reserve capacity.

Table 3.9 Skin Corrosion/Irritation

Skin Corrosion Category 1			Skin Irritation Category 2	Mild Skin Irritation Category 3
Destruction of dermal tissue: visible necrosis in at least one animal			Reversible adverse effects in dermal tissue Draize score: ≥ 2.3 < 4.0 or persistent inflammation	Reversible adverse effects in dermal tissue Draize score: ≥ 1.5 < 2.3
Subcategory 1A Exposure < 3 min. Observation < 1 hr,	Subcategory 1B Exposure < 1 hr. Observation < 14 days	Subcategory 1C Exposure < 4 hrs. Observation < 14 days		

3.2.3 Skin Irritation

Skin irritation means the production of reversible damage to the skin following the application of a test substance for up to 4 hours. Substances and mixtures in this hazard class are assigned to a single irritant category. For those authorities, such as pesticide regulators, wanting more than one designation for skin irritation, an additional mild irritant category is provided. See the Skin Corrosion/Irritation Table 3.9.

Several factors should be considered in determining the irritation potential before testing is initiated:

- Human experience or data showing reversible damage to the skin following exposure of up to 4 hours;
- Structure/activity or structure property relationship to a substance or mixture already classified as an irritant.

3.2.4 Eye Effects

Several factors should be considered in determining the *serious eye damage* or *eye irritation* potential before testing is initiated:

- Accumulated human and animal experience;
- Structure/activity or structure property relationship to a substance or mixture already classified;
- pH extremes like ≤ 2 and ≥ 11.5 that may produce serious eye damage.

Serious eye damage means the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the front surface of the eye, which is not fully reversible within 21 days of application. Substances and mixtures in this hazard class are assigned to a single harmonized category.

Table 3.10 Eye Effects

Category 1 Serious eye damage	Category 2 Eye Irritation	
Irreversible damage 21 days after exposure Draize score: Corneal opacity ≥ 3 Iritis > 1.5	Reversible adverse effects on cornea, iris, conjunctiva Draize score: Corneal opacity ≥ 1 Iritis ≥ 1 Redness ≥ 2 Chemosis ≥ 2	
	Irritant Subcategory 2A Reversible in 21 days	**Mild Irritant** Subcategory 2B Reversible in 7 days

Eye irritation means changes in the eye following the application of a test substance to the front surface of the eye, which are fully reversible within 21 days of application. Substances and mixtures in this hazard class are assigned to a single harmonized hazard category. For authorities, such as pesticide regulators, wanting more than one designation for eye irritation, one of two subcategories can be selected, depending on whether the effects are reversible in 21 or 7 days.

3.2.5 Sensitization

Respiratory sensitizer means a substance that induces hypersensitivity of the airways following inhalation of the substance. Substances and mixtures in this hazard class are assigned to one hazard category.

Skin sensitizer means a substance that will induce an allergic response following skin contact. The definition for "skin sensitizer" is equivalent to "contact sensitizer". Substances and mixtures in this hazard class are assigned to one hazard category. Consideration should be given to classifying substances which cause immunological contact urticaria (an allergic disorder) as contact sensitizers.

3.2.6 Germ Cell Mutagenicity

Mutagen means an agent giving rise to an increased occurrence of mutations in populations of cells and/or organisms. Substances and mixtures in this hazard class are assigned to one of two hazard categories. Category 1 has two subcategories. See the Germ Cell Mutagenicity (Table 3.11) below.

Table 3.11 Germ Cell Mutagenicity

<table>
<tr><th colspan="2">Category 1
Known / Presumed</th><th>Category 2
Suspected / Possible</th></tr>
<tr><td colspan="2">Known to produce heritable mutations in human germ cells</td><td rowspan="2">• May induce heritable mutations in human germ cells
• Positive evidence from tests in mammals and somatic cell tests
• In vivo somatic genotoxicity supported by in vitro mutagenicity</td></tr>
<tr><td>Subcategory 1A
Positive evidence from epidemiological studies</td><td>Subcategory 1B
Positive results in:
• In vivo heritable germ cell tests in mammals
• Human germ cell tests
• In vivo somatic mutagenicity tests, combined with some evidence of germ cell mutagenicity</td></tr>
</table>

3.2.7 Carcinogenicity

Carcinogen means a chemical substance or a mixture of chemical substances which induce cancer or increase its incidence. Substances and mixtures in this hazard class are assigned to one of two hazard categories. Category 1 has two subcategories. The Carcinogenicity Guidance Section in the GHS Document includes comments about IARC.

Table 3.12 Carcinogenicity

Category 1 **Known or Presumed Carcinogen**		**Category 2** **Suspected Carcinogen**
Subcategory 1A **Known Human Carcinogen** Based on human evidence	Subcategory 1B **Presumed Human Carcinogen** Based on demonstrated animal carcinogenicity	Limited evidence of human or animal carcinogenicity

3.2.8 Reproductive Toxicity

Reproductive toxicity includeds adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in offspring. Substances and mixtures with reproductive and/or developmental effects are assigned to one of two hazard categories, 'known or presumed' and 'suspected'. Category 1 has two subcategories for reproductive and developmental effects. Materials which cause concern for the health of breastfed children have a separate category, Effects on or Via Lactation.

Table 3.13 Reproductive Toxicity

Category 1		**Category 2** **Suspected**	**Additional Category**
Known or presumed to cause effects on human reproduction or on development		Human or animal evidence possibly with other information	Effects on or via lactation
Category 1A **Known** Based on human evidence	**Category 1B** **Presumed** Based on experimental animals		

3.2.9 Target Organ Systemic Toxicity (TOST): Single Exposure & Repeated Exposure

The GHS distinguishes between single and repeat exposure for Target Organ Effects. Some existing systems distinguish between single and repeat exposure for these effects and some do not. All significant health effects, not otherwise specifically included in the GHS, that can impair function, both reversible and irreversible, immediate and/or delayed are included in the non-lethal target organ/systemic toxicity class (TOST). Narcotic effects and respiratory tract irritation are considered to be target organ systemic effects following a single exposure.

Substances and mixtures of the single exposure target organ toxicity hazard class are assigned to one of three hazard categories in Table 3.14.

Table 3.14 TOST: Single Exposure

Category 1	**Category 2**	**Category 3**
Significant toxicity in humans - Reliable, good quality human case studies or epidemiological studies Presumed significant toxicity in humans • Animal studies with significant and/or severe toxic effects relevant to humans at generally low exposure (guidance)	Presumed to be harmful to human health - Animal studies with significant toxic effects relevant to humans at generally moderate exposure (guidance) - Human evidence in exceptional cases	Transient target organ effects - Narcotic effects - Respiratory tract irritation

Substances and mixtures of the repeated exposure target organ toxicity hazard class are assigned to one of two hazard categories in Table 3.15.

Table 3.15 TOST: Repeated Exposure

Category 1	Category 2
Significant toxicity in humans - Reliable, good quality human case studies or epidemiological studies Presumed significant toxicity in humans - Animal studies with significant and/or severe toxic effects relevant to humans at generally low exposure (guidance)	Presumed to be harmful to human health - Animal studies with significant toxic effects relevant to humans at generally moderate exposure (guidance) - Human evidence in exceptional cases

In order to help reach a decision about whether a substance should be classified or not, and to what degree it would be classified (Category 1 vs. Category 2), dose/concentration 'guidance values' are provided in the GHS. The guidance values and ranges for single and repeated doses are intended only for guidance purposes. This means that they are to be used as part of the weight of evidence approach, and to assist with decisions about classification. They are not intended as strict demarcation values. The guidance value for repeated dose effects refer to effects seen in a standard 90-day toxicity study conducted in rats. They can be used as a basis to extrapolate equivalent guidance values for toxicity studies of greater or lesser duration.

3.2.10 Aspiration Hazard

Aspiration toxicity includes severe acute effects such as chemical pneumonia, varying degrees of pulmonary injury or death following aspiration. Aspiration is the entry of a liquid or solid directly through the oral or nasal cavity, or indirectly from vomiting, into the trachea and lower respiratory system. Some hydrocarbons (petroleum distillates) and certain chlorinated hydrocarbons have been shown to pose an aspiration hazard in humans. Primary alcohols, and ketones have been shown to pose an aspiration hazard only in animal studies.

Table 3.16 Aspiration Toxicity

Category 1: Known (regarded) human	**Category 2:** Presumed human
- human evidence - hydrocarbons with kinematic viscosity ≤ 20.5 mm^2/s at 40° C.	- Based on animal studies - surface tension, water solubility, boiling point - kinematic viscosity ≤ 14 mm2/s at 40° C & not Category 1

Substances and mixtures of this hazard class are assigned to one of two hazard categories this hazard class on the basis of viscosity.

3.3 Environmental Hazards

3.3.1 Hazardous to the Aquatic Environment

The harmonized criteria are considered suitable for packaged goods in both supply and use in multi-modal transport schemes. Elements of it may be used for bulk land transport and bulk marine transport under MARPOL (International Convention for the Prevention of Pollution from Ships) insofar as this uses aquatic toxicity. Two Guidance Documents (Annexes 8 and 9 of the GHS Document) cover issues such as data interpretation and the application of the criteria to special substances. Considering the complexity of this endpoint and the breadth of the application, the Guidance Annexes are important in the application of the harmonized criteria.

3.3.1.1 Acute Aquatic Toxicity

Acute aquatic toxicity means the intrinsic property of a material to cause injury to an aquatic organism in a short-term exposure. Substances and mixtures of this hazard class are assigned to one of three toxicity categories on the basis of acute toxicity data: LC_{50} (fish) or EC_{50} (crustacea) or ErC_{50} (for algae or other aquatic plants). In some regulatory systems these acute toxicity categories may be subdivided or extended for certain sectors.

3.3.1.2 Chronic Aquatic Toxicity

Chronic aquatic toxicity means the potential or actual properties of a material to cause adverse effects to aquatic organisms during exposures that are determined in relation to the lifecycle of the organism. Substances and mixtures in this hazard class are assigned to one of four toxicity categories on the basis of acute data *and* environmental fate data: LC_{50} (fish) or EC_{50} (crustacea) or ErC_{50} (for algae or other aquatic plants) *and* degradation/bioaccumulation.

While experimentally derived test data are preferred, where no experimental data are available, validated Quantitative Structure Activity Relationships (QSARs) for aquatic toxicity and log KOW may be used in the classification process. The log K_{OW} is a surrogate for a measured Bioconcentration Factor (BCF), where such a measured BCF value would always take precedence.

Chronic Category IV is considered a "safety net" classification for use when the available data do not allow classification under the formal criteria, but there are some grounds for concern.

Table 3.17 Acute & Chronic Aquatic Toxicity

Acute Cat. I Acute toxicity ≤1.00 mg/l	**Acute Cat. II** Acute toxicity > 1.00 but ≤ 10.0 mg/l	**Acute Cat. III** Acute toxicity > 10.0 but < 100 mg/l	
Chronic Cat. I Acute toxicity ≤ 1.00 mg/l and lack of rapid degradability and log K_{ow} ≥ 4 unless BCF < 500	**Chronic Cat. II** Acute toxicity > 1.00 but ≤ 10.0 mg/l and lack of rapid degradability and log K_{ow} ≥ 4 unless BCF < 500 and unless chronic toxicity > 1 mg/l	**Chronic Cat. III** Acute toxicity > 10.0 but ≤ 100.0 mg/l and lack of rapid degradability and log K_{ow} ≥ 4 unless BCF < 500 and unless chronic toxicity > 1 mg/l	**Chronic Cat. IV** Acute toxicity > 100 mg/l and lack of rapid degradability and log K_{ow} ≥ 4 unless BCF < 500 and unless chronic toxicity > 1 mg/l

3.4 What is the GHS approach to classifying mixtures?

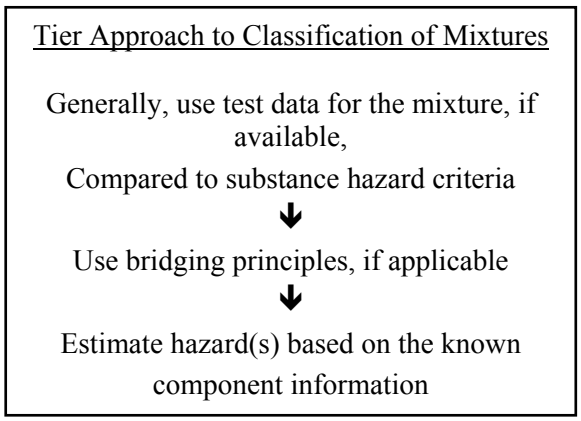
For consistency and understanding the provisions for classifying mixtures, the GHS defines certain terms. These working definitions are for the purpose of evaluating or determining the hazards of a product for classification and labeling.

> ***Substance*:** Chemical elements and their compounds in the natural state or obtained by any production process, including any *additive* necessary to preserve the stability of the product and any *impurities* deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.
>
> ***Mixture:*** Mixtures or solutions composed of two or more substances in which they do not react.
>
> ***Alloy:*** An alloy is a metallic material, homogeneous on a macroscopic scale, consisting of two or more elements so combined that they cannot be readily separated by mechanical means. Alloys are considered to be mixtures for the purpose of classification under the GHS.

Where impurities, additives or individual constituents of a substance or mixture have been identified and are themselves classified, they should be taken into account during classification if they exceed the cutoff value/concentration limit for a given hazard class.

As mentioned previously, the GHS physical hazard criteria apply to mixtures. It is assumed that mixtures will be tested for physical hazards. Each health and environmental endpoint chapter in the GHS contains specific criteria for classifying mixtures as well as substances. The GHS Document or "Purple Book" should be consulted for complete information on classifying mixtures.

Figure 3-4



The process established for classifying a mixture allows the use of (a) available data for the mixture itself and/or (b) similar mixtures and/or (c) data for ingredients of the mixture. The GHS approach to the classification of mixtures for health and environmental hazards is tiered, and is dependent upon the amount of information available for the mixture itself and for its components. The process for the classification of mixtures is based on the following steps:

(1) Where test data are available for the mixture itself, the classification of the mixture will be based on that data (See exception for carcinogens, mutagens & reproductive toxins in the GHS Document); (2) Where test data are not available for the mixture itself, then the appropriate bridging principles (as described below) in the specific chapter should be used; (3) If (i) test data are not available for the mixture itself, and (ii) the bridging principles cannot be applied, then use the calculation or cutoff values described in the specific endpoint to classify the mixture.

3.5 What are bridging principles?

Bridging principles are an important concept in the GHS for classifying untested mixtures. When a mixture has not been tested, but there are sufficient data on the components and/or similar tested mixtures, these data can be used in accordance with the following bridging principles:

- **Dilution**: If a mixture is diluted with a diluent that has an equivalent or lower toxicity, then the hazards of the new mixture are assumed to be equivalent to the original.
- **Batching**: If a batch of a complex substance is produced under a controlled process, then the hazards of the new batch are assumed to be equivalent to the previous batches.
- **Concentration of Highly Toxic Mixtures**: If a mixture is severely hazardous, then a concentrated mixture is also assumed to be severely hazardous
- **Interpolation within One Toxic Category: Mixtures** having component concentrations within a range where the hazards are known are assumed to have those known hazards.
- **Substantially Similar Mixtures**: Slight changes in the concentrations of components are not expected to change the hazards of a mixture and substitutions involving toxicologically similar components are not expected to change the hazards of a mixture
- **Aerosols**: An aerosol form of a mixture is assumed to have the same hazards as the tested, non-aerosolized form of the mixture unless the propellant affects the hazards upon spraying.

All bridging principles do not apply to every health and environmental endpoint. Consult each endpoint to determine which bridging principles apply.

When the bridging principles do not apply or ca not be used, the health and environmental hazards of mixtures are estimated based on component information. In the GHS, the methodology used to estimate these hazards varies by endpoint. The GHS Document or "Purple Book" should be consulted for more complete information on classifying mixtures. Figure 3.5 summarizes the GHS mixtures approach for the various health and environmental endpoints.

3.6 What testing is required?

The GHS itself does not include requirements for testing substances or mixtures. Therefore, there is no requirement under the GHS to generate test data for any hazard class. Some parts of regulatory systems may require data to be generated (e.g., for pesticides), but these requirements are not related specifically to the GHS. The GHS criteria for determining health and environmental hazards are test method neutral, allowing different approaches as long as they are scientifically sound and validated according to international procedures and criteria already

referred to in existing systems. Test data already generated for the classification of chemicals under existing systems should be accepted when classifying these chemicals under the GHS, thereby avoiding duplicative testing and the unnecessary use of test animals. The GHS physical hazard criteria are linked to specific test methods. It is assumed that mixtures will be tested for physical hazards.

Figure 3.5 GHS Mixtures

Hazard Endpoint	Classification Approach	Bridging Principles	Comments
Acute toxicity	Acute Toxicity Estimate (ATE): 2 formulas	All	Conversion values, relevant components usually at ≥ 1%
Serious Eye Damage & Eye Irritation	Mostly additivity approach, sometimes cutoffs	All	Relevant components usually at ≥ 1%, exceptions for certain chemical classes
Skin corrosion & Skin Irritation	Mostly additivity approach, sometimes cutoffs	All	Relevant components usually at ≥ 1%, exceptions for certain chemical classes
Skin Sensitization	Cutoffs with CA options	Dilution, Batching, Substantially similar mixtures, Aerosols	
Respiratory Sensitization	Cutoffs with CA options	Dilution, Batching, Substantially similar mixtures, Aerosols	
Germ Cell Mutagenicity	Cutoffs	Dilution, Batching, Substantially similar mixtures	Mixture test data only case-by case
Carcinogenicity	Cutoffs with CA options	Dilution, Batching, Substantially similar mixtures	Mixture test data only case-by-case
Reproductive Toxicity	Cutoffs with CA options	Dilution, Batching, Substantially similar mixtures	Mixture test data only case-by-case
Target Organ Systemic Toxicity	Cutoffs with CA options	All	
Aspiration Toxicity	Cutoffs	Dilution, Batching, Concentration of highly toxic mixtures, Interpolation within one toxicity category, Substantially similar mixtures	

Hazard Endpoint	Classification Approach	Bridging Principles	Comments
Hazardous to the Aquatic Environment	Additivity Formula (Acute only); Summation Method (Acute or Chronic); Combination of Additivity Formula & Summation Method	Dilution, Batching, Concentration of highly toxic mixtures, Interpolation within one toxicity category, Substantially similar mixtures	Relevant components usually at ≥ 1%, Mixture test data only case-by-case for chronic

4. HAZARD COMMUNICATION

Section 3, explained that classification is the starting point for the GHS. Once a chemical has been classified, the hazard(s) must be communicated to target audiences. As in existing systems, labels and Safety Data Sheets are the main tools for chemical hazard communication. They identify the hazardous properties of chemicals that may pose a health, physical or environmental hazard during normal handling or use. The goal of the GHS is to identify the intrinsic hazards found in chemical substances and mixtures, and to convey information about these hazards.

The international mandate for the GHS included the development of a harmonized hazard communication system, including labeling, Safety Data Sheets and easily understandable symbols, based on the classification criteria developed for the GHS.

4.1 What factors influenced development of the GHS communication tools?

Early in the process of developing the GHS communication tools, several significant issues were recognized. One of the most important was comprehensibility of the information provided. After all, the aim of the system is to present hazard information in a manner that the intended audience can easily understand and that will thus minimize the possibility of adverse effects resulting from exposure. The GHS identifies some guiding principles to assist in this process:

- Information should be conveyed in more than one way, e.g., text and symbols;
- The comprehensibility of the components of the system should take account of existing studies and literature as well as any evidence gained from testing;
- The phrases used to indicate degree (severity) of hazard should be consistent across the health, physical and environmental hazards.

Comprehensibility is challenging for a single culture and language. Global harmonization has numerous complexities. Some factors that affected the work include:

- Different philosophies in existing systems on how and what should be communicated;
- Language differences around the world;
- Ability to translate phrases meaningfully;
- Ability to understand and appropriately respond to symbols/pictograms.

These factors were considered in developing the GHS communication tools. The GHS Purple Book includes a comprehensibility-testing instrument in Annex 6.

4.2 Labels

4.2.1 What does a label look like?

Existing systems have labels that look different for the same product. We know that this leads to worker confusion, consumer uncertainty and the need for additional resources to maintain different systems. In the U.S. as well as in other countries, chemical products are regulated by sector/target audience. Different agencies regulate the workplace, consumers, agricultural chemicals and transport. Labels for these sectors/target audiences vary both in the U.S. and globally.

In order to understand the value of the GHS and its benefits to all stakeholders, it is instructive to look at the different labels for one fictional product. In the U.S. the product, ToxiFlam, which has a flash point of 120°F and has an oral LD50 of 275 mg/kg, has different labels for different sectors/target audiences. Label examples as seen in the U.S.A. are shown first, followed by international examples.

4.2.2 USA Examples:

Workplace and Workers

In the U.S., regulatory requirements for workplace labels are 'performance oriented'. This results at a minimum in a straightforward label that has a product identity, hazard statement and supplier identification (Figure 4.1). Some products can also have additional labeling requirements depending on their end use.

Figure 4.1

ToxiFlam
TOXIC
COMBUSTIBLE LIQUID AND VAPOR

My Company, My Street, MyTown NJ 00000
Tel: 444 999 9999

However, many companies follow the voluntary ANSI Z129.1 Precautionary Labeling Standard for workplace labeling and often use it also for labeling consumer products. The American National Standards Institute (ANSI) standard includes several label elements that are core to the GHS as well as other helpful elements to assist users in safe handling (Figure 4.2).

Figure 4.2

ToxiFlam (Contains XYZ)

WARNING ! HARMFUL IF SWALLOWED, FLAMMABLE LIQUID AND VAPOR

Do not taste or swallow. Do not take internally. Wash thoroughly after handling. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation.

FIRST AID: If swallowed, do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person.
In Case of Fire, use water fog, dry chemical, CO_2, or alcohol foam. Water may be ineffective.
Flash Point = 120°F. Residue vapor may explode or ignite on ignition; do not cut, drill, grind, or weld on or near this container.

See Material Safety Data Sheet for further details regarding safe use of this product.

My Company, My Street, MyTown NJ 00000, Tel: 444 999 9999

Consumer Products and Consumers

In several countries consumer products are regulated separately from workplace chemicals. In the U.S. the CPSC regulates consumer products. Consumer products have required label elements, but only the signal words are specified. The ANSI labeling standard is often used in developing consumer labels.

Figure 4.3

ToxiFlam
(Contains XYZ)
WARNING! TOXIC, COMBUSTIBLE LIQUID AND VAPOR

Do not taste or swallow. Do not take internally. Wash thoroughly after handling. Keep away from heat, sparks and flame. Keep container closed. Use only with adequate ventilation.

FIRST AID

If swallowed, do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person.

Keep out of the reach of children.

My Company, My Street, MyTown NJ 00000, Tel: 444 999 9999

Transport and Emergency Responders

For hazardous products being transported, outer containers have required label elements, product identifier and hazard symbols. Transportation requirements are in addition to workplace or end use label requirements.

Figure 4.4

Flammable liquids, toxic, n.o.s. (contains XYZ)
UN 1992

MyCompany, MyStreet NJ 00000

Agricultural Chemicals and Pesticides

In many systems, agricultural chemicals often have special label requirements. In the U.S. the EPA is the agency covering these chemicals. A pesticide product with the same hazards as ToxiFlam would have a label developed using FIFRA requirements. FIFRA has requirements for product identity, chemical identity, signal word, hazard statements, and precautionary measures including first aid.

Figure 4.5

ToxiFlam
Active/ Inerts: Contains XYZ %

KEEP OUT OF THE REACH OF CHILDREN

PRECAUTIONARY STATEMENTS - HAZARDS TO HUMANS AND DOMESTIC ANIMALS:
WARNING: May be fatal if swallowed. Wash thoroughly with soap and water after handling and before eating, drinking or using tobacco .
PHYSICAL AND CHEMICAL HAZARDS: Combustible. Do not use or store near heat or open flame.
FIRST AID:
If swallowed -Call a poison control center or doctor immediately for treatment advice.
-Have person sip a glass of water if able to swallow.
-Do not induce vomiting unless told to do so by a poison control center or doctor.
-Do not give anything by mouth to an unconscious person.

My Company, My Street, MyTown AZ 00000, Tel: 444 999 9999
EPA Est. No. 5840-AZ-1 EPA Reg. No. 3120-280

4.2.3 International Examples

All the previous examples are specific to the U.S. Many companies do business globally. So in addition to the U.S. regulations, these companies would need to comply with the corresponding regulations in the countries to which they export products. Canada and the EU are two existing systems that were considered in the development of the GHS. To illustrate the differences in labeling, it is interesting to examine an EU and Canadian label for ToxiFlam.

European Union Label

Labels in the EU have chemical identity, symbols, and R/S (Risk and Safety) phrases which are hazard statements, precautionary measures and first aid.

Figure 4.6

ToxiFlam (contains XYZ)

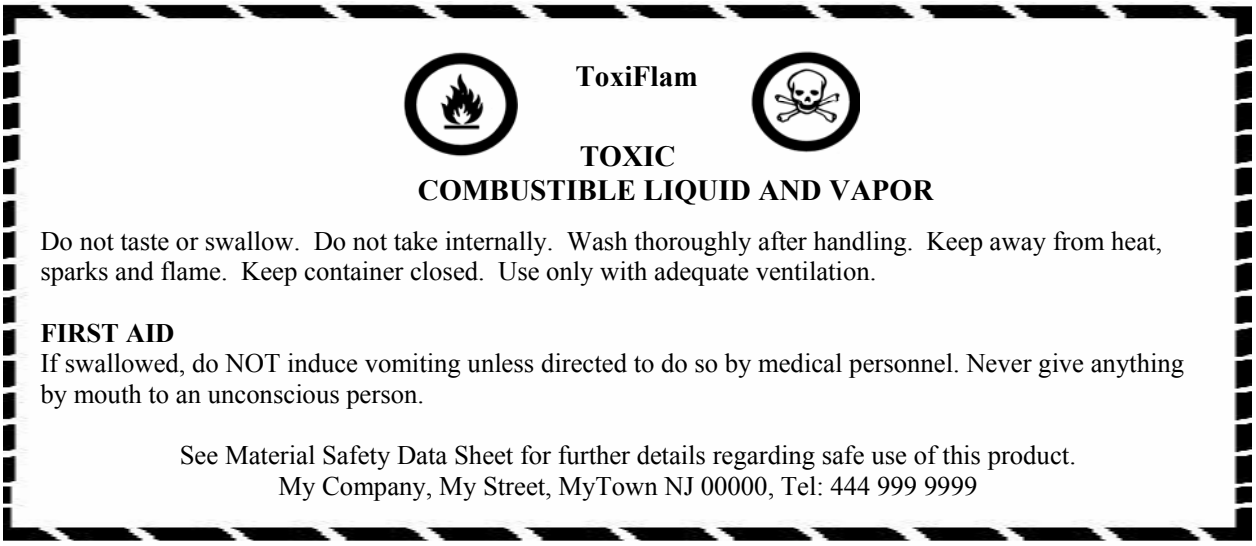
Harmful If Swallowed. (R22)
Flammable. (R10)
Keep away from food, drink and animal feedingstuffs. (S13)
Wear suitable protective clothing. (S36)
If swallowed, seek medical advice immediately and show this Container label. (S46)
In case of fire, use water, fog, CO2, or alcohol foam. (S43)

My Company, My Street, MyTown XX 00000 Tel: 44 22 999 9999

Canadian Workplace Hazardous Materials Identification System (WHMIS) Label

The WHMIS label requires product identifier, hazard symbol, hazard statement, precautionary measures, first aid, MSDS statement and supplier identification. In addition to these common label elements, WHMIS requires a hatched border.

Figure 4.7



4.3 What are the GHS label elements?

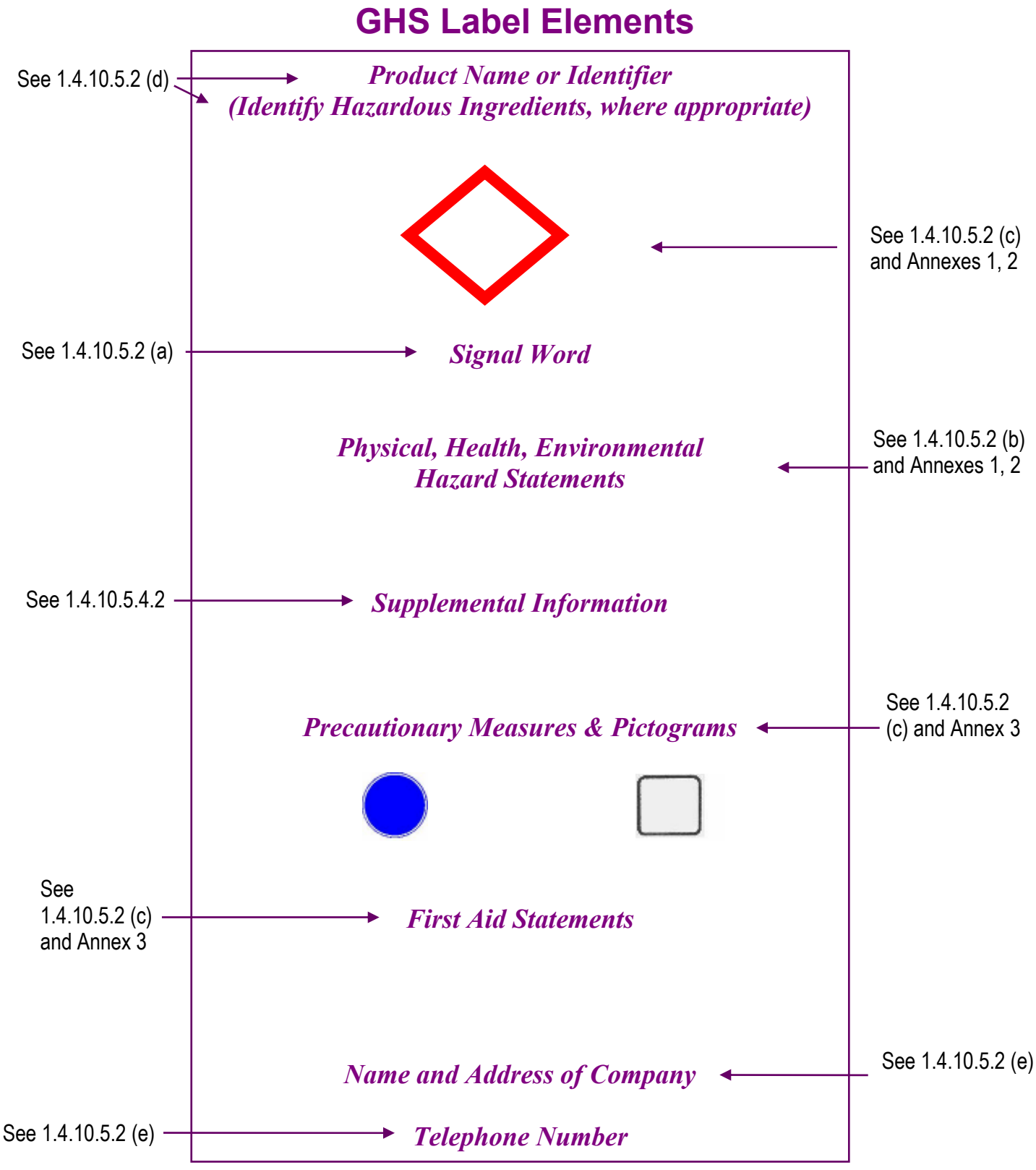
Some GHS label elements have been standardized (identical with no variation) and are directly related to the endpoints and hazard level. Other label elements are harmonized with common definitions and/or principles. See Figure 4.8 for an illustration of the GHS label elements.

The standardized label elements included in the GHS are:

- **Symbols (hazard pictograms)**: Convey health, physical and environmental hazard information, assigned to a GHS hazard class and category.
- **Signal Words:** "Danger" or "Warning" are used to emphasize hazards and indicate the relative level of severity of the hazard, assigned to a GHS hazard class and category**.**
- **Hazard Statements:** Standard phrases assigned to a hazard class and category that describe the nature of the hazard.

The symbols, signal words, and hazard statements have all been standardized and assigned to specific hazard categories and classes, as appropriate. This approach makes it easier for countries to implement the system and should make it easier for companies to comply with regulations based on the GHS. The prescribed symbols, signal words, and hazard statements can be readily selected from Annex 1 of the GHS Purple Book. These standardized elements are not subject to variation, and should appear on the GHS label as indicated in the GHS for each hazard category/class in the system. The use of symbols, signal words or hazard statements other than those that have been assigned to each of the GHS hazards would be contrary to harmonization.

Figure 4.8



The Section numbers refer to the sections in the GHS Document or "Purple Book".

4.3.1 Symbols/Pictograms

The GHS symbols have been incorporated into pictograms for use on the GHS label. Pictograms include the harmonized hazard symbols plus other graphic elements, such as borders, background patterns or colors which are intended to convey specific information. For transport, pictograms (Table 4.10) will have the background, symbol and colors currently used in the UN Recommendations on the Transport of Dangerous Goods, Model Regulations. For other sectors, pictograms (Table 4.9) will have a black symbol on a white background with a red diamond frame. A black frame may be used for shipments within one country. Where a transport pictogram appears, the GHS pictogram for the same hazard should not appear.

4.3.2 Signal Words

The signal word indicates the relative degree of severity a hazard. The signal words used in the GHS are

"Danger" for the more severe hazards, and

"Warning" for the less severe hazards.

Signal words are standardized and assigned to the hazard categories within endpoints. Some lower level hazard categories do not use signal words. Only one signal word corresponding to the class of the most severe hazard should be used on a label.

4.3.3 Hazard Statements

Hazard statements are standardized and assigned phrases that describe the hazard(s) as determined by hazard classification. An appropriate statement for each GHS hazard should be included on the label for products possessing more than one hazard.

The assigned label elements are provided in each hazard chapter of the Purple Book as well as in Annexes 1 & 2. Figure 4-11 illustrates the assignment of standardized GHS label elements for the acute oral toxicity categories.

Figure 4.9

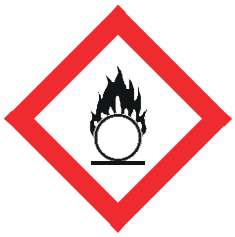
GHS Pictograms and Hazard Classes		
		
• Oxidizers	• Flammables • Self Reactives • Pyrophorics • Self-Heating • Emits Flammable Gas • Organic Peroxides	• Explosives • Self Reactives • Organic Peroxides
		
• Acute toxicity (severe)	• Corrosives	• Gases Under Pressure
		
• Carcinogen • Respiratory Sensitizer • Reproductive Toxicity • Target Organ Toxicity • Mutagenicity • Aspiration Toxicity	• Environmental Toxicity	• Irritant • Dermal Sensitizer • Acute toxicity (harmful) • Narcotic Effects • Respiratory Tract Irritation

Figure 4.10

Transport "Pictograms"		
 **Flammable Liquid Flammable Gas Flammable Aerosol**	 **Flammable solid Self-Reactive Substances**	 **Pyrophorics (Spontaneously Combustible) Self-Heating Substances**
 **Substances, which in contact with water, emit flammable gases (Dangerous When Wet)**	 **Oxidizing Gases Oxidizing Liquids Oxidizing Solids**	 **Explosive Divisions 1.1, 1.2, 1.3**
 **Explosive Division 1.4**	 **Explosive Division 1.5**	 **Explosive Division 1.6**
 **Compressed Gases**	 **Acute Toxicity (Poison): Oral, Dermal, Inhalation**	 **Corrosive**
 **Marine Pollutant**	 **Organic Peroxides**	

Figure 4.11

ACUTE ORAL TOXICITY – Annex 1					
	Category 1	**Category 2**	**Category 3**	**Category 4**	**Category 5**
LD_{50}	**≤ 5 mg/kg**	**> 5 < 50 mg/kg**	**≥ 50 < 300 mg/kg**	**≥ 300 < 2000 mg/kg**	**≥ 2000 < 5000 mg/kg**
Pictogram					**No symbol**
Signal word	**Danger**	**Danger**	**Danger**	**Warning**	**Warning**
Hazard statement	**Fatal if swallowed**	**Fatal if swallowed**	**Toxic if swallowed**	**Harmful if swallowed**	**May be harmful if swallowed**

Other GHS label elements include:

- **Precautionary Statements and Pictograms:** Measures to minimize or prevent adverse effects.
- **Product Identifier (ingredient disclosure):** Name or number used for a hazardous product on a label or in the SDS.
- **Supplier identification:** The name, address and telephone number should be provided on the label.
- **Supplemental information:** non-harmonized information.

4.3.4 Precautionary Statements and Pictograms

Precautionary information supplements the hazard information by briefly providing measures to be taken to minimize or prevent adverse effects from physical, health or environmental hazards. First aid is included in precautionary information. The GHS label should include appropriate precautionary information. Annex 3 of the GHS Purple Book includes precautionary statements and pictograms that can be used on labels.

Annex 3 includes four types of precautionary statements covering: prevention, response in cases of accidental spillage or exposure, storage, and disposal**.** The precautionary statements have been linked to each GHS hazard statement and type of hazard. The goal is to promote consistent use of precautionary statements. Annex 3 is guidance and is expected to be further refined and developed over time.

4.3.5 Product Identifier (Ingredient Disclosure)

A product identifier should be used on a GHS label and it should match the product identifier used on the SDS. Where a substance or mixture is covered by the UN Model Regulations on the Transport of Dangerous Goods, the UN proper shipping name should also be used on the package.

The GHS label for a substance should include the chemical identity of the substance (name as determined by IUPAC, ISO, CAS or technical name). For mixtures/alloys, the label should include the chemical identities of all ingredients that contribute to acute toxicity, skin corrosion or serious eye damage, germ cell mutagenicity, carcinogenicity, reproductive toxicity, skin or respiratory sensitization, or Target Organ Systemic Toxicity (TOST), when these hazards appear on the label. Where a product is supplied exclusively for workplace use, the Competent Authority may give suppliers discretion to include chemical identities on the SDS, in lieu of including them on labels. The Competent Authority rules for confidential business information (CBI) take priority over the rules for product identification.

4.3.6 Supplier Identification

The name, address and telephone number of the manufacturer or supplier of the product should be provided on the label.

4.3.7 Supplemental Information

Supplemental label information is non-harmonized information on the container of a hazardous product that is not required or specified under the GHS. In some cases this information may be required by a Competent Authority or it may be additional information provided at the discretion of the manufacturer/distributor. The GHS provides guidance to ensure that supplemental information does not lead to wide variation in information or undermine the GHS information. Supplemental information may be used to provide further detail that does not contradict or cast doubt on the validity of the standardized hazard information. It also may be used to provide information about hazards not yet incorporated into the GHS. The labeler should have the option of providing supplementary information related to the hazard, such as physical state or route of exposure, with the hazard statement.

4.4 How are multiple hazards handled on labels?

Where a substance or mixture presents more than one GHS hazard, there is a GHS precedence scheme for pictograms and signal words. For substances and mixtures covered by the UN Recommendations on the Transport of Dangerous Goods, Model Regulations, the precedence of symbols for physical hazards should follow the rules of the UN Model Regulations. For health hazards the following principles of precedence apply for symbols:

(a) if the skull and crossbones applies, the exclamation mark should not appear;

(b) if the corrosive symbol applies, the exclamation mark should not appear where it is used for skin or eye irritation;

(c) if the health hazard symbol appears for respiratory sensitization, the exclamation mark should not appear where it is used for skin sensitization or for skin or eye irritation.

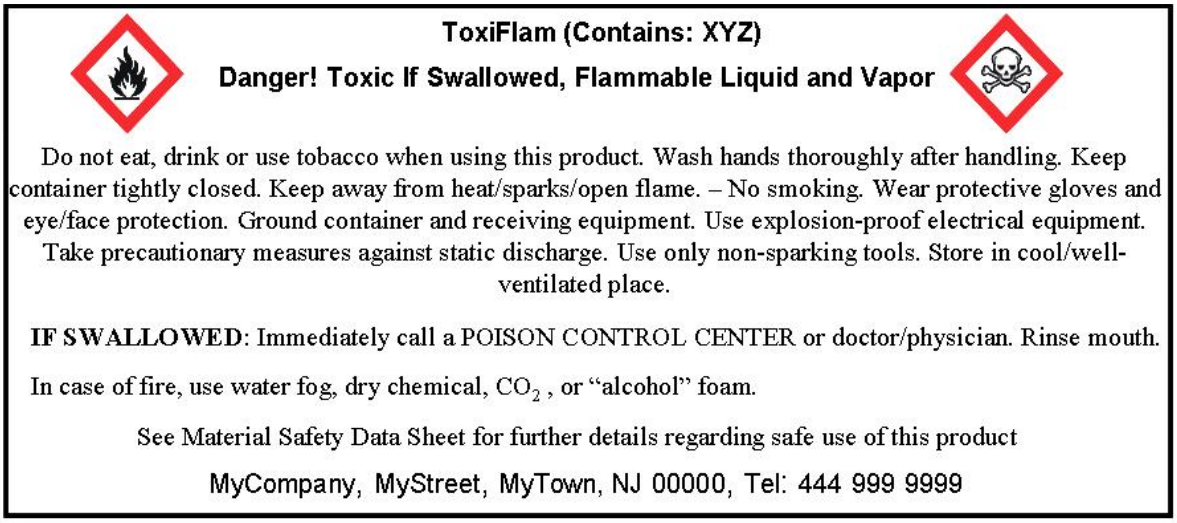
If the signal word 'Danger' applies, the signal word 'Warning' should not appear. All assigned hazard statements should appear on the label. The Competent Authority may choose to specify the order in which they appear.

4.5 Is there a specific GHS label format / layout?

The GHS hazard pictograms, signal word and hazard statements should be located together on the label. The actual label format or layout is not specified in the GHS. National authorities may choose to specify where information should appear on the label or allow supplier discretion.

Figure 4.12 shows an example of a GHS label for the fictional product 'ToxiFlam'. The core GHS label elements are expected to replace the need for the array of different labels shown earlier for ToxiFlam. (Figure 4.8 also illustrates the GHS label elements.)

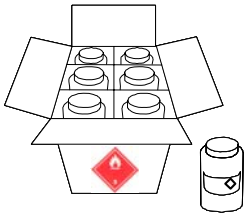
Figure 4.12 Example GHS Inner Container Label (e.g., bottle inside a shipping box)



There has been discussion about the size of GHS pictograms and that a GHS pictogram might be confused with a transport pictogram or "diamond". Transport pictograms (Table 4.10) are different in appearance than the GHS pictograms (Table 4.9). Annex 7 of the Purple Book explains how the GHS pictograms are expected to be proportional to the size of the label text. So that generally the GHS pictograms would be smaller than the transport pictograms.

Several arrangements for GHS labels are also provided in Annex 7 of the Purple Book. Figure 4.13 shows an arrangement for a combination packaging with an outer shipping box and inner bottles. The shipping box has a transportation

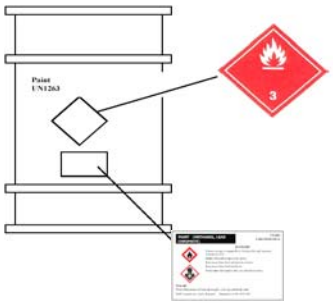
Figure 4.13 Combination Packaging (Outer box with inner bottles)



pictogram. The inner bottles have a GHS label with a GHS pictogram.

For a container such as a 55 gallon drum, the transport required markings and pictograms may be combined with the GHS label elements or presented separately. In Figure 4.14 a label arrangement for a single packaging such as a 55 gallon drum is shown. Pictograms and markings required by the transport regulations as well as GHS label and non-duplicative GHS pictogram are shown on the drum.

Figure 4.14 Combination Packaging (Outer box with inner bottles)



A label merging the transportation requirements and the GHS requirements into one label for the fictional product "ToxiFlam" is shown in Figure 4.15. This combined type label could also be used on a 55 gallon drum.

Figure 4.15 Example GHS Outer Container Label (55 gallon/200 liter drum)

ToxiFlam **Flammable liquids, toxic, n.o.s.**
Danger! Toxic If Swallowed **(contains XYZ)**
Flammable Liquid and Vapor **UN 1992**

Do not eat, drink or use tobacco when using this product. Wash hands thoroughly after handling. Keep container tightly closed. Keep away from heat/sparks/open flame. – No smoking. Wear protective gloves and eye/face protection. Ground container and receiving equipment. Use explosion-proof electrical equipment. Take precautionary measures against static discharge. Use only non-sparking tools. Store in cool/well-ventilated place.

IF SWALLOWED: Immediately call a POISON CONTROL CENTER or doctor/physician. Rinse mouth.

In case of fire, use water fog, dry chemical, CO_2 , or "alcohol" foam.

See Material Safety Data Sheet for further details regarding safe use of this product

MyCompany, MyStreet, MyTown NJ 00000, Tel: 444 999 9999

4.6 What about risk?

Competent Authorities may vary the application of the components of the GHS by the type of product (industrial, pesticide, consumer, etc.) or the stage in the lifecycle (workplace, farm, retail store, etc.). Once a chemical is classified, the likelihood of adverse effects may be considered in deciding what informational or other steps should be taken for a given product or use setting. Annex 5 of the GHS Purple Book includes a discussion of an example of how risk-based labeling could be considered for chronic health effects of consumer products in the consumer use setting.

4.7 Are workplace containers covered in the GHS ?

Products falling within the scope of the GHS will carry the GHS label at the point where they are supplied to the workplace, and that label should be maintained on the supplied container in the workplace. The GHS label or label elements can also be used for workplace containers (e.g., storage tanks). However, the Competent Authority can allow employers to use alternative means of giving workers the same information in a different written or displayed format when such a format is more appropriate to the workplace and communicates the information as effectively as the GHS label. For example, label information could be displayed in the work area, rather than on the individual containers. Some examples of workplace situations where chemicals may be transferred from supplier containers include: containers for laboratory testing, storage vessels, piping or process reaction systems or temporary containers where the chemical will be used by one worker within a short timeframe.

4.8 What is the GHS Safety Data Sheet (SDS)?

The (Material) Safety Data Sheet (SDS) provides comprehensive information for use in workplace chemical management. Employers and workers use the SDS as sources of information about hazards and to obtain advice on safety precautions. The SDS is product related and, usually, is not able to provide information that is specific for any given workplace where the product may be used. However, the SDS information enables the employer to develop an active program of worker protection measures, including training, which is specific to the individual workplace and to consider any measures that may be necessary to protect the environment. Information in a SDS also provides a source of information for other target audiences such as those involved with the transport of dangerous goods, emergency responders, poison centers, those involved with the professional use of pesticides and consumers.

The SDS should contain 16 headings (Figure 4.14). The GHS MSDS headings, sequence and content are similar to the ISO, EU and ANSI MSDS/SDS requirements, except that the order of sections 2 and 3 have been reversed. The SDS should provide a clear description of the data used to identify the hazards. Figure 4.14 and the GHS Purple Book provide the minimum information that is required in each section of the SDS. Examples of draft GHS SDSs are provided in Appendix B of this guidance document.

The revised Purple Book contains guidance on developing a GHS SDS (Annex 4). Other resources for SDSs include:

- ILO Standard under the Recommendation 177 on Safety in the Use of Chemicals at Work,
- International Standard 11014-1 (1994) of the International Standard Organization (ISO) and ISO Safety Data Sheet for Chemical Products 11014-1: 2003 DRAFT,
- American National Standards Institute (ANSI) Standard Z400.1,
- European Union SDS Directive 91/155/-EEC.

Figure 4.14

Minimum information for an SDS

1.	**Identification of the substance or mixture and of the supplier**	• GHS product identifier. • Other means of identification. • Recommended use of the chemical and restrictions on use. • Supplier's details (including name, address, phone number, etc.). • Emergency phone number.
2.	**Hazards identification**	• GHS classification of the substance/mixture and any national or regional information. • GHS label elements, including precautionary statements. (Hazard symbols may be provided as a graphical reproduction of the symbols in black and white or the name of the symbol, e.g., flame, skull and crossbones.) • Other hazards which do not result in classification (e.g., dust explosion hazard) or are not covered by the GHS.
3.	**Composition/information on ingredients**	**<u>Substance</u>** • Chemical identity. • Common name, synonyms, etc. • CAS number, EC number, etc. • Impurities and stabilizing additives which are themselves classified and which contribute to the classification of the substance. **<u>Mixture</u>** • The chemical identity and concentration or concentration ranges of all ingredients which are hazardous within the meaning of the GHS and are present above their cutoff levels. ***NOTE**: For information on ingredients, the competent authority rules for CBI take priority over the rules for product identification.*
4.	**First aid measures**	• Description of necessary measures, subdivided according to the different routes of exposure, i.e., inhalation, skin and eye contact, and ingestion. • Most important symptoms/effects, acute and delayed. • Indication of immediate medical attention and special treatment needed, if necessary.

5.	**Firefighting measures**	• Suitable (and unsuitable) extinguishing media. • Specific hazards arising from the chemical (e.g., nature of any hazardous combustion products). • Special protective equipment and precautions for firefighters.
6.	**Accidental release measures**	• Personal precautions, protective equipment and emergency procedures. • Environmental precautions. • Methods and materials for containment and cleaning up.
7.	**Handling and storage**	• Precautions for safe handling. • Conditions for safe storage, including any incompatibilities.
8.	**Exposure controls/personal protection**.	• Control parameters, e.g., occupational exposure limit values or biological limit values. • Appropriate engineering controls. • Individual protection measures, such as personal protective equipment.
9.	**Physical and chemical properties**	• Appearance (physical state, color, etc.). • Odor. • Odor threshold. • pH. • melting point/freezing point. • initial boiling point and boiling range. • flash point. • evaporation rate. • flammability (solid, gas). • upper/lower flammability or explosive limits. • vapor pressure. • vapor density. • relative density. • solubility(ies). • partition coefficient: n-octanol/water. • autoignition temperature. • decomposition temperature.
10.	**Stability and reactivity**	• Chemical stability. • Possibility of hazardous reactions. • Conditions to avoid (e.g., static discharge, shock or vibration). • Incompatible materials. • Hazardous decomposition products.

11.	**Toxicological information**	Concise but complete and comprehensible description of the various toxicological (health) effects and the available data used to identify those effects, including: • information on the likely routes of exposure (inhalation, ingestion, skin and eye contact); • Symptoms related to the physical, chemical and toxicological characteristics; • Delayed and immediate effects and also chronic effects from short- and long-term exposure; • Numerical measures of toxicity (such as acute toxicity estimates).
12.	**Ecological information**	• Ecotoxicity (aquatic and terrestrial, where available). • Persistence and degradability. • Bioaccumulative potential. • Mobility in soil. • Other adverse effects.
13.	**Disposal considerations**	• Description of waste residues and information on their safe handling and methods of disposal, including the disposal of any contaminated packaging.
14.	**Transport information**	• UN Number. • UN Proper shipping name. • Transport Hazard class(es). • Packing group, if applicable. • Marine pollutant (Yes/No). • Special precautions which a user needs to be aware of or needs to comply with in connection with transport or conveyance either within or outside their premises.
15.	**Regulatory information**	• Safety, health and environmental regulations specific for the product in question.
16.	**Other information including information on preparation and revision of the SDS**	

4.9 What is the difference between the GHS SDS and existing MSDSs/SDSs?

SDSs are in use globally. So it is useful to have an understanding of the similarities and differences in the existing MSDS/SDS content and format and the GHS SDS content and format. A table comparing MSDS/SDS content/format is provided in Appendix A of this guidance document.

4.10 When should SDSs and labels be updated?

All hazard communication systems should specify a means of responding in an appropriate and timely manner to new information and updating labels and SDS information accordingly. Updating should be carried out promptly on receipt of the information that necessitates the revision. The Competent Authority may choose to specify a time limit within which the information should be revised.

Suppliers should respond to "new and significant" information they receive about a chemical hazard by updating the label and safety data sheet for that chemical. New and significant information is any information that changes the GHS classification and leads to a change in the label information or information that may affect the SDS.

4.11 How does the GHS address Confidential Business Information (CBI)?

Confidential business information (CBI) will not be harmonized under the GHS. National authorities should establish appropriate mechanisms for CBI protection. The GHS established CBI principles which include:

- CBI provisions should not compromise the health and safety of users;
- CBI claims should be limited to the names of chemicals and their concentrations in mixtures;
- Mechanisms should be established for disclosure in emergency and non-emergency situations.

4.12 Does the GHS address training?

The GHS states in Chapter 1.4, Section1.4.9, the importance of training all target audiences to recognize and interpret label and/or SDS information, and to take appropriate action in response to chemical hazards. Training requirements should be appropriate for and commensurate with the nature of the work or exposure. Key target audiences include workers, emergency responders and also those responsible for developing labels and SDSs. To varying degrees, the training needs of additional target audiences have to be addressed. These should include training for persons involved in transport and strategies required for educating consumers in interpreting label information on products that they use.

5. REFERENCES

References for Section 1.

ANSI Z129.1: American National Standard for Hazardous Industrial Chemicals-Precautionary Labeling.

Australia: Australia Worksafe, National Occupational Health and Safety Commission, Approved Criteria for Classifying Hazardous Substances (1994).

CPSC FHSA: U.S. CPSC, 16 CFR 1500, FHSA regulations.

DOT: U.S. DOT, 49 CFR Part 173, Subpart D.

EPA FIFRA: U.S. EPA, 40 CFR Part 156, FIFRA regulations.

EU: Council Directive 92/32/European Economic Community, amending for the 7th time, Directive 67/548/European Economic Community, approximation of the laws, regulations and administrative provisions on the classification, packaging and labeling of dangerous preparations.

GHS: Globally Harmonized System of Classification and Labelling of Chemicals, United Nations, 1st Revised Edition 2005.

IATA: International Air Transport Association's Dangerous Goods Regulations.

ICAO: International Civil Aviation Organization's Technical Instructions for the Safe Transport Of Dangerous Goods By Air.

IMO: International Maritime Organization's International Maritime Dangerous Goods (IMDG) Code.

Japan: Japanese Official Notice of Ministry of Labor No. 60 "Guidelines for Labeling of the Danger and Hazards of Chemical Substances".

Korea: Korean Ministry of Labor Notice 1997-27 "Preparation of MSDS and Labelling Regulation".

Malaysia: Malaysian Occupational Safety and Health Act (1994), Act 514 and Regulations (1994).

Mexico: Dario Oficial (March 30, 1996) NORMA Oficial Mexicana NOM-114-STPS-1994.

NFPA: National Fire Protection Association, 704 Standard, System for the Identification of Fire Hazards of Materials, 2001.

NPCA HMIS: National Paint and Coatings Association, Hazardous Materials Identification System, 2001.

OSHA HCS: U.S. DOL, OSHA, 29 CFR 1910.1200.

WHMIS: Controlled Products Regulation, Hazardous Products Act, Canada Gazette, Part II, Vol. 122, No. 2, 1987.

References for Section 2.0:

GHS Chapter 1.1 Purpose, Scope and Application of the GHS.

GHS Chapter 1.3 Classification of Hazardous Substances and Mixtures.

References for Section 3.0:

GHS Chapter 1.3. Classification of Hazardous Substances and Mixtures.

GHS Part 2. Physical Hazards.

GHS Part 3. Health Hazards.

GHS Part 4. Environmental Hazards

GHS Annex 8. An Example of Classification in the GHS.

GHS Annex 9. Guidance on Hazards to the Aquatic Environment.

GHS Annex 10. Guidance on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media

References for Section 4:

GHS Chapter 1.4. Hazard Communication: Labelling.

GHS Chapter 1.5. Hazard Communication: Safety Data Sheets.

GHS Annex 1 Allocation of Label Elements.

GHS Annex 2 Classification and Labelling Summary Tables.

GHS Annex 3 Precautionary Statements and Precautionary Pictograms.

GHS Annex 4 Guidance on the preparation of Safety Data Sheets

GHS Annex 5 Consumer Product Labelling Based on the Likelihood of Injur.

GHS Annex 6 Comprehensibility Testing Methodology.

GHS Annex 7 Examples of Arrangements of GHS Label Elements.

References for Government and Private Standards:

Canada

Hazardous Products Act: Controlled Products Regulations; Consumer Chemical and Container Regulations, 2001 Pest Control Products Act; Transportation of Dangerous Goods Act.

Health Canada GHS Website: www.healthcanada.ca/ghs;

European Union (EU)

Directive 67/548/EEC (consolidated, 7th revision).

Directive 2001/59/EC adapting to technical progress for the 28th time Council Directive 67/548/EEC.

Manual of decisions, implementation for the sixth and seventh amendments to Directive 67/548/EEC on dangerous substances.

Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 related to the classification, packaging and labelling of dangerous preparations.

Commission Directive 91/155/EEC defining and laying down the detailed arrangements for the system of specific information relating to dangerous preparations (SDS.)

Directive 2001/58/EC (amending Directive 91/155/EEC) defining and laying down the detailed arrangements for the system of specific information relating to dangerous preparations (SDS).

EU GHS web site: http://europa.eu.int/comm/enterprise/reach/ghs_en.htm

Standards

American National Standard for Hazardous Industrial Chemicals - Precautionary Labeling (ANSI Z-129.1-2000).

American National Standard for Hazardous Industrial Chemicals - MSDS Preparation (ANSI Z400.1-2004).

ISO 11014-1:2003 DRAFT Safety Data Sheet for Chemical Products.

UN GHS

Globally Harmonized System of Classification and Labelling of Chemicals (GHS) ("The Purple Book"), United Nations, 2005 First Revised Edition, available at www.unece.org/trans/danger/publi/ghs/ghs_rev01/01files_e.html or from United Nations Publications (publications@un.org)

UN GHS website: www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.htm

UN TRANSPORT

UN Recommendations on the Transport of Dangerous Goods, Model Regulations (14th Revised Edition 2005).

UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria, 4th Revised Edition

USA

OSHA Hazard Communication Standard 29 CFR 1910.1200.

CPSC Consumer Product Safety Act (15 U.S.C. 2051 *et seq.*) and Federal Hazardous Substances Act (15 U.S.C. 1261 *et seq.*).

(FIFRA) Federal Insecticide, Fungicide, and Rodenticide Act (7 U.S.C. 136 *et seq.*).

US EPA Label Review Manual (3rd Edition, August 2003) EPA 735-B-03-001.

Federal Hazardous Materials Transportation Law (49 U.S.C. 5101 *et seq.*).

USA websites:

www.osha.gov/SLTC/hazardcommunications/global.html

www.epa.gov/oppfead1/international/globalharmon.htm

http://hazmat.dot.gov/regs/intl/globharm.htm

GHS Focal Point websites:

ILO - www.ilo.org/public/english/protection/safework/ghs/index.htm

OECD - www.oecd.org/department/0,2688,en_2649_34371_1_1_1_1_1,00.html

6.0 GLOSSARY

Aerosols means any non-refillable receptacles made of metal, glass or plastics and containing a gas compressed, liquefied or dissolved under pressure, with or without a liquid, paste or powder, and fitted with a release device allowing the contents to be ejected as solid or liquid particles in suspension in a gas, as a foam, paste or powder or in a liquid state or in a gaseous state. Aerosol includes aerosol dispensers.

Alloy means a metallic material, homogeneous the naked eye, consisting of two or more elements so combined that they cannot be readily separated by mechanical means. Alloys are considered to be mixtures for the purpose of classification under the GHS.

Aspiration means the entry of a liquid or solid chemical product into the trachea and lower respiratory system directly through the oral or nasal cavity, or indirectly from vomiting;

ASTM means the "American Society of Testing and Materials".

BCF means "bioconcentration factor".

BOD/COD means "biochemical oxygen demand/chemical oxygen demand".

CA means "competent authority".

Carcinogen means a chemical substance or a mixture of chemical substances which induce cancer or increase its incidence.

CAS means "Chemical Abstract Service".

CBI means "confidential business information".

Chemical identity means a name that will uniquely identify a chemical. This can be a name that is in accordance with the nomenclature systems of the International Union of Pure and Applied Chemistry (IUPAC) or the Chemical Abstracts Service (CAS), or a technical name.

Competent authority means any national body(ies) or authority(ies) designated or otherwise recognized as such in connection with the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Compressed gas means a gas which when packaged under pressure is entirely gaseous at -50°C; including all gases with a critical temperature ≤ -50°C.

Contact sensitizer means a substance that will induce an allergic response following skin contact. The definition for "contact sensitizer" is equivalent to "skin sensitizer".

Corrosive to metal means a substance or a mixture which by chemical action will materially damage, or even destroy, metals.

Criteria means the technical definition for the physical, health and environmental hazards;

Critical temperature means the temperature above which a pure gas cannot be liquefied, regardless of the degree of compression.

Dermal Corrosion: see *skin corrosion*; ***Dermal irritation***: see *skin irritation*.

Dissolved gas means a gas which when packaged under pressure is dissolved in a liquid phase solvent.

EC_{50} means the effective concentration of a substance that causes 50% of the maximum response.

EC Number or (ECN°) is a reference number used by the European Communities to identify dangerous substances, in particular those registered under EINECS.

ECOSOC means the "Economic and Social Council of the United Nations".

EINECS means "European Inventory of Existing Commercial Chemical Substances".

End Point means physical, health and environmental hazards;

ErC_{50} means EC_{50} in terms of reduction of growth rate.

EU means "European Union".

Explosive article means an article containing one or more explosive substances.

Explosive substance means a solid or liquid substance (or mixture of substances) which is in itself capable by chemical reaction of producing gas at such a temperature and pressure and at such a speed as to cause damage to the surroundings. Pyrotechnic substances are included even when they do not emit gases.

Eye irritation means the production of changes in the eye following the application of test substance to the front surface of the eye, which are fully reversible within 21 days of application.

Flammable gas means a gas having a flammable range with air at 20°C and a standard pressure of 101.3 kPa.

Flammable liquid means a liquid having a flash point of not more than 93°C.

Flammable solid means a solid which is readily combustible, or may cause or contribute to fire through friction.

Flash point means the lowest temperature (corrected to a standard pressure of 101.3 kPa) at which the application of an ignition source causes the vapors of a liquid to ignite under specified test conditions.

Gas means a substance which (i) at 50 °C has a vapor pressure greater than 300 kPa; or (ii) is completely gaseous at 20 °C at a standard pressure of 101.3 kPa.

GESAMP means "the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection of IMO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP."

GHS means "the Globally Harmonized System of Classification and # Labeling of Chemicals".

Hazard category means the division of criteria within each hazard class, e.g., oral acute toxicity includes five hazard categories and flammable liquids includes four hazard categories. These categories compare hazard severity within a hazard class and should not be taken as a comparison of hazard categories more generally.

Hazard class means the nature of the physical, health or environmental hazard, e.g., flammable solid carcinogen, oral acute toxicity.

Hazard statement means a statement assigned to a hazard class and category that describes the nature of the hazards of a hazardous product, including, where appropriate, the degree of hazard;

IARC means the "International Agency for the Research on Cancer".

ILO means the "International Labor Organization".

IMO means the "International Maritime Organization".

Initial boiling point means the temperature of a liquid at which its vapor pressure is equal to the standard pressure (101.3 kPa), i.e., the first gas bubble appears.

IOMC means the "Inter-organization Program on the Sound Management of Chemicals".

IPCS means the "International Program on Chemical Safety".

ISO means International Standards Organization.

IUPAC means the "International Union of Pure and Applied Chemistry".

Label means an appropriate group of written, printed or graphic information elements concerning a hazardous product, selected as relevant to the target sector(s), that is affixed to, printed on, or attached to the immediate container of a hazardous product, or to the outside packaging of a hazardous product.

Label element means one type of information that has been harmonized for use in a label, e.g., pictogram, signal word.

***LC_{50}* (50% lethal concentration)** means the concentration of a chemical in air or of a chemical in water which causes the death of 50% (one-half) of a group of test animals.

LD_{50} means the amount of a chemical, given all at once, which causes the death of 50% (one half) of a group of test animals.

L(E)C_{50} means LC_{50} or EC_{50}.

Liquefied gas means a gas which when packaged under pressure, is partially liquid at temperatures above –50°C. A distinction is made between.

(i) High pressure liquefied gas: a gas with a critical temperature between -50°C and +65°C; and

(ii) Low pressure liquefied gas: a gas with a critical temperature above +65°C.

Liquid means a substance or mixture which at 50°C has a vapor pressure of not more than 300 kPa (3 bar), which is not completely gaseous at 20 °C and at a standard pressure of 101.3 kPa, and which has a melting point or initial melting point of 20°C or less at a standard pressure of 101.3 kPa. A viscous substance or mixture for which a specific melting point cannot be determined shall be subjected to the ASTM D 4359-90 test; or to the test for determining fluidity (penetrometer test) prescribed in section 2.3.4 of Annex A of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR).

MARPOL means the "International Convention for the Prevention of Pollution from Ships".

Mixture means a mixture or a solution composed of two or more substances in which they do not react.

MSDS means "Material Safety Data Sheet" and in this document is used interchangeably with Safety Data Sheet (SDS).

Mutagen means an agent giving rise to an increased occurrence of mutations in populations of cells and /or organisms.

Mutation means a permanent change in the amount or structure of the genetic material in a cell;

NGO means "non-governmental organization".

NOEC means the "no observed effect concentration".

OECD means "The Organization for Economic Cooperation and Development".

Organic peroxide means a liquid or solid organic substance which contains the bivalent -0-0- structure and may be considered a derivative of hydrogen peroxide, where one or both of the hydrogen atoms have been replaced by organic radicals. The term also includes organic peroxide formulation (mixtures).

Oxidizing gas means any gas which may, generally by providing oxygen, cause or contribute to the combustion of other material more than air does.

Oxidizing liquid means a liquid which, while in itself not necessarily combustible, may, generally by yielding oxygen, cause, or contribute to, the combustion of other material.

Oxidizing solid means a solid which, while in itself not necessarily combustible, may, generally by yielding oxygen, cause, or contribute to, the combustion of other material.

QSAR means "quantitative structure-activity relationships".

Pictogram means a graphical composition that may include a symbol plus other graphic elements, such as a border, background pattern or color that is intended to convey specific information.

Precautionary statement means a phrase (and/or pictogram) that describes recommended measures that should be taken to minimize or prevent adverse effects resulting from exposure to a hazardous product, or improper storage or handling of a hazardous product.

Product identifier means the name or number used for a hazardous product on a label or in the SDS. It provides a unique means by which the product user can identify the substance or mixture within the particular use setting (e.g. transport, consumer or workplace).

Pyrophoric liquid means a liquid which, even in small quantities, is liable to ignite within five minutes after coming into contact with air.

Pyrophoric solid means a solid which, even in small quantities, is liable to ignite within five minutes after coming into contact with air.

Pyrotechnic article means an article containing one or more pyrotechnic substances;

Pyrotechnic substance means a substance or mixture of substances designed to produce an effect by heat, light, sound, gas or smoke or a combination of these as the result of non-detonative, self-sustaining exothermic (heat-related) chemical reactions.

Readily combustible solid means powdered, granular, or pasty substance or mixture which is dangerous if it can be easily ignited by brief contact with an ignition source, such as a burning match, and if the flame spreads rapidly.

Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria means the latest revised edition of the United Nations publication bearing this title, and any published amendment thereto.

Recommendations on the Transport of Dangerous Goods, Model Regulations means the latest revised edition of the United Nations publication bearing this title, and any published amendment thereto.

Refrigerated liquefied gas means a gas which when packaged is made partially liquid because of its low temperature.

Respiratory sensitizer means a substance that induces hypersensitivity of the airways following inhalation of the substance.

RID means The Regulations concerning the International Carriage of Dangerous Goods by Rail [Annex 1 to Appendix B (Uniform Rules concerning the Contract for International Carriage of Goods by Rail) (CIM) of COTIF (Convention concerning international carriage by rail)], as amended.

SAR means "Structure Activity Relationship".

SDS means "Safety Data Sheet" and in this document is used interchangeably with Material Safety Data Sheet (MSDS).

Self-Accelerating Decomposition Temperature (SADT) means the lowest temperature at which self-accelerating decomposition may occur with substance as packaged.

Self-heating substance means a solid or liquid substance, other than a pyrophoric substance, which, by reaction with air and without energy supply, is liable to self-heat; this substance differs from a pyrophoric substance in that it will ignite only when in large amounts (kilograms) and after long periods of time (hours or days).

Self-reactive substance means a thermally unstable liquid or solid substance liable to undergo a strongly exothermic decomposition even without participation of oxygen (air). This definition excludes substances or mixtures classified under the GHS as explosive, organic peroxides or as oxidizing.

Serious eye damage means the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the front surface of the eye, which is not fully reversible within 21 days of application.

Signal word means a word used to indicate the relative level of severity of hazard and alert the reader to a potential hazard on the label. The GHS uses 'Danger' and 'Warning' as signal words.

Skin corrosion means the production of irreversible damage to the skin following the application of a test substance for up to 4 hours.

Skin irritation means the production of reversible damage to the skin following the application of a test substance for up to 4 hours.

Skin sensitizer means a substance that will induce an allergic response following skin contact. The definition for "skin sensitizer" is equivalent to "contact sensitizer".

Solid means a substance or mixture which does not meet the definitions of a liquid or gas.

SPR means “Structure Property Relationship”.

Substance means chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

Substance which, in contact with water, emits flammable gases means a solid or liquid substance or mixture which, by interaction with water, is liable to become spontaneously flammable or to give off flammable gases in dangerous quantities.

Supplemental label element means any additional non-harmonized type of information supplied on the container of a hazardous product that is not required or specified under the GHS. In some cases this information may be required by other competent authorities or it may be additional information provided at the discretion of the manufacturer/distributor.

Symbol means a graphical element intended to succinctly convey information.

Technical name means a name that is generally used in commerce, regulations and codes to identify a substance or mixture, other than the IUPAC or CAS name, and that is recognized by the scientific community. Examples of technical names include those used for complex mixtures (e.g., petroleum fractions or natural products), pesticides (e.g., ISO or ANSI systems), dyestuffs (Color Index system) and minerals.

UNCED means the “United Nations Conference on Environment and Development”.

UNCETDG/GHS means the "United Nations Committee of Experts on the Transport of Dangerous Goods and on the Globally Harmonized System of Classification and Labelling of Chemicals".

UNITAR means the “United Nations Institute for Training and Research”;

UNSCEGHS means the "United Nations Sub-Committee of Experts on the Globally Harmonized System of Classification and Labelling of Chemicals".

UNSCETDG means the "United Nations Sub-Committee of Experts on the Transport of Dangerous Goods".

7.0 APPENDICES

A. Comparison of MSDS/SDS Elements

B. GHS MSDS Examples

Appendix A

Comparison of MSDS/SDS Elements

The following tables provide a comparison of MSDS elements for the following:

- **Globally Harmonized System[1]**
- **ISO Safety Data Sheet for Chemical Products 11014-1: 2003 DRAFT [2]**
- **ANSI MSDS Preparation Z400.1- 2004 [3]**
- **OSHA Hazard Communication Standard 29#CFR#1910.1200[4]**

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
1. Product and company identification	- GHS product identifier. - Other means of identification. - Recommended use of the chemical and restrictions on use. - Supplier's details (including name, address, phone number etc). - Emergency phone number.	- GHS product identifier. - Other means of identification. - Recommended use of the chemical and restrictions on use. - Supplier's details (including name, address, phone number etc). - Emergency phone number. .	- Product identity same as on label - Product name, product code - name, address and telephone number of supplier - emergency telephone number	- Product identity same as on label. - Name address and telephone number of the manufacturer, distributor, employer or other responsible party.
2. Hazards identification	- GHS classification of the substance/mixture and any regional information. - GHS label elements, including precautionary statements. (Hazard symbols may be provided as a graphical reproduction of the symbols in black and white or the name of the symbol, e.g., flame, skull and crossbones.) - Other hazards which do not result in classification (e.g., dust explosion hazard) or are not covered by the GHS.	- GHS classification of the substance/mixture and any regional information. - GHS label elements, including precautionary statements. (Hazard symbols may be provided as a graphical reproduction of the symbols in black and white or the name of the symbol, e.g., flame, skull and crossbones.) - Other hazards which do not result in classification (e.g., dust explosion hazard) or are not covered by the GHS.	- Emergency Overview (description of product and most significant immediate physical, health and environmental concerns) - OSHA Regulatory Status - Potential health effects (information on adverse human health effects and symptoms, relevant route(s) and length of exposure, type and severity of effects, target organs, medical symptoms that are aggravated by exposure) - if listed as a carcinogen by OSHA, IARC, NTP - environmental effects	- health hazards including acute and chronic effects, listing target organs or systems - signs & symptoms of exposure - conditions generally recognized as aggravated by exposure - primary routes of exposure - if listed as a carcinogen by OSHA, IARC, NTP - physical hazards, including the potential for fire, explosion, and reactivity

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
3. Composition/information on ingredients	***Substance*** - Chemical identity - Common name, synonyms, etc. - CAS number, EC number, etc. - Impurities and stabilizing additives which are themselves classified and which contribute to the classification of the substance. ***Mixture*** - The chemical identity and concentration or concentration ranges of all ingredients which are hazardous within the meaning of the GHS and are present above their cut-off levels. - Cut-off level for reproductive toxicity, carcinogenicity and category 1 mutagenicity is ≥ 0.1% - Cut-off level for all other hazard classes is ≥ 1% Note: For information on ingredients, the competent authority rules for CBI take priority over the rules for product identification	***Substance*** - Chemical identity - Common name, synonyms etc. - CAS number, EC number, etc. - Impurities and stabilizing additives which are themselves classified and which contribute to the classification of the substance. ***Mixture*** - The chemical identity and concentration or concentration ranges of all ingredients which are hazardous within the meaning of the GHS and are present above their cut-off levels. - Cut-off level for reproductive toxicity, carcinogenicity and category 1 mutagenicity is ≥ 0.1% - Cut-off level for all other hazard classes is ≥ 1%	- common chemical name(s) - generic name(s) - synonyms - CAS number(s) - components or impurities contributing to the hazard (name, concentration)	- Chemical and common name of ingredients contributing to known hazards - For untested mixtures, the chemical & common name of ingredients at 1% or more that present a health hazard and those that present a physical hazard in the mixture - Ingredients at 0.1% or greater, if carcinogens

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
4. First-aid measures	- Description of necessary measures, subdivided according to the different routes of exposure, i.e., inhalation, skin and eye contact and ingestion. - Most important symptoms/effects, acute and delayed. - Indication of immediate medical attention and special treatment needed, if necessary.	- Description of necessary measures, subdivided according to the different routes of exposure, i.e., inhalation, skin and eye contact and ingestion. - Most important symptoms/effects, acute and delayed. - Indication of immediate medical attention and special treatment needed, if necessary.	- first aid procedures by route of exposure, i.e., inhalation, skin contact, eye contact, ingestion - important symptoms and effects useful for diagnostic treatment - antidotes - notes to a physician	- emergency & first aid procedures
5. Firefighting measures	- Suitable (and unsuitable) extinguishing media. - Specific hazards arising from the chemical (e.g., nature of any hazardous combustion products). - Special protective equipment and precautions for fire-fighters.	- Suitable (and unsuitable) extinguishing media. - Specific hazards arising from the chemical (e.g., nature of any hazardous combustion products). - Special protective equipment and precautions for firefighters.	- Qualitative flammable and reactivity properties - suitable extinguishing media - unsuitable extinguishing media - Guidance to firefighters - Specific hazards arising from the chemical - Protective equipment and precautions for firefighters	- generally applicable control measures - flammable property information such as flashpoint - physical hazards including the potential for fire, explosion, and reactivity
6. Accidental release measures	- Personal precautions, protective equipment and emergency procedures. - Environmental precautions. - Methods and materials for containment and cleaning up.	- Personal precautions, protective equipment and emergency procedures. - Environmental precautions. - Methods and materials for containment and cleaning up.	- Clean-up technique - Personal Precautions - Environmental Precautions - containment technique - regulatory information	- procedures for clean up of spills and leaks

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
7. Handling and storage	- Precautions for safe handling. - Conditions for safe storage, including any incompatibilities.	- Precautions for safe handling. - Conditions for safe storage, including any incompatibilities.	*handling* - measures to prevent exposure and release, prevent fire or explosion and ensure precautions for safe handling *storage* - storage conditions and technical measures for safe storage - incompatibilities - suitable/non suitable packaging material	- Precautions for safe handling & use, including appropriate hygenic practices.
8. Exposure controls/ personal protection	- Control parameters (e.g., occupational exposure limit values or biological limit values). - Appropriate engineering controls. - Individual protection measures, such as personal protective equipment.	- Control parameters (e.g., occupational exposure limit values or biological limit values). - Appropriate engineering controls. - Individual protection measures, such as personal protective equipment.	- exposure guidelines (limit values) - engineering controls to minimize hazards - personal protective equipment (respiratory, hand, eye, skin and body protection) - General Hygiene Considerations	- General applicable control measures - appropriate engineering controls and work practices - protective measures during maintenance & repair - personal protective equipment - permissible exposure levels, threshold limit values, listed by OSHA, ACGIH, or established company limits.

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
9. Physical and chemical properties	- Appearance (physical state, colour, etc.) - Odour - Odour threshold - pH - melting point/freezing point - initial boiling point and boiling range - flash point: - evaporation rate - flammability (solid, gas) - upper/lower flammability or explosive limits - vapour pressure - vapour density - relative density: - solubility(ies) - partition coefficient: *n*-octanol/water - auto-ignition temperature - decomposition temperature	- Appearance (physical state, colour, etc.) - Odour - Odour threshold - pH - melting point/freezing point - initial boiling point and boiling range - flash point: - evaporation rate - flammability (solid, gas) - upper/lower flammability or explosive limits - vapour pressure - vapour density - relative density: - solubility(ies) - partition coefficient: *n*-octanol/water: - auto-ignition temperature - decomposition temperature	- appearance (color, physical form, shape) - odor/odor threshold - physical state - pH - melting/freezing point(specify which) - initial boiling point and boiling range - flash point - evaporation rate - flammability (solid, gas) - upper/lower flammability or explosive limits - vapor pressure - vapor density - specific gravity or relative density - solubility(ies) (specify solvent, e.g., water) - partition coefficient: n-octanol/water - auto-ignition temperature - decomposition temperature. - other relevant data	- characteristics of hazardous chemicals such as vapor pressure & density. - physical hazards including the potential for fire, explosion, and reactivity.
10. Stability and reactivity	- Chemical stability. - Possibility of hazardous reactions. - Conditions to avoid (e.g., static discharge, shock or vibration). - Incompatible materials, - Hazardous decomposition products.	- Chemical stability. - Possibility of hazardous reactions. - Conditions to avoid (e.g., static discharge, shock or vibration). - Incompatible materials. - Hazardous decomposition products.	- Physical hazards - chemical stability - conditions to avoid - Incompatible Materials - hazardous decomposition products - Possibility of Hazardous Reactions	- organic peroxides, pyrophoric, unstable # (reactive), or water-reactive hazards - physical hazards, including reactivity and hazardous polymerization

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
11. Toxicological information	- Concise but complete and comprehensible description of the various toxicological (health) effects and the available data used to identify those effects, including: - Information on the likely routes of exposure (inhalation, ingestion, skin and eye contact); - Symptoms related to the physical, chemical and toxicological characteristics; - Delayed and immediate effects and also chronic effects from short- and long-term exposure;. - Numerical measures of toxicity (such as acute toxicity estimates).	- Concise but complete and comprehensible description of the various toxicological (health) effects and the available data used to identify those effects, including: - Information on the likely routes of exposure (inhalation, ingestion, skin and eye contact); - Symptoms related to the physical, chemical and toxicological characteristics; - Delayed and immediate effects and also chronic effects from short- and long-term exposure; - Numerical measures of toxicity (such as acute toxicity estimates).	- Toxicological information: human, animal, and in vitro data, SAR - acute dose effects: single/ short-term exposures. (e.g., LD50, LC50). - Repeated dose effects: (e.g., NOAEL) - Irritation/Corrosivity - Sensitization (skin and respiratory) - Carcinogenicity - Neurological effects - Genetic effects (e.g., mutagenicity) - Reproductive effects - Developmental effects - Target organ effects	- See also Section 2 [health hazards Including acute and chronic effects, listing target organs or systems - signs & symptoms of exposure - primary routes of exposure - if listed as a carcinogen by OSHA, IARC, NTP]
12. Ecological information	- Ecotoxicity (aquatic and terrestrial, where available). - Persistence and degradability - Bioaccumulative potential - Mobility in soil - Other adverse effects	- Ecotoxicity (aquatic and terrestrial, where available). - Persistence and degradability - Bioaccumulative potential - Mobility in soil - Other adverse effects	- ecotoxicity acute and longterm (fish, invertebrates) - persistence / degradability - bioaccumulation / bioconcentration - mobilty: air, soil, water - Other adverse effects	- No present requirements.

MSDS Comparison				
MSDS Sections	**GHS SDS[1]**	**ISO MSDS[2]**	**ANSI MSDS[3]**	**OSHA MSDS[4]**
13. Disposal considerations	- Description of waste residues and information on their safe handling and methods of disposal, including any contaminated packaging.	- Description of waste residues and information on their safe handling and methods of disposal, including any contaminated packaging.	- safe and environmentally preferred waste management of the material and/or its container - classification under applicable law	- No present requirements, - See section 7,
14. Transport information	- UN number. - UN Proper shipping name. - Transport Hazard class(es). - Packing group, if applicable. - Marine pollutant (Y/N). - Special precautions which a user needs to be aware of or needs to comply with in connection with transport or conveyance either within or outside their premises.	- UN number. - UN Proper shipping name. - Transport Hazard class(es). - Packing group, if applicable. - Marine pollutant (Y/N). - Special precautions which a user needs to be aware of or needs to comply with in connection with transport or conveyance either within or outside their premises.	- proper shipping name - hazard class(es) - identification number - packing group - hazardous substances - marine pollutants (Y/N) - IMDG classification - TDG classification - ICAO/IATA classification - RID/ADR classification	- No present requirements,
15. Regulatory information	- Safety, health and environmental regulations specific for the product in question.	- Safety, health and environmental regulations specific for the product in question.	- U.S .federal regulations - international regulations - U.S. state regulations	- No present requirements.
16. Other information	- Other information including information on preparation and revision of the SDS.	- Other information including information on preparation and revision of the SDS.	- label text - hazard rating and rating system - information on preparation and revision of safety data sheet - Key/legend	- Date of preparation of MSDS or date of last change

1. Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, 2005.
2. ISO 11014-1:2003 DRAFT Safety Data Sheet for Chemical Products.
3. American National Standard for Hazardous Industrial Chemicals-MSDS Preparation (ANSI Z-400.1-2004).
4. U.S. DOL, OSHA, 29 CFR 1910.1200, HAZCOM.

Appendix B

MSDS Examples
(Fictional Products)

B-1: Bondit

B-2: Chemical Stuff

Appendix B-1

Bondit

(GHS MSDS Example)

Appendix B-1
MSDS for *BONDIT*

1. Identification

Name of the product: Bondit

Recommended use: General adhesive.

Producer: GHS Ltd., UK –
London, SE, Southwarkbridge 1

Telephone no. +44 171717 555.555 5,
Emergency no. +44 171717 333 333 3

2. Hazard(s) identification

Classification: Flammable liquid, Category 2
Eye irritation, Category 2A
Hazardous to the aquatic environment, Acute Category 3

Labelling:

Symbol: Flame, Exclamation mark
Signal word: Danger

Hazard statement:
Highly flammable liquid and vapour.
Causes severe eye irritation.
Harmful to aquatic life.

Precautionary statements:
Keep container tightly closed.
Keep away from heat/sparks/open flame. – No smoking.
Wear protective gloves and eye/face protection.
Ground/Bond container and receiving equipment.
Use explosion-proof electrical/ventilating/lighting/ equipment.
Take precautionary measures against static discharge.
Use only non-sparking tools.
Store in cool/well-ventilated place.
Avoid release to the environment.

3. Composition / Information on ingredients

Chemical identity: Component A 70-80%
Common name: Solvent A
Numbers of identity: CAS-Nr.:111111-11-1
Impurities: None

Chemical identity: Component C 20-25%
Common name: Not applicable
Numbers of identity: CAS-Nr.: 44444-44-4
Impurities: none

4. First-aid measures

Inhalation:
Remove person to fresh air. If respiratory irritation, dizziness, nausea, or unconsciousness occurs, seek immediate medical assistance. If breathing has stopped, give artificial respiration.

Skin contact:
Wash the contaminated area with soap and water. Remove contaminated clothing and wash before reuse. If irritation develops, get medical attention.

Eye contact:
Hold eyelids apart and flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Ingestion:
If swallowed, do NOT induce vomiting. Seek immediate medical attention.

5. Firefighting measures

Suitable extinguishing media: Foam, extinguishing powder, carbon dioxide, water fog. In case of fire, cool endangered containers with water fog.

Unsuitable extinguishing media: High pressure water jet.

Specific hazards in case of fire: None are known.

Special protective equipment and precaution for fire fighters: For fires in enclosed areas, wear self-contained breathing apparatus. Do not inhale combustion gases.

6. Accidental release measures

Personal precautions:
Depending on extent of release, consider the need for fire fighters/emergency responders with adequate personal protective equipment for cleaning up.

Do not eat, drink or smoke while cleaning up. Use a self-contained respirator, a mask with filter (type A class 3) or a filtering mask (e.g., EN 405). Wear protective clothing, safety glasses and impervious gloves (e.g., neoprene gloves). Ensure adequate ventilation. Avoid all sources of ignition, hot surfaces and open flames (see also Section 7).

Environmental precautions:
Prevent spills from entering storm sewers or drains and contact with soil.

Methods and materials for containment and cleaning up:
Eliminate all ignition sources. Runoff may create fire or explosion hazard in sewer system. Absorb on fire retardant, liquid-absorbing material (treated sawdust, diatomaceous earth, sand). Shovel up and dispose of at an appropriate waste disposal facility in accordance with current applicable laws and regulations, and product characteristics at time of disposal (see also Section 13).

7. Handling and storage

Precautions for safe handling:
Avoid contact with eyes. Avoid prolonged repeated skin contact and breathing mists/vapours.

Use in well-ventilated area away from all ignition sources. Switch off all electrical devices such as parabolic heaters, hotplates, storage heaters etc. in good time for them to have cooled down before commencing work. Do not smoke; do not weld. Do not empty waste into sanitary drains. Take measures to prevent the build up of electrostatic charge.

Conditions for safe storage, including incompatibilities:
Storage containers must be grounded and bonded. Store away from all ignition sources in a cool area equipped with an automatic sprinkling system. Ensure adequate ventilation. Store at temperatures between +5 and +50°C. Store only in the original container.

8. Exposure controls / personal protection

Information on the system design:
Draw off vapours directly at the point of generation and exhaust from the work area. In the case of regular work, provide bench-mounted extraction equipment.

Exposure Limits:

Component Name (CAS-No.)	Reference	TWA		STEL	
		ppm	mg/m3	ppm	mg/m3
	UK OEL	500	1200	--	--
Component C (4444-44-4)	German MAK	200	950	--	--

Ventilation:
Use in well-ventilated area with local exhaust.

Respiratory protection:
Approved respiratory equipment must be used when airborne concentrations are unknown or exceed the exposure limits. When processing large amounts, use a light duty construction compressed air line breathing apparatus (e.g., in accordance with EN1835), a mask with filter (type A class 3, colour brown) or a filtering half mask (e.g., in accordance with EN 405) when there is inadequate ventilation.

Eye protection:
Safety glasses with side shields or chemical goggles must be worn.

Skin protection:
If prolonged or repeated skin contact is likely, neoprene gloves should be worn. Good personal hygiene practices should always be followed.

9. Physical and chemical properties

Physical state:	Liquid
Colour:	Colourless, transparent
Odour:	Solvent, ester-like
Odour threshold:	Not available
pH-value:	Not applicable
Melting point:	Not available
Freezing Point:	Not available
Initial boiling point:	56°C
Flash point:	- 22°C DIN 51755
Evaporation rate:	Not available
Flammability (solid, gas):	Not applicable

Explosion limits:	lower limit = 1.4 Vol%; upper limit 13.0 Vol% (literature)
Vapour pressure:	240 mbar (highest partial vapour pressure) at 20°C
Vapour density:	Not available
Relative density:	0.89 g/cm3 at 20°C
Solubility:	Partially soluble in water at 20°C
Partition coefficient:	Log Kow = 3.3
Auto-ignition temperature:	Not available
Decomposition temperature:	Not available

10. Stability and reactivity

Chemical stability: No decomposition, if used according to specifications.
Possibility of hazardous reactions: None are known.
Conditions to avoid: Heat, sparks, flame and build up of static electricity.
Materials to avoid: Halogens, strong acids, alkalies and ozidizers.
Hazardous decomposition products: None are known.

11. Toxicological information

Acute Toxicity:

Test	Results	Basis
Oral Toxicity (Rats)	Not Classified	Based on Ingredients
Dermal Toxicity (Rats)	Not Classified	Product Test Data
Inhalation Toxicity, Vapor (Rats)	Not Classified	Based on Testing of Similar Materials
Eye Irritation (Rabbits)	Eye Irritant Category 2A	Based on Testing of Similar Materials
Dermal Irritation (Rabbits)	Not Classified	Product Test Data

Summary Comments: May cause severe eye irritation like ocular lesions, which are reversible.

Subchronic/Chronic Toxicity:

Test	Results	Comments
Dermal Sensitization (Guinea Pig)	Not Classified: Negative response in Bueller, guinea pig test. 0% animals considered positive.	Product Test Data

Summary Comments: Component A may have a drying effect on the skin; frequent or prolonged contact may cause flaking or cracking of the skin.

12. Ecological information

Persistence and degradability: The total of the organic components contained in the product is not classified as "readily biodegradable" (OECD-301 A-F). However, this product is expected to be inherently biodegradable.

Bio-accumulative potential;
There is no evidence to suggest bioaccumulation will occur.

Mobility: Accidental spillage may lead to penetration in the soil and groundwater. However, there is no evidence that this would cause adverse ecological effects.

Aquatic Toxicity:

Test	Results	Comments
Acute Toxicity	Acute Category 3: 96 hr. LC_{50} = 65 mg/L	Product Test Data

13. Disposal considerations

Waste Disposal:
Product is suitable for burning in an enclosed, controlled burner for fuel value or disposal by supervised incineration. Such burning may be limited by local regulation. The product is suitable for processing at an appropriate government waste disposal facility. Use of these methods is subject to user compliance with applicable laws and regulations and consideration of product characteristics at time of disposal.

Recommended European waste code (EWC): 080406

14. Transport information

UN-number: 1993
UN proper shipping name: Flammable Liquid, N.O.S. (Contains Component C)
Transport hazard class: 3
Packing group: II
Marine Pollutant: No

15. Regulatory information

Inventory Status:
All components are on TSCA, EINECS/ELINCS, AICS, and DSL.

German:

Regulations governing combustible liquids (German-VbF) class: AI

German water endangering class (WGK) = 1, slightly water-endangering product (manufacturer classification.)

Australian Regulations:

AS 1940 Class: PGII
Poisons Schedule: S5

U.S. Regulations:

U.S. Superfund Amendments and Reauthorization Act (SARA) Title III:

SARA (311/312) HAZARD CATEGORIES:
FIRE, ACUTE

SARA 313: This product contains the following SARA 313 Toxic Release Chemicals.

Chemical Name	CAS Number	Concentration
Component A	111111-11-1	70-80%
Component C	4444-44-4	20-25%

The following product components are cited on the lists below:

Chemical Name	CAS Number	List Citations
Component A	111111-11-1	NJ RTK, TSCA 12(b)
Component C	4444-44-4	Prop. 65, NJ RTK

16. Other information

Abbreviations and acronyms:

UK OES = United Kingdom Occupational Exposure Standards
German MAK = Germany Maximum Allowable Concentration

MSDS Prepration date: July 1, 2005

The information contained herein is accurate to the best of our knowledge. My Company makes no warranty of any kind, express or implied, concerning the safe use of this material in your process or in combination with other substances.

Appendix B-2

Chemical Stuff

(GHS MSDS Example)

Appendix B-2

MSDS for Chemical Stuff

GHS SAFETY DATA SHEET

1. Identification

Product Name: **Chemical Stuff**
Synonyms: Methyltoxy Solution
CAS Number: 000-00-0
Product Use: Organic Synthesis
Manufacturer/Supplier: My Company
Address: My Street, Mytown, TX 00000

General Information: 713-000-0000
Transportation Emergency Number: CHEMTREC: 800-424-9300

2. Hazards Identification

GHS Classification:

Health	Environmental	Physical
Acute Toxicity – Category 2 (inhalation), Category 3 (oral/dermal) Eye Corrosion - Category 1 Skin Corrosion – Category 1 Skin Sensitization – Category 1 Mutagenicity – Category 2 Carcinogenicity – Category 1B Reproductive/Developmental – Category 2 Target Organ Toxicity (Repeated) – Category 2	Aquatic Toxicity – Acute 2	Flammable Liquid – Category 2

GHS Label:

Symbols: flame, skull and crossbones, corrosion, health hazard

Hazard Statements	Precautionary Statements
DANGER! Highly Flammable Liquid and Vapor. Fatal if inhaled. Causes severe skin burns and eye damage. May cause allergic skin reaction. Toxic if swallowed and in contact with skin May cause cancer. Suspected of damaging the unborn child. Suspected of causing genetic defects. May cause damage to cardiovascular, respiratory, nervous, and gastrointestinal systems and liver and blood through prolonged or repeated exposure. Toxic to aquatic life.	Do not eat, drink or use tobacco when using this product. Do not breathe mist/vapors. Keep container tightly closed. Keep away from heat/sparks/open flame. – No smoking. Wear respiratory protection, protective gloves and eye/face protection. Use only in a well-ventilated area. Take precautionary measures against static discharge. Use only non-sparking tools. Store container tightly closed in cool/well-ventilated place. Wash thoroughly after handling.

3. Composition / Information on Ingredients

Component	CAS Number	Weight %
Methyltoxy	000-00-0	80

(See Section 8 for Exposure Limits)

4. First Aid Measures

Eye: Eye irritation. Flush immediately with large amounts of water for at least 15 minutes. Eyelids should be held away from the eyeball to ensure thorough rinsing. Get immediate medical attention.

Skin: Itching or burning of the skin. Immediately flush the skin with plenty of water while removing contaminated clothing and shoes. Get immediate medical attention. Wash contaminated clothing before reuse.

Inhalation: Nasal irritation, headache, dizziness, nausea, vomiting, heart palpitations, breathing difficulty, cyanosis, tremors, weakness, red flushing of face, irritability. Remove exposed person from source of exposure to fresh air. If not breathing, clear airway and start cardiopulmonary resuscitation (CPR). Avoid mouth-to-mouth resuscitation.

Ingestion: Get immediate medical attention. Do not induce vomiting unless directed by medical personnel.

5. Fire Fighting Measures

Suitable Extinguishing Media: Use dry chemical, foam, or carbon dioxide to extinguish fire. Water may be ineffective but should be used to cool fire-exposed containers, structures and to protect personnel. Use water to dilute spills and to flush them away from sources of ignition.

Fire Fighting Procedures: Do not flush down sewers or other drainage systems. Exposed firefighters must wear NIOSH-approved positive pressure self-contained breathing apparatus with full-face mask and full protective clothing.

Unusual Fire and Explosion Hazards: Dangerous when exposed to heat or flame. Will form flammable or explosive mixtures with air at room temperature. Vapor or gas may spread to distant ignition sources and flash back. Vapors or gas may accumulate in low areas. Runoff to sewer may cause fire or explosion hazard. Containers may explode in heat of fire. Vapors may concentrate in confined areas. Liquid will float and may reignite on the surface of water.

Combustion Products: Irritating or toxic substances may be emitted upon thermal decomposition. Thermal decomposition products may include oxides of carbon and nitrogen.

6: Accidental Release Measures

Keep unnecessary people away; isolate hazard area and deny entry. Stay upwind; keep out of low areas. (Also see Section 8).

Vapor protective clothing should be worn for spills and leaks. Shut off ignition sources; no flares, smoking or flames in hazard area. Small spills: Take up with sand or other noncombustible absorbent material and place into containers for later disposal. Large spills: Dike far ahead of liquid spill for later disposal.

Do not flush to sewer or waterways. Prevent release to the environment if possible. Refer to Section 15 for spill/release reporting information.

7. Handling and Storage

Handling
Do not get in eyes, on skin or on clothing. Do not breathe vapors or mists. Keep container closed. Use only with adequate ventilation. Use good personal hygiene practices. Wash hands before eating, drinking, smoking. Remove contaminated clothing and clean before re-use. Destroy contaminated belts and shoes and other items that cannot be decontaminated.

Keep away from heat and flame. Keep operating temperatures below ignition temperatures at all times. Use non-sparking tools.

Storage
Store in tightly closed containers in cool, dry, well-ventilated area away from heat, sources of ignition and incompatibles. Ground lines and equipment used during transfer to reduce the possibility of static spark-initiated fire or explosion. Store at ambient or lower temperature. Store out of direct sunlight. Keep containers tightly closed and upright when not in use. Protect against physical damage.

Empty containers may contain toxic, flammable and explosive residue or vapors. Do not cut, grind, drill, or weld on or near containers unless precautions are taken against these hazards.

8. Exposure Controls / Personal Protection

Exposure Limits

Component	OSHA	
	TWA	STEL
Methyltoxy	3 ppm (skin)	C 15 ppm (15 min.)

Engineering Controls: Local exhaust ventilation may be necessary to control air contaminants to their exposure limits. The use of local ventilation is recommended to control emissions near the source. Provide mechanical ventilation for confined spaces. Use explosion-proof ventilation equipment.

Personal Protective Equipment (PPE)

Eye Protection: Wear chemical safety goggles and face shield. Have eye-wash stations available where eye contact can occur.

Skin Protection: Avoid skin contact. Wear gloves impervious to conditions of use. Additional protection may be necessary to prevent skin contact including use of apron, face shield, boots or full body protection. A safety shower should be located in the work area. Recommended protective materials include:
Butyl rubber and for limited contact Teflon.

Respiratory Protection: If exposure limits are exceeded, NIOSH approved respiratory protection should be worn. A NIOSH approved respirator for organic vapors is generally acceptable for concentrations up to 10 times the PEL. For higher concentrations, unknown concentrations and for oxygen deficient atmospheres, use a NIOSH approved air-supplied respirator. Engineering controls are the preferred means for controlling chemical exposures. Respiratory protection may be needed for non-routine or emergency situations. Respiratory protection must be provided in accordance with OSHA 29 CFR 1910.134.

9. Physical and Chemical Properties

Flashpoint: 2^{o}C (35^{o}F)
Autoignition Temperature: 480^{o}C (896^{o}F)
Boiling Point: 77^{o}C (170.6^{o}F) @ 760 mm Hg
Melting Point: -82^{o}C
Vapor Pressure: 100.0 mm Hg @ 23^{o}C

Vapor Density(Air=1): 1.7; air = 1
% Solubility in Water: 10 @ 20ºC

Pour Point: NA
Molecular Formula: Mixture

Lower Flammability Limit: >3.00%
Upper Flammability Limit: <15.00%
Specific Gravity: 0.82g/ml @ 20^{o}C
% Volatile: 100
Evaporation Rate (Water=1): 5(Butyl Acetate =1)
Viscosity: 0.3 cP @ 25^{o}C
Octanol/Water Partition Coefficient: log K_{ow}: 0.5
pH: 7, 8% aqueous solution
Molecular Weight: Mixture

Odor/Appearance: Clear, colorless liquid with mild, pungent odor.

10. Stability and Reactivity

Stability/Incompatibility: Incompatible with ammonia, amines, bromine, strong bases and strong acids.

Hazardous Reactions/Decomposition Products: Thermal decomposition products may include oxides of carbon and nitrogen.

11. Toxicological Information

Signs and Symptoms of Overexposure: Eye and nasal irritation, headache, dizziness, nausea, vomiting, heart palpitations, difficulty breathing, cyanosis, tremors, weakness, itching or burning of the skin.

Acute Effects:

Eye Contact: may cause severe conjunctival irritation and corneal damage.

Skin Contact: may cause reddening, blistering or burns with permanent damage. Harmful if absorbed through the skin. May cause allergic skin reaction.

Inhalation: may cause severe irritation with possible lung damage (pulmonary edema).

Ingestion: may cause severe gastrointestinal burns.

Target Organ Effects: May cause gastrointestinal (oral), respiratory tract, nervous system and blood effects based on experimental animal data. May cause cardiovascular system and liver effects.

Chronic Effects: based on experimental animal data, may cause changes to genetic material; adverse effects on the developing fetus or on reproduction at doses that were toxic to the mother. Methyltoxy is classified by IARC as group 2B and by NTP as reasonably anticipated to be a human carcinogen. OSHA regulates Methyltoxy as a potential carcinogen.

Medical Conditions Aggravated by Exposure: preexisting diseases of the respiratory tract, nervous system, cardiovascular system, liver or gastrointestinal tract.

Acute Toxicity Values

Oral LD_{50} (Rat) = 100 mg/kg
Dermal LD_{50} (Rabbit) = 225-300 mg/kg
Inhalation LC_{50} (Rat) = 200 ppm/4 hr., 1100 ppm vapor/1 hr

12. Ecological Information

LC_{50} (Fathead Minnows) = 9 mg/L/96 hr.
EC_{50} (Daphnia) = 8.6 mg/L/48 hr.

Bioaccumulation is not expected to be significant. This product is readily biodegradable.

13. Disposal Considerations

As sold, this product, when discarded or disposed of, is a hazardous waste according to Federal regulations (40 CFR 261). It is listed as Hazardous Waste Number Z000, listed due to its toxicity. The transportation, storage, treatment and disposal of this waste material must be conducted in compliance with 40 CFR 262, 263, 264, 268 and 270. Disposal can occur only in properly permitted facilities. Refer to state and local requirements for any additional requirements, as these may be different from Federal laws and regulations. Chemical additions, processing or otherwise altering this material may make waste management information presented in the MSDS incomplete, inaccurate or otherwise inappropriate.

14. Transport Information

U.S. Department of Transportation (DOT)
Proper Shipping Name: Methyltoxy
Hazard Class: 3, 6.1
UN/NA Number: UN0000
Packing Group: PG 2
Labels Required: Flammable Liquid and Toxic

International Maritime Organization (IMDG)
Proper Shipping Name: Methyltoxy
Hazard Class: 3 Subsidiary 6.1
UN/NA Number: UN0000

Packing Group: PG 2
Labels Required: Flammable Liquid and Toxic

15. Regulatory Information

U.S. Federal Regulations

Comprehensive Environmental Response and Liability Act of 1980 (CERCLA):
The reportable quantity (RQ) for this material is 1000 pounds. If appropriate, immediately report to the National Response Center (800/424-8802) as required by U.S. Federal Law. Also contact appropriate state and local regulatory agencies.

Toxic Substances Control Act (TSCA): All components of this product are included on the TSCA inventory.

Clean Water Act (CWA): Methyltoxy is a hazardous substance under the Clean Water Act. Consult Federal, State and local regulations for specific requirements.

Clean Air Act (CAA): Methyltoxy is a hazardous substance under the Clean Air Act. Consult Federal, State and local regulations for specific requirements.

Superfund Amendments and Reauthorization Act (SARA) Title III Information:

SARA Section 311/312 (40 CFR 370) Hazard Categories:

Immediate Hazard: X	Delayed Hazard: X	Fire Hazard: X
Pressure Hazard:	Reactivity Hazard:	

This product contains the following toxic chemical(s) subject to reporting requirements of SARA Section 313 (40 CFR 372)

Component:	CAS Number:	Maximum %
Methyltoxy	000-00-0	80

State Regulations

California: This product contains the following chemicals(s) known to the State of California to cause cancer, birth defects or reproductive harm:

Component:	CAS Number:	%
Methyltoxy	000-00-0	80

International Regulations

Canadian Environmental Protection Act: All of the components of this product are included on the Canadian Domestic Substances list (DSL).

Canadian Workplace Hazardous Materials Information System (WHMIS):

Class B-2	Flammable Liquid
Class D-1-B	Toxic
Class D-2-A	Carcinogen
Class D-2-B	Chronic Toxin
Class E	Corrosive

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

European Inventory of Existing Chemicals (EINECS): All of the components of this product are included on EINECS.

EU Classification: F Highly Flammable; T Toxic; N Dangerous to the Environment
EU Risk (R) and Safety (S) Phrases:

R11: Highly flammable.
R23/24/25: Toxic by inhalation, in contact with skin and if swallowed.
R37/38: Irritating to respiratory system and skin.
R41: Risk of serious damage to eyes.
R43: May cause sensitization by skin contact.
R45: May cause cancer.
R51/53: Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
S53: Avoid exposure - obtain special instructions before use.
S16: Keep away from sources of ignition - No Smoking.
S45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
S9: Keep container in a well-ventilated place.
S36/37: Wear suitable protective clothing and gloves.
S57: Use appropriate container to avoid environmental contamination.

16. Other Information

National Fire Protection Association (NFPA) Ratings: This information is intended solely for the use of individuals trained in the NFPA system.

Health: 3 **Flammability: 3** **Reactivity: 0**

Revision Indicator: New MSDS

Disclaimer: The information contained herein is accurate to the best of our knowledge. My Company makes no warranty of any kind, express or implied, concerning the safe use of this material in your process or in combination with other substances.

Ref for FDA Voluntary Cosmetic Registration Program available at
https://www.fda.gov/Cosmetics/RegistrationProgram/default.htm

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Skin absorption and human exposure estimation of three widely discussed UV filters in sunscreens – In vitro study mimicking real-life consumer habits

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ABSTRACT

Due to health concerns about safety, three UV-filters (Benzophenone-3, BP3, 10%; Ethylhexyl Methoxycinnamate, EHMC, 10%; Butyl Methoxydibenzoylmethane, BMDBM; 5%) were examined *in vitro* for absorption on full-thickness pig-ear skin, mimicking human in-use conditions. Kinetic profiles confirmed the rapid permeation of BP3; after the first hour of skin (frozen-stored) exposure to 2 mg/cm^2 (W/O sunscreen; recommended but unrealistic amount), about 0.5% of the applied dose passed into the receptor fluid. The absorption rate of filters was higher from W/O than from O/W emulsions. The fresh/frozen-stored skin permeability coefficient (0.83–0.54) for each UV filter was taken into account. Systemic Exposure Dosage of BP3, EHMC, BMDBM for humans as a consequence of (i) whole-body and (ii) face treatment with 0.5 mg/cm^2 of W/O sunscreen for 6-h skin exposure followed by washing and subsequent 18-h permeation (a realistic scenario) were estimated to be (i) 4744, 1032 and 1036 μg/kg-bw/day, and (ii) 153, 33 and 34 μg/kg-bw/day, respectively. From Margin of Safety for BP3, EHMC and BMDBM (i) 42, 485 and 192 as well as (ii) 1307; 15,151 and 5882, respectively, only the value of 42 (<100) for BP3 indicated a possible health risk. Escalation of a phobia towards all organic UV filters is undesirable.

1. Introduction

Topical application of personal care products (PCPs) containing ultraviolet (UV) filters is a preferred protection against various detrimental effects associated with excessive sun exposure, including sunburn, immunosuppression, photoageing, and skin cancer. Historically, UV filters (initially UV-B filters) were designed to be used by adults and children in sun protection products. Modern sunscreens contain one or several UV-B filters enriched with UV-A filters. At present, UV filters are not only used in sunscreens but are also important ingredients of various leave-on PCPs for daily use such as skin-, lip-, and hair-care, as well as makeup preparations. This often results in daily application of products containing UV filters without the user making a conscious decision to use any sunscreen agent.

For many years, attention has focused on the effectiveness of UV filters to mitigate the negative impact of solar radiation. Currently, the safety and usefulness of sunscreens is being questioned. Opinions that sunscreens may be dangerous are supported by certain media, and according to Nohynek and Schaefer (2001), also by controversial interpretations of some scientific studies, resulting in a "sunscreen phobia". It should be emphasized that at present, before a new UV filter is allowed on the market in the European Union (EU), a stringent toxicological safety evaluation is carried out. Safety and efficacy requirements for UV filters are comparable with those of human dermatological drugs (Nohynek et al., 2010). Only those

List of symbols: AD, applied dose (sunscreen); BMDBM, Butyl Methoxydibenzoylmethane; BP3, Benzophenone-3; CID, compound identifier; EC, European Commission; EHMC, Ethylhexyl Methoxycinnamate; EP, exposure period (skin); FTS, full-thickness skin; HPLC, high performance liquid chromatography; INCI, International Nomenclature of Cosmetic Ingredients; LoQ, Limit of quantification; MoS, Margin of Safety; MW, molecular weight; NOAEL, No Observed Adverse Effect Level; OECD, Organisation for Economic Co-operation and Development; O/W, oil-in-water (emulsion); PCPs, personal care products; Po/w, partition coefficient n-octanol/water (log); RF, receptor fluid; SC, stratum corneum; SCCP, Scientific Committee on Consumer Products (EC); SCCS, Scientific Committee on Consumer Safety (EC); SED, Systemic Exposure Dosage; SPF, Sun Protection Factor; TEC, Transcutaneous Electrical Conductivity; USFDA, United States Food and Drug Administration; UV, ultraviolet; WHO, World Health Organisation; W/O, water-in-oil (emulsion).

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compounds that are safe and effective may obtain approval for human use by the competent authorities (Nohynek et al. 2010; SCCS, 2012). Similar safety assessment procedures before registration of UV filters are necessary in the United States (US-FDA, 2014), Australia (ARGS, 2012), and Japan (MHW, 2000). So, the new *UV filters* introduced in the last decades, have improved safety and efficacy.

However, some synthetic UV-absorbing filters have been in use for several decades, but their safety, efficacy and toxicological profile are still not clear. *Especially* there is an increasing concern regarding possible harmful consequences of exposure to Benzophenone-3, Ethylhexyl Methoxycinnamate, 3-Benzylidene Camphor, 4-Methylbenzylidene Camphor, Homosalate, Ethylhexyl Dimethyl-PABA, and Butyl Methoxydibenzoylmethane (Krause et al., 2012; Axelstad and Hass, 2013; Oz̀aez et al., 2013; Urek et al., 2013; Kim et al., 2014). Three possible side effects are the most problematic: (i) permeation into the viable layers of the skin; (ii) interference with the endocrine system in humans; (iii) photounstability (Klimová et al., 2013). This article is focused on the first of these hot issues.

In principle, sunscreens are intended for external application to the skin. To ensure effectiveness, UV filters should adhere to the skin surface like a protective film and have a high affinity for the *stratum corneum* (SC). But, to avoid toxicity, UV filters should permeate the skin as little as possible. Ideally, no amount of UV filters should be accumulated in the viable skin and be systemically available through the vascular system (lymph and/or blood vessels) (Klinubol et al., 2008; Scalia et al., 2011; Klimová et al., 2013).

Using a wide variety of *in vitro* and *in vivo* assay systems, many studies have demonstrated that certain organic UV filters can be absorbed into and across the skin, further metabolized in the body and excreted (Giokas et al., 2007; Kim et al., 2014). These processes may result in some local adverse effects, e.g. allergic contact dermatitis (Heurung et al., 2014), and some systemic effects, e.g. mutagenic and estrogenic activity (Chisvert et al., 2012; Oz̀aez et al., 2013; Roussel et al., 2015). That is the reason why studies that monitor the transport of these chemicals from the outer surface of the skin both into the skin and into the systemic circulation are important.

The filters studied in this work were three UV-absorbing synthetic chemicals: Benzophenone-3 (BP3); Ethylhexyl Methoxycinnamate (EHMC); Butyl Methoxydibenzoylmethane (BMDBM). The compounds were chosen because they have been (i) worldwide used in sunscreens and other PCPs for decades; (ii) authorized by legislation in many countries, although to a different maximum allowable concentration (see Table 1); (iii) often used together in the same PCP; (iv) the subject of increasing debate about their possible adverse effects on humans.

Nowadays, there is a growing concern regarding potentially harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. As the BP3 and EHMC structure are similar to estrogens, a number of experimental studies in a variety of screening systems (Schneider et al., 2005; Calafat et al., 2008; Schlumpf et al., 2008a,b; Zhang et al., 2011, 2013; Bluthgen et al., 2012; Oz̀aez et al., 2013; Kerdivel et al., 2013; Liao and Kannan, 2014; Watanabe et al., 2015) as well as review articles (SCCP, 2008; Axelstad et al., 2011, 2013; WHO, 2012; Krause et al., 2012; Urek et al., 2013; Kim and Choi, 2014; Manová et al., 2013) dealing with their influence on the endocrine system (oestrogen activity, progesterone activity, effect on reproduction, and other) were published in the past years. It should be emphasized, that conclusions on the potentially hormone-like activities of these filters were somewhat conflicting. One of the reasons may be variable designs and endpoints, the particular *in vitro* studies. Despite this, there is no doubt that for assessment of the systemic human exposure to both compounds via the PCPs, the rate of dermal absorption is important.

The formation of reactive intermediates with adverse side effects as a result of significant photounstability (Yang et al., 2008; Hojerová et al., 2011; Gaspar et al., 2013; Alfonso et al., 2014; Benevenuto et al., 2015) is the most undesirable side effect associated with the skin absorption of BMDBM.

Over the past years, several articles regarding dermal absorption of BP3, EHMC and BMDBM have been published. Studies have included experiments on human volunteers and animals *in vivo*, experiments on excised human, rodent, mouse, baby-mouse, pig, pig-ear, guinea-pig, etc. skin *in vitro*, and, more recently, *in vitro* experiments on a synthetic skin as well as a prediction using mathematical models. Several authors have demonstrated that BP3 can pass through the skin in significant amount (Gupta et al., 1999; Potard et al., 1999; Fernandez et al., 2000; Kurul and Hekimoġlu, 2001; Janjua et al., 2004; Gonzales et al., 2006; Calafat et al., 2008; Klinubol et al., 2008; SCCP, 2008; Gulbake et al., 2010; Kunisue et al., 2010, 2012; Liao and Kannan, 2014; Watanabe et al., 2015). Both significant (Gupta et al., 1999; Janjua et al., 2004; Jiménez et al., 2004; Iannuccelli et al., 2008; Klinubol et al., 2008; Montenegro et al., 2008; Durand et al., 2009; Vettor et al., 2010; Scalia et al., 2011) and insignificant (Potard et al., 1999; Simeoni et al., 2004) skin permeation of EHMC or BMDBM were reported. However, the experimental conditions used in some of the cited studies were far from the real-life habits of consumers (see Sections 3.3 and 3.4).

So, the aim of this work was to estimate the extent to which the three widely discussed UV filters in the sunscreens pose a health risk regarding dermal absorption under experimental conditions *in vitro* that mimic the real conditions *in vivo* as closely as possible.

Table 1
Physicochemical characteristics of the ultraviolet filters evaluated for dermal absorption.

INCI name[a]	INN name[b]	CAS no[c]	Molecular formula	Log Ko/w[d]	Molecular weight (g mol^{-1})	Absorption efficiency[e]	Maximum absorbance (nm)	Max. level in the EU[f] (%)
Benzophenone-3	Oxybenzone	131-57-7	$C_{14}H_{12}O_3$	3.79	228.25	UV-A II, UV-B	287.5	10
Ethylhexyl Methoxycinnamate	Octinoxate	5466-77-3	$C_{18}H_{26}O_3$	5.80	290.40	UV-B	308	10
Butyl Methoxydibenzoylmethane	Avobenzone	70356-09-1	$C_{20}H_{22}O_3$	4.51	310.39	UV-A I	358	5

[a] The name according to the International Nomenclature of Cosmetic Ingredients (CosIng, 2015).
[b] The International non-proprietary name recommended by the World Health Organisation (CosIng, 2015).
[c] The code number according to the Chemical Abstracts Service (CosIng, 2015).
[d] The partition coefficient n-octanol/water (PubChem, 2015).
[e] The main absorption efficiency in UV region (UV-B 290–320 nm; UV-A II 320–340 nm; UV-A I 340–420 nm).
[f] The maximum level allowed for cosmetic products in the European Union (EC, 2009; CosIng, 2015).

Experiments were focused on the accumulation of the UV filters in the skin and the transdermal permeation following their administration under non-occluded conditions, separately of (i) two doses of the sunscreens, (ii) three skin exposure periods to the sunscreens, and (iii) two emulsion types (W/O and O/W) containing all three UV filters. The purpose of the study was also to estimate the Systemic Exposure Dosage and the Margin of Safety values of the assessed UV filters for humans.

2. Materials and methods

2.1. Chemicals

BP3 (PubChem CID: 4632), EHMC (PubChem CID: 51040) and BMBDM (PubChem CID: 5355130) from Merck KGaA (Darmstadt, Germany) were used without further purification (a purity by certificates of analysis of 99.9%, 99.8% and 98.6% for BP3, EHMC and BMDBM, respectively). Physicochemical properties of the filters are reported in Table 1. Other ingredients for two laboratory-produced sunscreens were received from local producers of cosmetics. Polyethylene glycol 20 oleyl ether (PEG-20 oleyl ether; PubChem CID: 5364713), from Comercial Química Massó (Prague, the Czech Republic) and chemicals for phosphate-buffered saline from Mikrochem (Pezinok, the Slovak Republic) were of reagent grade. Methanol, acetonitrile and water were purchased from Fisher Scientific (Leicester, the United Kingdom) in high performance liquid chromatography (HPLC) grade. The solubility in the receptor fluid (RF) and stability at the temperature of 20–75 °C of all the filters studied were checked prior to the beginning of the experiments. The three filters were readily soluble in the RF and stable under the conditions of the study.

2.2. Sunscreens

Two different emulsions, a hydrophilic cream (oil-in-water emulsion, O/W) and a hydrophobic cream (water-in-oil emulsion, W/O), as vehicles for the filters were chosen in this study, whereas the emulsion-based sunscreens are the most common types (our unpublished market research conducted in 2013 on the Slovak Republic). The sunscreens were prepared in a conventional laboratory manner. Briefly, the oil- and aqueous-soluble ingredients (Table 2) were heated separately at 75 °C. Since the fat soluble, the filters were incorporated into the oil phase in each case at the maximum level allowed for cosmetics in the EU (EC, 2009). Under agitation at 650 rpm for 3 min with a stirrer (VEB MLW ER-10, Medingen, Germany) the dispersed phase was added to the dispersion medium and cooled to room temperature over approximately 30 min. The sunscreens were stored at 22 ± 1 °C until use.

Table 2
Components of the laboratory-prepared sunscreens under study.

Ingredients (INCI name)	% (w/w) of ingredient	
	Emulsion W/O	Emulsion O/W
Oil phase		
Cetyl PEG/PPG-10/1 dimethicone	2.60	–
Glyceryl stearate	–	2.60
Diethylhexyl carbonate	5.00	5.00
Paraffinum liquidum	3.00	4.00
Dimethicone	1.00	–
Cetyl alcohol	–	3.50
Isohexadecane	5.00	–
Isopropyl myristate	–	5.00
Benzophenone-3	**10.0**	**10.0**
Ethylhexyl Methoxycinnamate	**10.0**	**10.0**
Butyl Methoxydibenzoylmethane	**5.00**	**5.00**
Aqueous phase		
Propylene glycol	5.00	5.00
C12–14 pareth-12	–	3.00
Sodium chloride	0.70	–
Methylparaben	0.05	0.05
Propylparaben	0.05	0.05
Aqua	qs 100.00	qs 100.00
pH[a]	6.38	6.32
Dynamic viscosity (Pa s)[a]	0.105	0.091
Sun Protection Factor[b]	25–30	25–30

INCI: International Nomenclature of Cosmetic Ingredients (CosIng, 2015).
[a] The measurement conducted as described in Materials and methods.
[b] The values estimated using the BASF Sunscreen Simulator (BASF, 2014).

A pH meter (HI8520, Hanna Instruments, Woonsocket, the US) was used to measure pH values of the sunscreens at room temperature. The dynamic viscosity values of both sunscreens were quantified by a viscometer (VT 550, Haake, Karlsruhe, Germany) using a concentric cylinder MV-I system at shear rate 10 s^{-1} and room temperature. The Sun Protection Factor (SPF) of the sunscreens was estimated according to the BASF Sunscreen Simulator (BASF, 2014).

2.3. Dermal absorption study

The *in vitro* skin permeation experiments were performed according to OECD (2004a,b), WHO (2006), EC (2008), and SCCS (2012) guidelines as closely as possible.

2.3.1. Skin preparation

Excised pig-ear skin was used to mimic human skin. Fresh ears of around 6 months old domestic pigs (the Slovak large white) were obtained from a local slaughterhouse immediately postmortem and prior to steam cleaning. After subsequent transport to the laboratory ears were cleaned under cold running water and dried with a soft tissue. The dorsal skin of the upper half region of the ear was carefully removed from the underlying cartilage using a scalpel. The resultant full-thickness skin (FTS), incorporating the SC, viable epidermis, and dermis, was visually inspected for physical damage, excluding tattooed and otherwise unsuitable skin. Hairs were cropped to a length of 3 mm with an electric hair clipper. Whole FTS sheets were wrapped individually in an aluminum foil and stored at −20 °C for up to 6 weeks until use. The experiment described in Section 2.4.1, the left halves of four FTS sheets were used immediately (the fresh FTS) whereas the right halves were frozen (the frozen-stored FTS). One hour prior to the experiment, the whole frozen FTS sheets were allowed to thaw at ambient temperature and cut into discs of about a 3-cm diameter. The thickness of each disc was measured at six different locations using a digital micrometre (SKW 1/0.001, Helios Messtechnik, Niedernhall, Germany).

2.3.2. Diffusion apparatus

For these investigations, static glass diffusion cells (JM-Glass, Bratislava, the Slovak Republic) based on the Franz design (Franz, 1975), consisting of a donor chamber (an available diffusion area of 2.00 cm^2) and a receptor chamber (a volume from 5.6 to 6.0 mL) were used. The FTS disc was placed horizontally between the donor and the receptor chamber with the SC open to the air and fixed with a metal clip.

2.3.3. Skin barrier integrity

The integrity of the skin barrier, crucial for the experiment, was examined by measuring the Transcutaneous Electrical Conductivity (TEC) across the FTS disc mounted in the diffusion cell, as reported in our previous papers (Klimová et al., 2012; Lucová et al., 2013). The donor and the receptor chamber were filled with degassed RF pre-warmed at 32 ± 1 °C. The FTS discs within the acceptable conductivity range of ≤0.9 mS/cm (SCCP, 2008) were used. Following evaluation of the skin integrity, the diffusion cell was

dismantled. The FTS disc was blotted with a soft tissue and immediately used for the further experiment.

2.3.4. Dosing and dermal absorption conditions

The *in vitro* dermal studies were performed to mimic the "in use" conditions in humans as closely as possible. Two laboratory-prepared sunscreens were applied separately at a dose of 2.0 mg/cm^2 or 0.5 mg/cm^2 on the SC of the FTS disc outside the cell. The sunscreen was distributed with a small plastic spatula (Boehringer, Mannheim, Germany) evenly over the whole diffusion area of 2.00 cm^2. Borders of the diffusion area were highlighted as the result of the metal clip pressure during the previous measurement of the TEC value. The exact amount applied (4 ± 0.01 mg or 1 ± 0.01 mg) was calculated by the weight difference of the spatula using a balance (Mettler AE 240, Mettler Toledo, Greifensee, Switzerland; readability of 0.01 mg, reproducibility of 0.02 mg). The FTS disc was then mounted back in the same position of the diffusion cell.

The RF in the receptor chamber consisted of degassed phosphate buffered saline (pH 7.4; own preparation) with a non-ionic surfactant PEG-20 oleyl ether (6%, w/v; HLB 15) to ensure solubility of hydrophobic UV filters (Bronaugh et al., 1986; OECD, 2004a,b). After removing air bubbles, the cell was placed into a temperature-controlled water bath (Julabo Labortechnik, Seelbach, Germany) on a magnetic stirrer plate (Variomag 15, Thermo Scientific, Karlsruhe, Germany). The content of the receptor chamber was continuously agitated throughout the diffusion by a teflon-coated magnetic bar at 600 rpm and thermostated in order to ensure the skin surface temperature 32 ± 1 °C corresponding with the *in vivo* skin temperature (EC, 2008).

The sunscreen remained on the surface of FTS for 24 h or 6 h under non-occluded condition mimicking two cases of the human skin exposure to a sunscreen. Because of EHMC and BMDBM photounstability (Hojerová et al., 2011), all the experiments were performed avoiding light exposure.

2.4. Experimental design

Four sets of *in vitro* diffusion experiments were carried out using both sunscreens containing the three filters as follows. The 1st set was aimed at the effect of storage by freezing on the pig-ear skin permeability. The 2nd set focused on 12-h absorption-time profiles of the filters permeated through the frozen-stored FTS. The 3rd set of the experiments was aimed at the effect of the applied dose (AD) and the 4th set at the effect of the exposure period (EP); both with respect to the fate of UV filters after individual exposure of frozen-stored FTS to one of the two sunscreens.

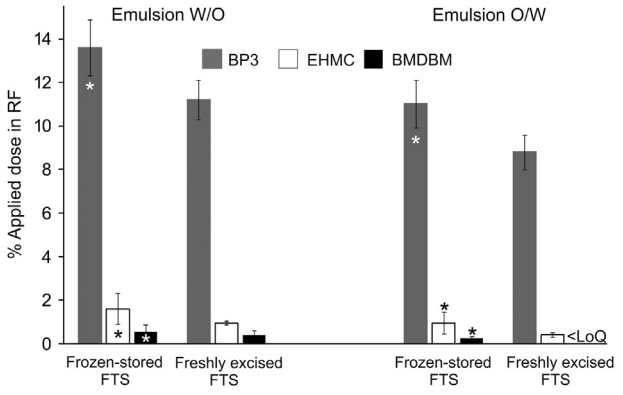

Fig. 1. The percentage of the UV filters detected in the receptor fluid after 24-h exposure to the applied dose (2.0 mg/cm^2) of the sunscreen containing 10% of BP3 (200 μg/cm^2), 10% of EHMC (200 μg/cm^2) and 5% of BMDBM (100 μg/cm^2), using freshly excised and frozen-stored (at −20 °C for 2 weeks) full-thickness skin from the same pig ear. Values are the mean ± SD (n = 6). *Value for the frozen-stored skin significantly different ($p < 0.05$) from the value of the same UV filter for the freshly excised skin from the same pig ear. <LoQ: below the Limit of quantification in the RF for BMDBM (0.406 μg/cm^2).

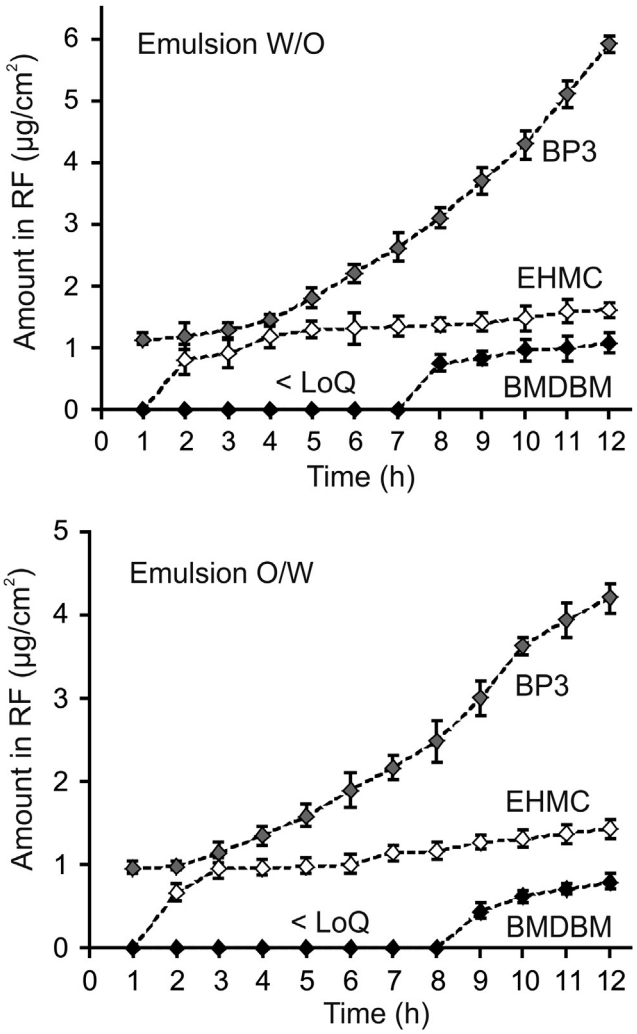

Fig. 2. The absorption-time profiles of the UV filters permeated through the frozen-stored full-thickness pig-ear skin from the 2.0 mg/cm^2 of the sunscreen (containing 10% of BP3 (200 μg/cm^2), 10% of EHMC (200 μg/cm^2), and 5% of BMDBM (100 μg/cm^2). Values are the mean ± SD (n = 3). <LoQ: below the Limit of quantification in the RF for BMDBM (0.406 μg/cm^2).

2.4.1. The 1st set: the effect of storage by freezing on the pig-ear skin permeability

Three discs were obtained from the left half of each fresh FTS sheet. In the same day, a 24-h permeation study through six discs from two FTS sheets for each sunscreen at 2.0 mg/cm^2 was started. At the end of the exposure, only the RF was analyzed for the UV filters. The same schedule was repeated after 2 weeks using the right halves of the frozen-stored FTS sheets (at −20 °C for 2 weeks). In this case, all the compartments of the diffusion system were analyzed for the UV filters (see Section 2.5). Thus, six replicates of the fresh FTS and six replicates of the frozen-stored FTS were used per sunscreen.

2.4.2. The 2nd set: the absorption-time profiles of the UV filters

This study was carried out using the frozen-stored FTS

(at −20 °C for max 6 weeks) exposed separately to both sunscreens at 2.0 mg/cm^2. The RF (100 μL) was withdrawn through the sampling port of the receptor chamber at various time intervals over 12 h (see Fig. 2) and replaced with the fresh RF equilibrated to the experimental temperature. Samples were assayed immediately for the UV filters by the HPLC. This procedure was done in triplicate per sunscreen.

2.4.3. The 3rd set: the effect of the sunscreen dose on the UV filters skin permeation

The declared SPF on the label is based on the use of a sunscreen layer of 2.0 mg/cm^2 (EC, 2006; Bendová et al., 2007; US-FDA, 2014). Therefore, in the first part of the 3rd set of the experiments, a sunscreen at 2.0 mg/cm^2 was applied for 24 h to frozen-stored FTS discs (at −20 °C for max 6 weeks).

But, it has been shown that the sunscreen quantity of 2.0 mg/cm^2 is higher than that usually applied by the consumers (EC, 2006), most often in the range of 0.5–1.3 mg/cm^2 (BfR, 2003; Autier et al., 2007; SCCP, 2008; SCCS, 2012). According to Faurschou and Wulf (2007) and Kim et al. (2010) only around a quarter (0.5 mg/cm^2) of the recommended sunscreen layer is generally applied by sunbathers. Isedeh et al. (2013) stated that in real life consumers used from 0.39 to 0.79 mg/cm^2 of a sunscreen independent of skin type and even less of everyday cosmetics containing UV filters. Lademann et al. (2014) reported that sunscreens are applied by humans in amounts that are even lower by a factor of almost 10 than are defined in the regulations for determining the SPF. So, in the second part of the 3rd set of the experiments, a sunscreen dose of 0.5 mg/cm^2 was applied to the frozen-stored FTS discs. After 24-h exposure, the excess of the sunscreen on the FTS surface (the unabsorbed dose) was carefully removed and the compartments of the diffusion cell were processed as described in Section 2.5. Six cells were used per sunscreen.

2.4.4. The 4th set: the skin reservoir effect on the UV filters skin permeation

Sunscreens are applied by the consumers to the skin for a period which depends on the SPF value of the product, skin phototype and photosensitivity as well as many other factors. In this set of the experiments, 6-h skin exposure to the sunscreen was chosen as a representative period for the summertime. To mimic typical "in-use" conditions in humans (SCCS, 2012) a sunscreen dose of 0.5 mg/cm^2 (EC, 2006) was applied to the FTS discs of the twelve diffusion cells for each sunscreen (twenty four in total). At the end of the 6-h skin exposure, the unabsorbed sunscreen was carefully removed from all the FTS discs with an aqueous soap solution (see Section 2.5), so the time of the wash-off determined the exposure period.

The first part of this set of the experiments focused on skin distribution of the filters immediately after 6-h skin exposure to the sunscreen. Six diffusion cells per sunscreen were dismounted and processed as described in Section 2.5. The second part of this set was aimed at the skin reservoir effect regarding permeation of the UV filters after the 6-h skin exposure. The remaining six cells for each sunscreen were left to run for another 18 h during which any UV filter already absorbed into the skin could continue to diffuse across the skin and into the receptor fluid. Then these cells were processed like those before.

2.5. Processing of the diffusion system compartments

At the end of the 3rd and the 4th set of experiments (24 h or 6 h), the FTS surface was sequentially washed by the application of a cotton swab imbibed with 0.5% (w/w) aqueous solution of sodium lauryl sulfate in order to mimic human skin cleansing with a liquid soap (a syndet). This procedure was next repeated three times. The cotton swabs were flooded into a centrifuge tube with 4 mL of acetonitrile–methanol solution (80:20, v/v), vigorously shaken with a vortex for 30 min and centrifuged for 10 min at 6000 rpm. The supernatant was filtered through a 0.45-μm-pore-size filter (MCE, Merck Millipore, Billerica, US) and analyzed for the UV filters by the HPLC. Then the FTS disc was removed from the cells. In view of the fact that we were not able to sufficiently separate the SC from the rest of the FTS after the experiment (see Section 3.3), the full epidermis was separated from the dermis by the heating method (Lucová et al., 2013). Briefly, the aluminum foil with the FTS disc was placed on the thermostatic electric plate with the SC upward and subjected to heat at 60 °C. After 30 s, the epidermis was easily separated from the dermis with a scalpel. Both skin layers were cut into small pieces using a scissors, separately flooded into a well sealable centrifuge tube with 5 mL of acetonitrile–methanol solution (80:20, v/v) and subsequently processed as the washing solution, with the exception of the vortexing time (6 h).

Verification of the extraction method assay was carried out by spiking the FTS disc (n = 3 for each concentration) with 20 μL of

Table 3
Skin distribution of the UV filters after 24-h exposure of two doses of sunscreens (containing 10% of BP3, 10% of EHMC and 5% of BMDBM) to the frozen-stored skin.

Full thickness pig-ear skin *ex-vivo*		Compartment of the diffusion system	Emulsion W/O			Emulsion O/W		
			BP3[b]	EHMC[b]	BMDBM[b]	BP3[b]	EHMC[b]	BMDBM[b]
TEC[a] 0.30–0.58 (mS/cm)	2.0 mg/cm^2 of the sunscreen	Applied amount (μg/cm^2)	200	200	100	200	200	100
Thickness[a] 0.89 ± 0.16 (mm)		Surface (μg/cm^2)	112.7 ± 4.1*	135.1 ± 6.3*	67.2 ± 2.9	129.7 ± 4.4	137.8 ± 6.1	61.1 ± 3.9
		Epidermis (μg/cm^2)	10.5 ± 1.8	10.7 ± 1.2	8.3 ± 1.5*	8.6 ± 3.1	10.2 ± 1.5	14.8 ± 1.7
		Dermis (μg/cm^2)	37.5 ± 2.7	24.1 ± 1.4	11.2 ± 1.6	28.6 ± 3.8	24.3 ± 1.8	10.8 ± 1.5
		Receptor fluid (μg/cm^2)	27.2 ± 1.3*	3.2 ± 0.7*	1.1 ± 0.3	22.1 ± 1.1	1.9 ± 0.8	0.5 ± 0.09
		Recovery (%, w/w)	**93.9 ± 2.0**	**87.6 ± 1.1**	**87.8 ± 1.8**	**94.5 ± 4.7**	**88.1 ± 5.3**	**87.2 ± 2.4**
TEC[a] 0.39–0.51 (mS/cm)	0.5 mg/cm^2 of the sunscreen	Applied amount (μg/cm^2)	50	50	25	50	50	25
Thickness[a] 0.95 ± 0.10 (mm)		Surface (μg/cm^2)	15.9 ± 0.3*	27.6 ± 2.2	17.3 ± 0.7	18.7 ± 2.3	27.2 ± 1.5	17.5 ± 1.7
		Epidermis (μg/cm^2)	9.4 ± 0.4*	10.3 ± 0.9*	3.1 ± 0.7*	11.2 ± 1.0	8.8 ± 1.3	2.7 ± 1.2
		Dermis (μg/cm^2)	10.3 ± 2.1	7.5 ± 0.5*	2.2 ± 0.3	11.2 ± 1.3	11.1 ± 0.3	3.4 ± 1.6
		Receptor fluid (μg/cm^2)	12.7 ± 2.8*	2.1 ± 0.3*	<LoQ	9.2 ± 0.8	1.2 ± 0.07	<LoQ
		Recovery (%, w/w)	**96.6 ± 3.1**	**95.0 ± 4.0**	**90.4 ± 2.8**	**100.6 ± 3.3**	**96.6 ± 2.4**	**94.4 ± 3.4**

BP3: Benzophenone-3; EHMC: Ethylhexyl Methoxycinnamate; BMDBM: Butyl Methoxydibenzoylmethane; TEC: Transcutaneous Electrical Conductivity; <LoQ: below the Limit of quantification.
LoQ for BP3 and EHMC: 0.216 μg/mL (LoQ of 0.432 μg/cm^2 on the surface; 0.540 μg/cm^2 in the skin and on average of 0.615 μg/cm^2 in the receptor fluid).
LoQ for BMDBM: 0.140 μg/mL (LoQ of 0.280 μg/cm^2 on the surface; 0.350 μg/cm^2 in the skin and on average of 0.406 μg/cm^2 in the receptor fluid).
*Value for W/O emulsion significantly different ($p < 0.05$) from the value of the same UV filter for O/W emulsion.
[a] Values are the mean ± SD (n = 12).
[b] Values are the mean ± SD (n = 6); no significant differences were found ($p \geq 0.05$) in a given compartment of the diffusion system after 24-h exposure.

Table 4
Skin distribution of the UV filters from the sunscreen dose of 0.5 mg/cm^2 (containing 10% of BP3, i.e. 50 μg/cm^2; 10% of EHMC, i.e. 50 μg/cm^2 and 5% of BMDBM, i.e. 25 μg/cm^2) after 6-h exposure to the frozen-stored skin.

Full thickness pig-ear skin *ex-vivo*		Compartment of the diffusion system	Emulsion W/O			Emulsion O/W		
			BP3[b]	EHMC[b]	BMDBM[b]	BP3[b]	EHMC[b]	BMDBM[b]
TEC[a] 0.37–0.61 (mS/cm)	Promptly after	Surface (μg/cm^2)	41.3 ± 2.8	42.5 ± 5.3	21.3 ± 0.7	42.3 ± 1.9*	42.9 ± 1.3	21.5 ± 0.7
Thickness[a] 0.83 ± 0.11 (mm)	6-h exposure	Epidermis (μg/cm^2)	4.9 ± 0.8*	4.8 ± 0.7*	1.6 ± 0.4	4.1 ± 0.6*	2.7 ± 0.6	1.8 ± 0.5
		Dermis (μg/cm^2)	6.1 ± 0.6*	1.2 ± 0.08*	1.5 ± 0.9	4.9 ± 0.3	0.8 ± 0.07*	1.3 ± 0.04
		Receptor fluid (μg/cm^2)	1.3 ± 0.07*	<LoQ	<LoQ	1.0 ± 0.04*	<LoQ	<LoQ
		Recovery (%, w/w)	**107.2 ± 2.3**	**97.0 ± 1.4**	**97.6 ± 2.7**	**104.6 ± 3.0**	**92.8 ± 2.4**	**98.4 ± 1.8**
TEC[a] 0.42–0.54 (mS/cm)	After following	Surface (μg/cm^2)	40.5 ± 1.1	41.2 ± 3.4	21.1 ± 0.9	41.5 ± 0.4	41.9 ± 1.3	21.7 ± 1.3
Thickness[a] 0.88 ± 0.13 (mm)	18-h permeation	Epidermis (μg/cm^2)	3.3 ± 0.4	3.4 ± 0.6	1.2 ± 0.3	2.7 ± 0.2	1.7 ± 0.2	1.1 ± 0.1
		Dermis (μg/cm^2)	2.7 ± 0.7	2.1 ± 0.4	2.4 ± 0.3	4.0 ± 0.9	2.3 ± 0.03	1.5 ± 0.2
		Receptor fluid (μg/cm^2)	7.1 ± 1.0	0.9 ± 0.06	<LoQ	5.5 ± 1.2	<LoQ	<LoQ
		Recovery (%, w/w)	**107.2 ± 3.6**	**95.2 ± 1.7**	**98.8 ± 2.2**	**107.4 ± 2.9**	**91.8 ± 2.2**	**97.2 ± 4.1**

BP3: Benzophenone-3, EHMC: Ethylhexyl Methoxycinnamate, BMDBM: Butyl Methoxydibenzoylmethane.
[a,b]See Table 3 for other key abbreviation.
*Value of the 6-h skin exposure significantly different ($p < 0.05$) from the value of the 6-h skin exposure + 18-h permeation of the same UV filter.

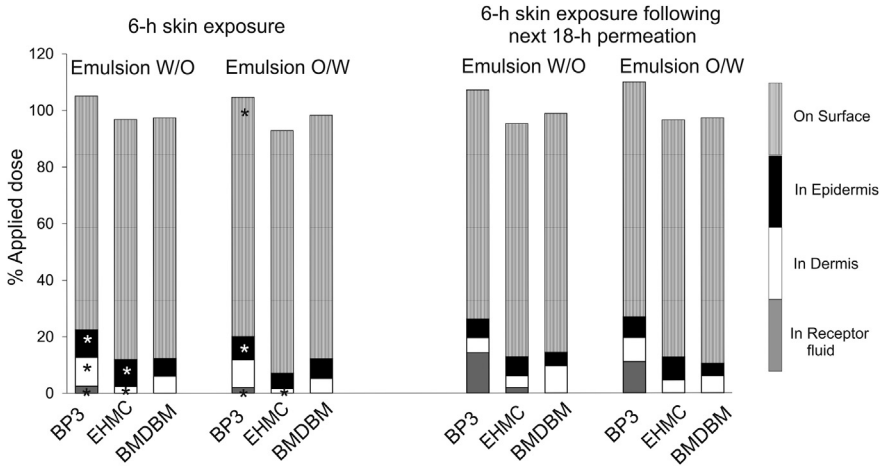

Fig. 3. Skin distribution of the UV filters after permeation through the frozen-stored full-thickness pig-ear skin (i) promptly after 6-h skin exposure; (ii) 24-h from the start of the 6-h skin exposure, in both cases to 0.5 mg/cm^2 of the sunscreen containing 10% of BP3, 10% of EHMC, and 5% of BMDBM. Values are the mean ± SD ($n = 6$). *Value for the 6-h skin exposure significantly different ($p < 0.05$) from the value for the 6-h skin exposure following 18-h permeation of the same UV filter.

Table 5
Estimated Daily Systemic Exposure Dosage (SED) of the UV filters for humans as a result of skin treatment with a sunscreen product (based on W/O emulsion containing 10% of BP3, 10% of EHMC and 5% of BMDBM).

Skin surface area involved	SSA[a] (cm^2)	F[a] (day^{-1})	UV filter	24 h from the start of the 6-h skin exposure to a sunscreen (0.5 mg/cm^2)		NOAEL (mg/kg bw/day)	MoS[g]
				DA[b] (μg/cm^2)	SED[c] (mg/kg bw/day)		
Total body area	17,500	2/day	BP3	8.13	4.74	200[d]	**42**
			EHMC	1.77	1.03	500[e]	485
			BMDBM	1.78	1.04	200[f]	192
Face	565	2/day	BP3	8.13	0.153	200[d]	1307
			EHMC	1.77	0.033	500[e]	15,151
			BMDBM	1.78	0.034	200[f]	5882

BP3: Benzophenone-3; EHMC: Ethylhexyl Methoxycinnamate; BMDBM: Butyl Methoxydibenzoylmethane; SSA: Skin Surface Area; F: Frequency of application of sunscreen products; DA: dermal absorption; NOAEL: No Observed Adverse Effect Level; MoS: Margin of Safety.
[a] Value complies with the SCCS (2012).
[b] Experimental value from Table 4 (the sum of the UV filter' amount in the dermis and receptor fluid, considered as systemically available in this study, corrected by the fresh/frozen-stored skin permeability' coefficient of 0.83, 0.59, 0.74 for BP3, EHMC and BMDBM, respectively).
[c] Value calculated from the experimental value (Eq. (1)).
[d] Value complies with the SCCP (2008).
[e] Value complies the BfR (2003).
[f] Value complies with the SCCNFP (2001).
[g] Value calculated from the NOAEL and SED values (Eq. (2)).

acetonitrile–methanol solution (80:20, v/v) containing (i) 1.25, 1.25, and 0.625 μg/mL as well as (ii) 25, 25, and 12.5 μg/mL of BP3, EHMC, and BMDBM, respectively. Then the FTS disc was processed as above and analyzed for the UV filters. Ranges of 93.6–106.0%, 87.6–97.2%, 87.2–102.0% recovery for BP3, EHMC, and BMDBM, respectively were observed for the extraction from both emulsions. The RF in all sets of the experiments were filtered through a 0.45-μm-pore-size filter and analyzed for the UV filters by the HPLC.

2.6. HPLC analysis

The HPLC system (Ecom, Prague, the Czech Republic) consisted of one Kappa 10 PP piston pump equipped with a 20 μL loop injector, and an UV detector (Philips PU-4225, Spectra-Physics, Darmstadt, Germany). The analyses were carried out on a reversed-phase column (5 μm Kromasil C18, 150 × 4.6 mm; Macherey&Nagel, Düren, Germany) fitted with a guard column (5 μm particles, VK100 C18, 10 × 4 mm). The mobile phase consisting of acetonitrile, methanol, and water (80:10:10, v/v) was eluted isocratically at a flow rate of 0.8 mL/min. BP3 and EHMC were determined by simultaneous assay at 308 nm (retention times of 3.7 ± 0.1 and 9.9 ± 0.1 min, respectively), and BMDBM by separate assay at 354 nm (retention time of 9.5 ± 0.1 min).

The calibration curves were linear over the measured range for all the filters ($R^2 > 0.994$). The repeatability of the method was established for two concentrations (0.2 and 80 μg/mL) of BP3, EHMC, and BMDBM in the sample solution and five injections per concentration. The relative standard deviation values were <1.0%. The limits of quantification (LoQ) were 0.216, 0.216, and 0.140 μg/mL for BP3, EHMC, and BMDBM, respectively.

The quantities of the filters detected based on the HPLC-calibration curves as well as the LoQ values were recalculated to (i) the volume of 4 mL for the extract from the unabsorbed sunscreen; (ii) the volume of 5 mL for the extracts from the epidermis and dermis; and (iii) the precise receptor chamber volume (from 5.6 to 6.0 mL) for the RF sample. The LoQ values for BP3, EHMC, and BMDBM were (i) 0.432, 0.432, and 0.280; (ii) 0.540, 0.540, and 0.350; and (iii) an average of 0.615, 0.615, and 0.406 μg/cm^2, respectively (Table 3).

The data were expressed in the amount of the UV filter per square centimetre (μg/cm^2) of the skin (Fig. 2, Tables 3 and 4) or as a percentage of the applied dose (% AD) of the UV filter (Figs. 1 and 3).

2.7. Statistical analysis

Differences in the concentrations of the UV filters for both sunscreens in each set of this study were compared by the one-way analysis of variance (ANOVA) using the software program Microsoft® Office Excel 2007 for Windows. A value $p < 0.05$ was considered statistically significant.

3. Results and discussion

First of all we would like to express our opinion on the use of *ex-vivo* pig-ear skin as a model membrane. There is no doubt that human skin would be the obvious choice for the *in vitro* dermal absorption study mimicking the real use conditions of sunscreens. But the human skin obtained from surgery or cadavers is normally not frequently available, is quite often already damaged and there are large differences in the skin quality between different human donors. Because of their similarity in terms of epidermal thickness and composition, dermal structure, lipid content, and general morphology, the pig skin, mainly pig-ear skin has been recognized by the international authorities and scientists as a practical alternative and relevant model for predicting permeability of cosmetic ingredients in humans (Fernandez et al., 2000; Jacobi et al., 2007; SCCP, 2008; SCCS, 2010; Klang et al., 2012; Lau et al., 2012; Barel et al., 2015).

3.1. Results of the 1st set of experiments: the effect of the storage by freezing on the pig-ear skin permeability; (i) the fresh skin; (ii) the frozen-stored skin; 24-h skin exposure; 2.0 mg/cm^2 of the sunscreen (Fig. 1)

After 24-h skin exposure to the W/O or O/W sunscreen emulsion (2.0 mg/cm^2), the BP3, EHMC, and BMDBM amount of 27.2 ± 1.3, 3.2 ± 0.7, and 1.1 ± 0.3 μg/cm^2 (W/O) as well as 22.1 ± 1.1, 1.9 ± 0.8, and 0.5 ± 0.1 μg/cm^2 (O/W) passed through the frozen-stored skin. The amounts of 22.4 ± 0.9, 1.9 ± 0.1, and 0.8 ± 0.2 μg/cm^2 (W/O) as well as 17.6 ± 0.8, 1.0 ± 0.1, and <LoQ μg/cm^2 (O/W), respectively, passed through the fresh skin.

The quantities of the filters expressed in % AD absorbed into the RF are presented in Fig. 1. Briefly, significant differences ($p < 0.05$) were found in all transdermal permeation of the filters through both types of the skin. Compared to the frozen-stored skin from the same pig ear, the quantities of filters in the RF permeated through freshly excised skin were somewhat lower in all cases; i.e. 83–80% for BP3, 59–54% for EHMC, and from 74% to incalculable % for BMDBM, in W/O and O/W emulsions, respectively. Therefore, when the values of the daily Systemic Exposure Dosage were estimated (see Section 3.3–3.5, and Table 5), the coefficients of fresh/frozen-stored FTS permeability for BP3, EHMC, and BMDBM 0.83, 0.59 and 0.74, respectively (W/O) and 0.80, 0.54, and an incalculable value, respectively (O/W) were taken into account.

The results are entirely consistent with our previous experimental findings on the permeability of the fresh and frozen-stored pig-ear skin (also at −20 °C for up to 6 weeks) within the *in vitro* dermal absorption study aimed at parabens (Pažoureková et al., 2013). Experiments in the cited study confirmed that the freezing of pig-ear skin slightly increases the skin permeability and slightly decreases hydrolysis of the ester (methylparaben). Certain increasing effect of freezing on permeation of various substances through human, rat or pig-ear skin have also been confirmed by other authors (Kasting and Bowman, 1990; Hadzija et al., 1992; Babu et al., 2003; Brain et al., 2005; Barel et al., 2015).

Nevertheless, the frozen-stored skin was used for further experiments in this study. It is undisputed that the fresh skin best mimics conditions in the human skin *in vivo*. But when long-term experiments are performed, it is impossible to obtain day-to-day fresh pig ears of the same qualities. According to OECD (2004[a]) and SCCS (2010), the experiments can be carried out using the freshly excised or frozen-stored skin, but for the assessment of the metabolism the fresh skin is preferred. Bronaugh et al. (1986) and Steiling et al. (2001) also reported that the skin tissue need not necessarily be freshly excised as a percutaneous permeation occurs by passive diffusion and the barrier function resides in the dead cells of the SC. Harrison et al. (1984) reported that appropriate storage (for several months at −20 °C) has no relevant effect on the *in vitro* permeability of both human and animal types of the skin. Based on two studies cited (Bronaugh et al., 1986; Steiling et al., 2001), there is stated in the WHO criteria for dermal absorption that provided the samples are in their normal state of hydration when cooled, animal and human skin can be stored for up to 1 year at −20 °C (WHO, 2006). According to OECD (2004a) animal and human skin can be stored for several months at −20 °C, but it is inadvisable to refreeze and thaw skin specimens as this can increase the permeability (OECD, 2004a). Frozen stored skin may not be suitable for some metabolism studies (OECD, 2004a,b; WHO, 2006). It has also been shown that skin should not be stored at

very low temperatures since the storage of the skin at −80 °C can enhance permeability (Hawkins and Reifenrath, 1984).

Despite WHO (2006) and OECD (2004a) authorization for an extended period of skin storage, the FTS sheets used in this study were stored at −20 °C for max. 6 weeks, in accordance with our previous experimental studies (Lucová et al., 2013; Pažoureková et al., 2013).

3.2. Results of the 2nd set of experiments: the absorption-time profiles: 12-h skin exposure; 2.0 mg/cm^2 of the sunscreen (Fig. 2)

The absorption-time profiles were obtained from the cumulative amounts of the UV filters (μg/cm^2) permeated from the sunscreen dose of 2.0 mg/cm^2 through the frozen-stored FTS into the RF over 12 h, corrected for the sampling dilution and exact volume of the receptor chamber. Fig. 2 shown the results plotted against time.

The BP3 amount in the RF from both sunscreens were clearly detectable (i.e., ≥LoQ of 0.615 μg/cm^2) already after the first hour of the exposure. Janjua et al. (2008) determined the presence of BP3 in human plasma already 1–2 h after the *in vivo* administration of sunscreen.

Approximately constant increase in the portion of BP3 from both sunscreens was achieved 5 h after application. The amounts of EHMC in the RF from both sunscreens were clearly detectable (i.e., ≥LoQ of 0.615 μg/cm^2) already after the second hour of the skin exposure followed by a low increase over the next ten hours. However, BMDBM was undetectable in the RF in the first seven hours from W/O emulsion and in the first eight hours from O/W emulsion (<LoQ of 0.406 μg/cm^2).

Among the filters under study, BP3 showed the highest capacity to permeate the skin. After 12-h exposure, the BP3 amount measured in the RF was 3.7-fold and 5.5-fold greater than that of EHMC and BMDBM, respectively from W/O vehicle as well as 2.9-fold and 5.3-fold greater than that of EHMC and BMDBM, respectively from O/W vehicle. We consider that BP3 is more able to permeate the skin than the other two filters due to its physico-chemical characteristics, in particular the molecular weight and hydrophobicity. The skin permeation process requires both lipid and aqueous solubility with an optimal Po/w (the partition coefficient of octanol–water); poor absorption is more likely when the log Po/w is greater than 4 or lower than −1, and when the molecular weight is greater than 500 (Roberts and Walters, 1998; OECD, 2004b; Durand et al., 2009; Pažoureková et al., 2013). The log Po/w value (3.79) and molecular weight (228.25 g/mol) of BP3 allow easy permeability of the filter through the skin.

The cumulative amounts of EHMC permeated into the RF were 1.5-fold (W/O) and 1.8-fold (O/W) greater than that of BMDBM. However, taking into account the different percentages of both agents in the sunscreens (10% of EHMC, 5% of BMDBM), the permeation rate of BMDBM was greater than that of EHMC. This can be explained by the partition coefficient for BMDBM (log Po/w 4.51) which is closer to the optimal range for the skin permeation of a compound (from −1 to 4) than more hydrophobic EHMC (log Po/w 5.80), wherein the differences in the molecular weight are not large (Table 1).

The results show that the W/O emulsion allows somewhat greater permeation of all three filters in the RF than O/W emulsion; e.g. after 12 h permeation was of 1.4, 1.1, and 1.4-fold greater for BP3, EHMC and BMDBM, respectively, although the viscosities of the emulsions were similar.

3.3. Results of the 3rd set of experiments: the effect of the sunscreen dose; (i) 2.0 mg/cm^2; (ii) 0.5 mg/cm^2; 24-h skin exposure (Table 3)

To obtain the full efficacy associated with a SPF, a sunscreen dose of 2.0 mg/cm^2 has to be applied to the skin surface (EC, 2006; US-FDA, 2014). This is a large amount, and thus it is needed to provide a homogeneous distribution of the product. So, in the first part of this set of experiments, a sunscreen dose of 2.0 mg/cm^2 ($AD_{2.0}$) was applied to the frozen-stored FTS.

However, it has been shown that people tend to apply a lower amount. This is due to various reasons; on one hand, there is lack of information about the recommended amount, and on the other hand, if this data was known, taking into account the body of an average adult person as a model, the recommended amount would equate to 6 teaspoons of sunscreens (ca 36 g), that is usually quite a higher amount than the common dose people apply themselves (Chisvert et al., 2012; SCCS, 2012). Therefore, in the second part of this set of experiments, a dose 0.5 mg/cm^2 of the sunscreen ($AD_{0.5}$), as more realistic amount in view of the living habits of consumers (SCCS, 2012), was applied to the frozen-stored FTS.

At the end of 24-h skin exposure, all the filters were detected in the unabsorbed dose, skin extracts, and BP3 and EHMC also in the RF, irrespective of the investigated test item (O/W or W/O). The results in Table 3 summarize the skin distribution of each UV filter (μg/cm^2) after 24-h skin exposure separately to both sunscreens.

As expected, for both doses and both emulsion types, the significant amount of each filter remained on the skin surface (i.e., it was recovered from the washing solution); 56% $AD_{2.0}$ and 32% $AD_{0.5}$ (W/O), 65% $AD_{2.0}$ and 37% $AD_{0.5}$ (O/W) for BP3; 68% $AD_{2.0}$ and 55% $AD_{0.5}$ (W/O), 69% $AD_{2.0}$ and 54% $AD_{0.5}$ (O/W) for EHMC; 67% $AD_{2.0}$ and 69% $AD_{0.5}$ (W/O), 61% $AD_{2.0}$ and 70% $AD_{0.5}$ (O/W) for BMDMB.

From both sunscreens at both applied doses clearly detectable amounts of all the filters were also accumulated in the epidermis (Table 3). In a classical *in vitro* dermal absorption setting, the total amount measured in the epidermis (without the SC), the dermis and the RF is considered to be systemically available (SCCS, 2010, 2012; OECD, 2011). As mentioned in Section 2.5, after our experiments we were unable to quantitatively eliminate the SC from the FTS disc by the tape-stripping procedure. During this process the rest of the skin was damaged, mainly due to its hair follicles. Also OECD GD 28 (OECD, 2011) states that fractionation of the skin after experiments can be difficult in some cases. But comparing the distribution of all the filters studied in different parts of the skin system (epidermis, dermis, RF) it is evident that their accumulation in the epidermis is not significant (Table 3). Based on these results, we can conclude that the observed UV filters do not have a high affinity for the SC and do not meet the requirement for the primary accumulation in the SC.

In view of the above, the cumulative amount of the UV filter only in the dermis and the RF was expected to be systemically available in this study. In addition, based on the experimental results in Section 3.1, the amounts were corrected using the coefficients of fresh/frozen-stored FTS permeability for each UV filter separately in W/O emulsion and O/W emulsion. So, 24 h after the application of the sunscreen at $AD_{2.0}$ and $AD_{0.5}$ (containing the maximum level of each filter allowed for cosmetics in the EU), approximately these quantities could enter the systemic circulation in humans: 53.7 μg/cm^2 and 19.1 μg/cm^2 (W/O), 40.6 and 16.3 μg/cm^2 (O/W) for BP3; 16.1 and 5.7 μg/cm^2 (W/O), 14.1 and 6.6 μg/cm^2 (O/W) for EHMC; 9.1 and about 1.6 μg/cm^2 (W/O), and incalculable values (O/W) for BMDBM, respectively. The total recovery of all the filters in this set of experiments ranged from 87.2% to 100.6% AD, which are values within the range of 85–115% AD recommended by SCCS (2012).

It is evident that an increased dose of the sunscreen had a significant impact on increasing the systemically available amount of all the filters studied. However, this increase was not linearly dose-dependent. Although the $AD_{2.0}$ was 4-fold greater as the $AD_{0.5}$, the cumulative amounts of BP3 or EHMC in the dermis and the RF were only from 2.8 to 2.1-fold higher. We consider that the results are due to the limited skin-accumulation capacity at a certain time of

absorption. This is also confirmed by greater sunscreen excess on the FTS surface (the unabsorbed dose) of each UV filter for the $AD_{2.0}$ than $AD_{0.5}$ (e.g. about 1.8-fold for BP3). The dose-dependent comparison for BMDBM cannot be done; even if some amount of UV filter permeated into the RF after skin exposure to the $AD_{0.5}$ of the sunscreen, this amount was below the LoQ.

Durand et al. (2009) applied 2.0 mg/cm^2 of W/O emulsion containing five UV filters including 9.5% of EHMC onto human skin *in vitro* for 24 h. The authors measured about 8% AD of EHMC as cutaneously permeated, which is almost identical to the finding in this set of experiments; 8.1% $AD_{2.0}$ of EHMC (10%) was measured as systemically available in our study. Gupta et al. (1999), studied *in vitro* absorption of two sunscreens through dermatomed micro-yucatan pig skin stored under refrigeration at 4 °C. After 10-h skin treatment with low realistic sunscreen dose (about 5.5 mg/cm^2) containing 6.0% of BP3 and 7.5% of EHMC in a hydroalcoholic and an oil based (diisopropyl adipate) vehicle, the cumulative amounts approximately 11% AD and 4% AD of BP3, and 8% AD and 3% AD of EHMC, respectively were observed in the RF plus viable skin. Also Klinubol et al. (2008) applied a dose of 4.4 mg/cm^2 to *ex-vivo* baby-mouse skin for 24 h and confirmed the significant permeation of EHMC (3.0% AD) and BMDBM (0.8% AD) into the RF. Jiménez et al. (2004), studying the permeability of EHMC (5%) from various vehicles to the *ex-vivo* flank pig skin, used 8 mg/cm^2 of the sunscreen for 24 h and reported that the cumulative amounts of EHMC in the dermis and the RF were 1.7% AD and 1.2% AD in W/O, and O/W emulsions, respectively. Montenegro et al. (2008) evaluated *in vitro* skin permeation of EHMC (5%) and BMDBM (1%) using a high dose of sunscreen (266 mg/cm^2) administrated to the human skin surface for 22 h. The cumulative amounts of EHMC and BMDBM absorbed from six different vehicles ranged from 0.06 to 0.38% AD and from 0.06 to 0.28% AD, respectively.

Here we would like to express three opinions. Firstly, we consider that a dose exceeding 2.0 mg/cm^2 per application is not very realistic, unsuitable to mimic the real-life habits of consumers and in turn poorly represents their systemic exposure to the UV filters (see Section 2.4.3). On the other hand, consumers may apply products several times per day. Sunscreen re-application has not been considered in this study. Secondly, we believe that the percentage comparison of accumulated amounts of the filters from different sunscreen doses would be misleading, since, as AD increases, % of unabsorbed residue also increases and % of compound in the RF decreases. Thirdly, when the dermal absorption of hydrophobic substances is studied, it is necessary to use an appropriate solubilizer, since the composition of the RF significantly affects a percutaneous permeation (OECD, 2004a,b; WHO, 2006; SCCS, 2012). This was not always respected in some of the cited studies. According to Bronaugh et al., 1986, maximum *in vitro* absorption of hydrophobic compounds was obtained with the RF containing a 6% solution in water of the non-ionic surfactant PEG-20 oleyl ether. This finding was also confirmed in our previously unpublished study. It should be noted that even higher absorption of hydrophobic compound was achieved using the RF consisting of ethanol: water (50:50, v/v), but we consider that this is very far from the imitation of a physiological fluid.

The affinity of the filters for the vehicle is also important. In agreement with above cited papers, the dermal absorption of the UV filters studied was formulation-dependent. The least influence of the vehicle on the cumulative filter amount in our study was noted with the most hydrophobic filter (EHMC). According to Roberts et al. (2004), if a hydrophobic vehicle is used (here W/O emulsion), the release of compound may decrease as log Po/w increases. The more soluble the compound in the vehicle, the more likely it is to be retained within the vehicle (WHO, 2006).

3.4. Results of the 4th set of experiments: the skin reservoir effect; (i) 6-h skin exposure; (ii) 6-h skin exposure + 18-h free permeation; 0.5 mg/cm^2 of the sunscreen (Table 4, Fig. 3)

In the final set of experiments, we tried to mimic the real-life habits of consumers when applying sunscreens as closely as possible. Therefore, a sunscreen dose of 0.5 mg/cm^2 under non-occluded conditions was applied. According to Fernandez et al. (2000) in line with our view, the exposure period used for *in vitro* studies should also reflect in-use conditions; long skin exposures to sunscreens (24 h or 48 h) are inappropriate and not realistic. The cited authors applied different sunscreen vehicles to pig-ear skin for 8 h. In our study, the 6-h pig-ear skin exposure period (EP_6) was chosen.

The results presented in Table 4 demonstrate the skin distribution (μg/cm^2) of the three filters at the same applied sunscreen dose (0.5 mg/cm^2) after the 6-h exposure of frozen stored pig-ear skin. From Fig. 3 it is possible to visualize the distribution of each UV filter in the skin (% AD).

It has long been understood that a chemical substance, instead of passing entirely through the skin, can remain partly in the skin and can act as a reservoir, being released (or not) at a later time (Roberts et al., 2004; WHO, 2006). The duration of the reservoir depends on the nature of the permeant, the vehicle used, the temperature of the skin, and the relative humidity to which the skin is exposed (WHO, 2006). Doan et al. (2010), studying the relationship between *in vitro* and *in vivo* skin absorption of lipophilic cosmetic ingredients after 24 h and 7 h from the application of O/W emulsion, observed that lipophilic chemicals initially form a reservoir in the skin and then diffuse out of the skin within the next 72 h. Unfortunately, sporadic studies focused on a reservoir effect in relation to the UV filters are mainly aimed at reservoir capacity of the SC only (Fernandez et al., 2000; Teichmann et al., 2005). Therefore, we were interested in a reservoir effect and a potential release of the filters accumulated in the skin after 24 h (EP_{6+18}) from the start of the skin exposure to a sunscreen. The total recovery of all the filters under this set of the study ranged from 91.8 to 107.4% AD; the values are within the range 85–115% AD recommended by SCCS (2012).

For both cases and both emulsion types, consistent with our results presented in Section 3.3, the highest amount of the filters remained on the skin surface; expressed in % AD, approximately 81–86% for BP3, 82–85% for EHMC, and 84–87% for BMDBM. The amounts of BP3 and EHMC in the RF immediately after 6-h skin exposure were significantly different from the ones after the following 18-h permeation from both emulsions. For BMDBM such a comparison cannot be done; if a certain amount permeated into the RF after EP_6 or EP_{6+18}, it was below the LoQ.

To compare the systemically available amounts of the filters after EP_6 and EP_{6+18}, the amounts in the dermis and the RF (Table 4) were summed and recalculated using the correction coefficient for each UV filter in the same manner as in Section 3.3. The results showed that after EP_6 and EP_{6+18} approximately these amounts of UV filters could enter the systemic circulation in humans: 6.1 and 8.1 μg/cm^2 (W/O), 4.7 and 7.6 μg/cm^2 (O/W) for BP3; about 0.7 and about 1.8 μg/cm^2 (W/O), about 0.4 and about 1.2 μg/cm^2 (O/W) for EHMC; about 1.1 and 1.8 μg/cm^2 (W/O) and incalculable values (O/W) for BMDMB, respectively. Expressed through the multiple, there was a significant increase of the amount of systemically available filters as a result of the following 18-h permeation without any other sunscreen on the skin; i.e. about 1.3–1.6-fold for BP3 and 2.6–3.0-fold for EHMC.

The findings confirmed our assumption that the skin absorption of UV filters does not occur instantaneously, but the compound concentration in the deeper levels of the skin increases over time.

Although most studies have emphasized the SC as a reservoir for lipophilic compounds, we agree with Roberts et al. (2004), that the viable avascular tissue (viable epidermis), and in the dermis may themselves act as reservoirs. The UV filters contained therein are gradually released and diffuse into the dermis and the RF after removal of the sunscreen from the skin surface. In our view when assessing dermal absorption of UV filters in relation to the systemic availability it is not sufficient to evaluate the score immediately at the end of the exposure period (i.e., promptly after washing off a sunscreen), but it is necessary to follow the subsequent release of the compounds. We intend to continue this topic in the future.

3.5. The Systemic Exposure Dosage and the Margin of Safety (Table 5)

Finally, based on our experimental findings, we made an estimate of the Systemic Exposure Dosage (SED) for each filter on humans treated with finished sunscreens similar to the formulation used in this study. The SED of a cosmetic substance is the amount expected to enter the blood stream (and therefore be systemically available) per kg body weight and per day. It is expressed in mg/kg body weight/day. For this definition, the human body weight of 60 kg is commonly accepted (SCCS, 2012).

It is understandable that conditions *in vitro* cannot completely simulate those carried out on living creatures. *In vivo* studies in humans are the gold standard. Today, however, *in vivo* dermal absorption testing is not any more an option for cosmetic substances and finished cosmetic products in the European context (EC, 2009), as the animal testing deadline of 11 March 2013 has passed. Therefore, when the SED value is estimated, the results of *in vitro* experiments are accepted. Of course, experimental factors affecting dermal absorption *in vitro* (inter alia, a species and thickness of skin sample, composition of the RF, dose of the test substance, occlusion or non-occlusion of the test area and the exposure period) should be chosen as closely as possible to the "in-use" conditions *in vivo* and be defined.

The SED values in this study were estimated for one reasonably predictable situation mimicking the behavior of consumers, i.e. the human skin treatment with a W/O sunscreen at 0.5 mg/cm^2, followed by taking shower after 6 h. Prediction of total skin absorption of the UV filters was made 24 h after the application of a sunscreen (i.e., after 6-h skin exposure followed by 18-h permeation). In a classical *in vitro* dermal absorption setting, the total amount measured in the epidermis (without the SC), dermis and the RF is considered to be systemically available and taken into account for further calculations (SCCS, 2010, 2012; OECD, 2011). The reason specified in Section 3.3, the cumulative amounts of the filter absorbed only in the dermis and the RF were considered to be systemically bioavailable in this study. Moreover, based on our experimental results in Section 3.1, the amounts absorbed through frozen-stored FTS were corrected using the fresh/frozen-stored FTS permeability coefficient for W/O emulsion (0.83, 0.59, and 0.74 for BP3, EHMC, and BMDBM, respectively).

The SED values were calculated under the "Notes of guidance for testing of cosmetic ingredients and their safety evaluation in Europe" (SCCS, 2012).

$$\mathrm{SED} = \frac{\mathrm{DA}\ (\mu g/cm^2) \times \mathrm{SSA}\ (cm^2) \times \mathrm{F}\ (day^{-1})}{60\ (kg)}, \quad (1)$$

where the SED (μg/kg bw/day) is the Systemic Exposure Dosage; DA is dermal absorption of the substances reported as amount (μg/cm^2); SSA (cm^2) is the skin surface area expected to be treated with the cosmetic product; F (day^{-1}) is the usual frequency of application of the cosmetic product; 60 kg is default human body weight (SCCS, 2012).

It is necessary to note that the DA values were measured in this study with a frequency of application of 1. According to the SCCS (2012), for calculating the SED, the frequency (F) of application of 2 per day for the sun care cosmetics is recommended as normal foreseeable use value. Therefore, the value of F = 2 was taken into account when calculating the SED also in this study (Table 5). So the SED value is based on the assumption that the total DA would double in going from 1 to 2 applications.

Our scenarios consisted of two cases (i) a whole-body treatment with a sunscreen on a sunbathing day; (ii) a face treatment with a sunscreen on a non-specific day (Table 5). The calculated SED values of BP3, EHMC, and BMDBM for humans as a consequence of (i) whole-body treatment were 4744, 1032, and 1036 μg/kg bw/day; and for (ii) face treatment were 153, 33, and 34 μg/kg bw/day, respectively.

Based on the SED values the Margin of Safety (MoS) values for each UV filter were calculated according to Eq. (2) (SCCS, 2012).

$$\mathrm{MoS} = \frac{\mathrm{NOAEL}\ (\mathrm{mg/kgbw/day})}{\mathrm{SED}\ (\mathrm{mg/kgbw/day})}, \quad (2)$$

where the NOAEL is the No Observable Adverse Effect Level, and the SED is the Systemic Exposure Dosage value during normal foreseeable use.

The NOAEL-values taken into calculation (from the dermal repeated dose studies) were 200 mg/kg body weight/day for BP3 (SCCP, 2008), 500 mg/kg body weight/day for EHMC (SCCNFP, 2001) and 200 mg/kg body weight/day for BMDBM (BfR, 2003).

The relation of the NOAEL to the SED, i.e. the value of the MoS, should be at least 100-fold. If the MoS value exceeds 100, the compound is regarded as safe for use (SCCP, 2008). The factor of 100 is empirical and takes into account differences between susceptibility of a man and test species (interspecies differences) as well as differences between individuals (intraspecies differences).

The Margin of Safety (MoS) values for BP3, EHMC, and BMDBM were estimated (i) 42, 485, and 192 in the case of a whole body sunscreen treatment twice per day over 6 h and (ii) 1307;15,151, and 5882 in the case of a face sunscreen treatment twice per day over 6 h, respectively (Table 5). With one exception, all of the MoS values were higher than 100 and would consequently be acceptable. However, if the PCPs containing BP3 at the maximum concentration authorized in the EU and Australia (10%) for use in the PCPs would be applied on the total area of the human body in an amount of 0.5 mg/cm^2 twice daily for 6 h, the MoS value of 42 indicates a possible health risk.

However, it should be emphasized once again: the SED values were calculated on the assumption that the total dermal absorption of the UV filters would double in going from 1 to 2 applications (i.e., twice a day) of a sunscreen. If that were not quite, it is likely that the true SED value would be lower and therefore the actual MoS value would be higher, but certainly less than 100 for BP3, so the overall conclusions would not change.

The SCCP in 2008 assessed the study of the Chemie Wirtschaftsförderungs GmbH (CWFG, 2007; unpublished data) concerning the *in vitro* percutaneous absorption of BP3 in standard O/W and W/O sunscreen formulations through viable porcine-ear skin, which was recognized as scientifically acceptable. Based on the mean dermal absorption level of 19.3 μg/cm^2 of the applied dose (W/O), there was determined the MoS value of 112 for BP3 as a UV filter in sunscreens up to 6% in the SCCP (2008). Since the MoS was >100, the SCCP stated the opinion that "The use of Benzophenone-3 as a UV filter up to 6% in cosmetic sunscreen products and up to 0.5% in all types of cosmetic products to

protect the formulation does not pose a risk to the health of the consumer, apart from its contact allergenic and photoallergenic potential (SCCP, 2008). The 10% maximum concentration in ready for use preparations of BP3 however, remained unchanged. We are of the opinion that it would be appropriate to reconsider the maximum permissible concentration of BP3 in the final PCPs in the EU.

However, in the context of the results of this study it is necessary to express a few comments. On the one hand, PCPs containing BP3 and other UV filters are re-applied several times during the days of the hot season (Wu et al., 2010; Biesterbos et al., 2013) in the course of long years of life. On the other hand, generally, consumers apply sunscreens to less than 100% of their body surface area and sunscreens are lost from the skin by washing off, sweating, skin desquamation, as well as loss through absorption to clothes. Furthermore, by Benech-Kieffer et al. (2003) and Nohynek et al. (2010) the *in vitro* skin penetration test tends to overestimate actual human systemic exposure when compared side-by-side with actual human internal exposure data. Maximizing exposure data and accumulating the worst-possible scenarios may then create imaginary health risks where actually none exist. Since skin absorption of chemicals is dependent on the anatomical site, skin condition, and hydration state of the skin, there can be major differences in permeability of UV filters among different persons (Benson et al., 2005). The special situation concerns children; compared with adults, children have a three-fold higher ratio of body surface to body weight (BfR, 2003; Nohynek et al., 2010).

Although UV filters are key ingredients used in PCPs at levels greater than many other ingredients, there are still insufficient data regarding their actual metabolism and accumulation in the human body. Generally, UV filters absorbed through the skin into the vascular system reach to kidneys, where are either metabolized or remain intact. Urine is the first route and faeces are the second route for excretion of parent UV filters and alternatively also their metabolites. Concerning BP3 some studies have shown that the intact compound and its derivatives or metabolites were found widely in various human bodily fluids, such as urine (Calafat et al., 2008; Kunisue et al., 2010, 2012; León et al., 2010; Zhang et al., 2013; Kim and Choi, 2014), blood (Zhang et al., 2013), breast milk (Ye et al., 2008), and semen (León et al., 2010) in correlation with the frequency of use of PCPs. For example, according to Kunisue et al. (2012), there were found BP3 and its derivatives in 99,9% of urine collected from 625 US women, significantly in higher concentration during the summertime than in other months of the year. In addition, these authors reported an association between exposure to high concentrations of BP3 and its derivatives and endometriosis as a gynecological disorder that affects reproductive-age women and is typically defined as an estrogen-dependent disease. Krause et al. (2012) demonstrated a link between high concentrations of BP3 in mothers' urine and decreased birth weight in girls and increased birth weight and head circumference in boys. Zhang et al. (2013) stated that in China, the use of BP3 in cosmetics has been rapidly increasing over the past decade, but body burdens of BP3 in Chinese were lower than those reported for the US populations. The authors detected BP3 level in 83% of adults, 35% of pregnant women and 30% of children. Females had higher urinary concentrations of BP3 than males; the concentration ratio of the BP3 between blood and urine in adults was 0.21.

Finally, it must be observed that human exposure to BP3 and its metabolites as well as to other UV filters and their metabolites can also occur indirectly via the food chain, for example, through the fish tissues originating from the lakes contaminated with UV filters (Kim et al., 2014; Manová et al., 2015).

4. Conclusions

Personal care products containing UV filters have two different toxicological aspects. On the one hand, they should protect the consumer against adverse effects of the solar radiation. On the other hand, some UV filters can have side effects with potential health risks to the consumer.

Usage patterns of PCPs are important factors for risk assessment. In this study, the potential of BP3, EHMC, and BMDBM for systemic absorption in humans was investigated using the *in vitro* technique mimicking the real skin treatment with sunscreens by the consumer as closely as possible. Taking into account all the above, we would like to summarize our key findings.

Firstly, the exposure to EHMC and BMDBM (at the maximum level allowed for cosmetics in the EU and Australia) poses low health risk from systemic absorption when applied to the intact skin in a sunscreen product similar to the model formulations under conventional consumer use conditions. The estimated Margin of Safety values of both compounds for these conditions should be considered acceptable (>100). However, based on the results of this study, it can be assumed that neither of these two filters have a high affinity for the SC and do not meet the general requirement for the primary accumulation of UV filter in it. We consider this finding worrying, taking into account, on the one hand, the possible undesirable side effects of the compounds and, on the other hand, the large body area and frequency of skin treatment with a sunscreen or everyday-use cosmetic products containing them, perhaps several times a day (e.g. in the summertime) for many years. We would like to study the impact of the sunscreen re-application to an intact and slightly damaged skin in the near future.

Secondly, BP3 showed significant potential to pass through the skin and be systemically available. Even in the case of an administration of a low (realistic) dose of a sunscreen product (0.5 mg/cm^2, containing 10% of BP3) on the whole-body area twice per day over 6 h, the MoS value of 42 (<100) indicates a possible health risk. Given the above findings, and also in view of its suspected endocrine-disrupting activity (although weak), we consider that it would be appropriate to reduce the maximum authorized concentration of BP3 (up to 10%) for use in the final PCPs in the European Union, may be up to 6% (as in the US) or to up to 5% (as in Japan).

Thirdly, the increased dose of the sunscreen had a significant impact on increasing the systemically available amount of all three UV filters. However, this increase is not linearly dose-dependent. Although the $AD_{2.0}$ was 4-fold greater than the $AD_{0.5}$, the systemically available amounts of BP3 or EHMC were only from 2.8 to 2.1-fold greater. As it has been shown by other researchers, sunbathers tend to apply only about a quarter (0.5 mg/cm^2) of the recommended sunscreen dose (2.0 mg/cm^2) that is necessary to obtain the full efficacy associated with a SPF. Although this is not the cause of a low sunscreen dose use by a consumer, in our view, the concerns about extremely increased skin permeation in connection with the higher dose are unfounded.

Fourthly, based on our findings referred to in Section 3.4, we consider that when assessing the dermal absorption of UV filters in relation to the potential systemic availability, it is not a sufficient score at the end of the exposure (i.e., immediately after washing off sunscreen), but it is necessary to monitor the subsequent release of the compound absorbed in the dermis. We also intend to study in the near future the skin reservoir effect on permeation of the UV filters.

Fifthly, there is no doubt that, given the recent developments, the safety and toxicological profiles of some UV-absorbing filters used for long decades are still not clear. But the new UV filters, before marketing, are subjected to a stringent safety assessment. Therefore we would like to emphasize that the aim of this study was not to escalate a phobia towards all UV filters, leading to an

undesirable increase in consumer concerns about using sunscreens, but to contribute to the knowledge as certain "in-use" conditions affect the dermal absorption of the assessed UV filters. We agree with Nohynek and Schaefer (2001) that sunscreen phobia may lead to a decrease in sunscreen use, higher exposure to ultraviolet radiation, and subsequent adverse health effects, including increasing rates of melanoma and other skin cancers.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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The stripping technique: in vitro absorption and penetration of five UV filters on excised fresh human skin.

Potard G1, Laugel C, Schaefer H, Marty JP.

Abstract
This article gives the results of a study whose aim was to compare the compartmental distribution and absorption of 5 UV filters, in vitro, by fresh human skin, after exposure times of 30 min and 16 h. These UV filters from BASF (octyl methoxycinnamate, benzophenone 4, benzophenone 3, octyl triazone and octocrylene) were incorporated separately in a simple oil-in-water emulsion. The composition of the emulsions was designed in order to obtain a sun protection factor of 5. Therefore the UV filters were introduced into the emulsions at different concentrations. We show that the affinity for each skin level [stratum corneum (SC), viable epidermis, dermis and receptor fluid] is different according to the test substance used. Some substances accumulated in the SC, whereas others passed through the skin very quickly and were quantified in the receptor fluid. The stripping technique allowed us to see that more than 94% of the chemical compound in the SC was in the first eight tapes. The problem of individual values below the limit of detection was raised, a correlation between the two exposure times was found ($y = 1.702x - 0.105$; $R = 0.94$) and a classification of products according to their affinity for the SC was determined.

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Sunscreens in human plasma and urine after repeated whole-body topical application.

Janjua NR1, Kongshoj B, Andersson AM, Wulf HC.

Abstract
BACKGROUND:
The three chemical ultraviolet absorbers benzophenone-3 (BP-3), octyl-methoxycinnamate (OMC) and 3-(4-methylbenzylidene) camphor (4-MBC) are commercially used in sunscreens worldwide. Apart from sun protection, they may possess endocrine-disrupting effects in animals and in vitro. For all three compounds, only sporadic measurements of percutaneous absorption and excretion after topical application in humans have been described.
METHODS:
In this study, 32 healthy volunteers, 15 young males and 17 postmenopausal females, were exposed to daily whole-body topical application of 2 mg/cm(2) of sunscreen formulation at 10% (w/w) of each for 4 days. Blood concentrations were measured at 0, 1, 2, 3, 4, 24 and 96 h and urine concentrations at 0, 24, 48, 72 and 96 h.

RESULTS:
Almost all three sunscreens were undetectable in plasma and urine before the first application. One to 2 h after the first application, all three sunscreens were detectable in plasma. The maximum median plasma concentrations were 187 ng/mL BP-3, 16 ng/mL 4-MBC and 7 ng/mL OMC for females and 238 ng/mL BP-3, 18 ng/mL 4-MBC and 16 ng/mL OMC for men. In the females, urine levels of 44 ng/mL BP-3 and 4 ng/mL of 4-MBC and 6 ng/mL OMC were found, and in the males, urine levels of 81 ng/mL BP-3, 4 ng/mL of 4-MBC and OMC were found. In plasma, the 96-h median concentrations were higher compared with the 24-h concentrations for 4-MBC and OMC in men and for BP-3 and 4-MBC in females.

23) J Microencapsul. 2010 May;27(3):253-62. doi: 10.3109/10717540903097770.

Skin absorption studies of octyl-methoxycinnamate loaded poly(D,L-lactide) nanoparticles: estimation of the UV filter distribution and release behaviour in skin layers.

Vettor M1, Bourgeois S, Fessi H, Pelletier J, Perugini P, Pavanetto F, Bolzinger MA.

Abstract
New formulation strategies have to be developed to limit the skin penetration of UV-filter. Nanoparticles (NP) are very suitable for that purpose. In this study, the skin distribution, at different times (1, 2 and 3 h), of octyl-methoxycinnamate (OMC) from loaded PLA-nanoparticles was compared to a classical formulation containing non-encapsulated OMC, using the Franz cell method. The results showed that the OMC penetration was clearly impeded by stratum corneum and that the major part of the OMC-NP was accumulated at the skin surface (> 80%). A significant lower OMC amount was quantified in viable skin with NP compared to the OMC emulgel. To accurately determine the real OMC amount in close contact with viable skin layers two solvents were used to extract OMC from the skin compartments. Acetone (ACET) allowed quantifying both OMC in NP and OMC released from the particles, while isopropylmyristate (IPM), a non-solvent of the NP polymer (PLA), allowed quantifying only OMC released from the particles. Using IPM as an extraction solvent, it appeared that the OMC released from NP, in contact with viable skin, was 3-fold lower than free OMC diffused from the emulgel. Lastly, a sustained release was observed when nanoparticles were used.

J Invest Dermatol. 2004 Jul;123(1):57-61.

Systemic absorption of the sunscreens benzophenone-3, octyl-methoxycinnamate, and 3-(4-methyl-benzylidene) camphor after whole-body topical application and reproductive hormone levels in humans.

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Abstract
Recent in vitro and animal studies have reported estrogen-like activity of chemicals used in sunscreen preparations. We investigated whether the three sunscreens benzophenone-3 (BP-3), octyl-methoxycinnamate (OMC), and 3-(4-methylbenzylidene) camphor (4-MBC) were absorbed and influenced endogenous reproductive hormone levels in humans after topical application. In this 2-wk single-blinded study 32 healthy volunteers, 15 young males and 17 postmenopausal females, were assigned to daily whole-body topical application of 2 mg per cm(2) of basic cream formulation without (week 1) and with (week 2) the three sunscreens at 10% (wt/wt) of each. Maximum plasma concentrations were 200 ng per mL BP-3, 20 ng per mL 4-MBC, and 10 ng per mL OMC for females and 300 ng per mL BP-3, 20 ng per mL 4-MBC, and 20 ng per mL OMC for men. All three sunscreens were detectable in urine. The reproductive hormones FSH, LH were unchanged but minor differences in testosterone levels were observed between the 2 wk. A minor difference in serum estradiol and inhibin B levels were observed in men only. These differences in hormone levels were not related to sunscreen exposure.

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REVIEW

An overview of UV-absorbing compounds (organic UV filters) in aquatic biota

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Abstract The purpose of this article is to summarize biological monitoring information on UV-absorbing compounds, commonly referred as organic UV filters or sunscreen agents, in aquatic ecosystems. To date a limited range of species (macroinvertebrates, fish, and birds), habitats (lakes, rivers, and sea), and compounds (benzophenones and camphors) have been investigated. As a consequence there is not enough data enabling reliable understanding of the global distribution and effect of UV filters on ecosystems. Both liquid chromatography and gas chromatography coupled with mass spectrometry-based methods have been developed and applied to the trace analysis of these pollutants in biota, enabling the required selectivity and sensitivity. As expected, the most lipophilic compounds occur most frequently with concentrations up to 7112 ng g^{-1} lipids in mussels and 3100 ng g^{-1} lipids (homosalate) in fish. High concentrations have also been reported for 4-methylbenzilidenecamphor (up to 1800 ng g^{-1} lipids) and octocrylene (2400 ng g^{-1} lipids). Many fewer studies have evaluated the potential bioaccumulation and biomagnification of these compounds in both fresh and marine water and terrestrial food webs. Estimated biomagnification factors suggest biomagnification in predator–prey pairs, for example bird–fish and fish–invertebrates. Ecotoxicological data and preliminary environmental assessment of the risk of UV filters are also included and discussed.

Keywords UV filters · Biota · Chromatography · Mass spectrometry · Bioaccumulation · Toxicity

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Introduction

UV filters, including both inorganic and organic sunscreen agents, constitute a group of emerging environmental pollutants, potentially hazardous compounds that have been receiving steadily growing attention over the last decade as society has become aware of the dangerous effects of UV solar radiation. These chemicals can be found not only in cosmetics but also in other personal care products, food packaging, pharmaceuticals, plastics, textiles, and vehicle-maintenance products to prevent photodegradation of polymers and pigments [1, 2].

Incomprehensibly, there are scarce data about, and limited understanding of, the environmental occurrence, fate, distribution and effects of many UV filters and their metabolites and other transformation products, despite their extensive use. According to market studies, sunscreen product sales were higher than half a billion US dollars in 2005, and it is estimated that 10,000 tons of UV filters are produced annually for the global market [3].

It is likely that usage of sunscreen agents is going to increase in the future, because of the recommendations of health authorities on the prevention of skin cancer. One of the main reasons for the scarcity of data was the lack of suitable analytical methods capable of detecting emerging

pollutants at trace levels in, usually, complex environmental matrices. However, as a consequence of increasing concern about the potential effects of sunscreens on ecosystems and humans, in last five years environmental analytical scientists have developed sensitive and selective analytical methods.

Eco-toxicological data on both UV filters and their degradation products is also missing. Despite the small amount of information available about their toxicity, the low environmental concentrations reported so far suggest a low potential risk. However, the long-term risk associated with the pseudo-persistence of these chemicals in the environment is largely unknown.

The purpose of this review is to summarize scarce and scattered information about the profiles of UV filters in aquatic organisms, analytical methods, bioaccumulation/biomagnification, ecotoxicity, and environmental analysis and risk assessment (ERA). Finally, the article identifies current gaps in our knowledge and potential future research needs in ERA.

Physicochemical properties

UV filters are substances with almost null absorption of visible radiation but important light absorption in the UVA (315–400 nm) and UVB (280–315 nm) ranges [4]. Sunscreens can be classified into organic (chemical) absorbers and inorganic (physical) blockers on the basis of their mechanism of action. Organic UV filters absorb UV radiation with excitation to a higher energy state. Excess energy is dissipated by emission of higher wavelengths or relaxation by photochemical processes, for example isomerization and heat release. They include camphors, benzophenones, cinnamates, triazines, among others. Inorganic sunscreens, i.e. titanium dioxide and zinc oxide, protect the skin by reflecting and scattering UV radiation.

The focus of this review is on organic UV filters. A feature common to all of these is the presence of an aromatic moiety with a side-chain with different degrees of unsaturation. Their structures and other physicochemical properties are listed in Table 1. Some, for example 4-methylbenzylidene camphor (4MBC), ethylhexylmethoxy cinnamate (EHMC), and octocrylene (OC), are chiral compounds. Although the enantiomers of these compounds are not expected to have different physicochemical properties, isomers and enantiomers may differ in biological behavior. Commercial formulations contain mainly geometrical (*E*) isomers, although some UV filters (e.g., methoxycinnamates) contain both the (*E*) and the (*Z*) isomers. Because of the high lipophilicity and poor biodegradability of many UV filters (mostly with log K_{ow} 4–8) they end up in sewage sludge during wastewater treatment [5–8], and accumulate in river sediments [9–12] and biota [13–20].

Analytical methodology

Sampling and sample preparation

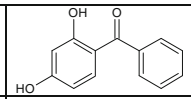
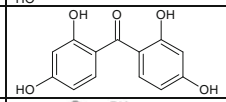
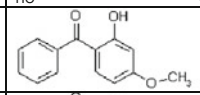
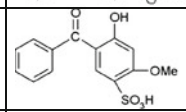
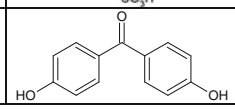
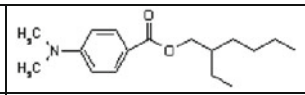
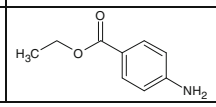
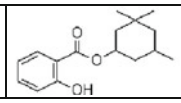
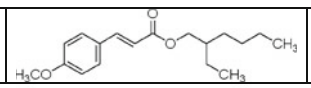
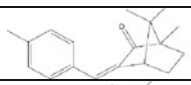
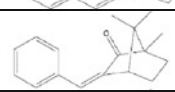
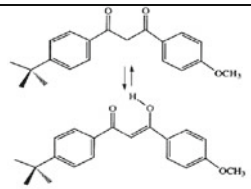
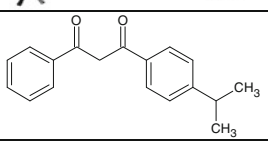
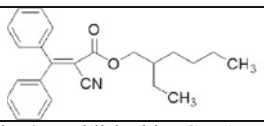
Sampling procedures for analysis of residues of UV filters in aquatic biota mainly involve traditional fishing, either by native fishers or by electric fishing, for which special permission is often needed. Unlike other matrices, there is the added difficulty of the availability of samples of the desired species, which often depends on external factors which are difficult to control. Moreover, the variability between individuals of the same species (size and living cycle) hinders comparison of results. Most studies have focused on fish, a representative matrix of the aquatic environment assumed to be able to retain and bioaccumulate UV filters because of the lipophilicity of the compounds. The most usual sample analyzed is muscle, probably because of its low lipid content in comparison with other tissues and because it is part of the human diet. Studies have also been conducted on macrozoobenthos, mussels, and birds. Selected tissues are homogenized by blending and often freeze-dried before extraction.

Extraction and clean up

Extraction of UV filters from tissues has been achieved by conventional Soxhlet extraction (which has become less attractive because of the time and solvent consumed) [16, 18], pressurized-liquid extraction (PLE) [14], solid–liquid extraction [13–15, 17, 19], and microwave-assisted extraction (MAE) [20]. These techniques lead to coextraction of a lipid fraction that must be removed before determination of the UV filters. Clean-up of biota sample extracts is usually a two-stage process. The sample extracts can first be subjected to gel-permeation chromatography (GPC), primarily to remove lipids, followed by adsorption chromatography on silica or Florisil columns. Quite often RP-HPLC has also been used for extraction and purification.

In the first work published on UV filter levels in biota [18], benzophenone-3 (BP3), 4MBC, homosalate (HMS), EHMC, ethylhexyl dimethyl PABA (OD-PABA), isopropyl dibenzoyl methane (IDM), and butyl methoxy dibenzoyl methane (BM-DBM) were extracted from fish tissue by Soxhlet extraction. The tissue was first homogenized and dried with sodium sulfate, then extracted with petroleum ether–ethyl acetate 2:1 (*v*/*v*). Lipids and other potential matrix interferences were removed by GPC (Bio Beads SX-3) with cyclohexane–acetone 3:1 (*v*/*v*) as mobile phase. For analysis of IDM and BM-DBM, CH_3I/NaH was added to half of the extract to form their derivatives for further GC–MS analysis. This half of the extract was then purified on a silica column (elution with hexane–ethyl acetate 7:3 (*v*/*v*)). The other half was also loaded on to a silica column and

Table 1 Physicochemical properties of the organic UV filters addressed in this review. In parentheses, the key system adopted herein

Name (INCI nomenclature)[a]	CAS no.	Structure	Molecular weight (g mol^{-1})	Log K_{ow}	Solubility (g L^{-1})[b]
Benzophenones					
Benzophenone-1 (BP1)	131-56-6		214.22	3.15[c]	0.39[c]
Benzophenone-2 (BP2)	131-55-5		246.22	2.78[d]	0.98[c]
Benzophenone-3 (BP3)	131-57-7		228.24	3.79[d]	0.10[c]
Benzophenone-4 (BP4)	4065-45-6		308.31	0.993[c]	11[c]
4,4′-Dihydroxybenzophenone (4DHB)	611-99-4		214.22	2.19[d]	0.6[c]
p-Aminobenzoic acid derivatives					
Ethylhexyldimethyl PABA (OD-PABA)	21245-02-3		277.4	5.412[c]	4.7 × 10^{-3}[c]
Ethyl-PABA (Et-PABA)	94-09-7		165.19	1.86[d]	1.31[d]
Salicylates					
Homosalate (HMS)	118-56-9		262.35	5.947[c]	0.021[c]
Cinnamates					
Ethylhexyl methoxycinnamate (EHMC)	5466-77-3		290.4	5.8	6.4 × 10^{-3}[c]
Camphor derivatives					
4-Methylbenzylidene camphor (4MBC)	36861-47-9		254.37	4.95	0.017
3-Benzylidene camphor (3BC)	15087-24-8		240.34	2.84	0.034
Dibenzoylmethane derivatives					
Butylmethoxydibenzoylmethane (BM-DBM)	70356-09-1		310.39	4.191	4.3 × 10^{-3}[c]
Isopropyldibenzoylmethane (IDM)	63250-25-9		266.33	4.382	0.027
Crylenes					
Octocrylene (OC)	6197-30-4		361.49	6.88	3.6 × 10^{-4}[d]

[a] INCI (International Nomenclature for Cosmetic Ingredient) established by CTFA and COLIPA

[b] In water at 25 °C

[c] Calculated by use of Advanced Chemistry Development (ACD/Labs) Software V11.02 (1999–2011 ACD/Labs)

[d] Experimental values from database of physicochemical properties; Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm

the same solvent mixture in a different proportion (91:9 *v*/*v*) was used for elution.

A similar method was developed by Meinerling and Daniels [16] for analysis of 4MBC, BP3, EHMC, and OC in the muscle of rainbow trout. In this case Soxhlet extraction with *n*-hexane–acetone 9:1 (*v*/*v*) was followed by GPC (Bio Beads SX-3) with cyclohexane–ethyl acetate 1:1 (*v*/*v*) as eluent. In a further clean-up step, a Florisil column was used to remove polar compounds.

In the procedure followed by Balmer et al. [14] for analysis of 4MBC, BP3, EHMC, and OC, fish samples were homogenized with sodium sulfate and column extracted or PLE extracted with dichloromethane (DCM)–cyclohexane 1:1 (*v*/*v*). The extracts were then cleaned by GPC on a Biobeads S-X3 column with DCM–cyclohexane 35:65 (*v*/*v*) as eluent, followed by silica purification. Buser et al. [15] extracted 4MBC and OC by successive extraction with potassium oxalate (2 mL, 35 %), ethanol (100 mL), diethyl ether (50 mL), and *n*-pentane (70 mL). After extraction, matrix components were removed by GPC and silica purification.

The methods described above are only suitable for extracting UV filters with similar physicochemical properties. A method for simultaneous determination of nine UV filters, from polar to lipophilic, in fish has been reported by Zenker et al. [17]. Mid-polar and lipophilic UV filters were extracted from homogenized tissue by solvent extraction with ethyl acetate–*n*-heptane–water 1:1:1 (*v*/*v*) and further purified by reversed-phase RP-HPLC. The fraction containing mid-polarity UV filters was analyzed by HPLC–MS whereas the fraction containing the lipophilic ones was determined by GC–MS. Polar and medium-polarity UV filters were extracted with a mixture of methanol (MeOH) and acetonitrile (ACN), followed by HPLC–MS analysis. This is the procedure requiring the smallest amount of sample (4 g); good limits of detection are achieved for most compounds. The same method proved to be suitable for analysis of macrozoobenthos and bird samples also.

Bachelot et al. [20] developed a method for determination of EHMC, OC, and OD-PABA in marine mussels. MAE was performed with 25 mL acetone–heptane 1:1 (*v*/*v*). After extraction, the liner was rinsed with the same solvent mixture. The extracts were percolated through anhydrous sodium sulfate. Further purification was performed by RP-HPLC on a RP Spherisorb ODS2 column (4.6 mm×150 mm, 5.0 μm) following a procedure adapted from Zenker et al. [17].

GPC or column purification with silica or Florisil is useful whenever compounds with similar physicochemical properties must be separated from interfering matrix substances, for example lipids, present in the sample. When these methods are used for a mixture of compounds with different physicochemical properties they are less effective. RP-HPLC is a suitable alternative when UV filters with a large range of physicochemical properties must be analyzed.

Instrumental analysis

LC is the technique of choice for the analysis of UV filters in cosmetic products. In contrast, GC is preferred for their environmental analysis. Nevertheless, both techniques have been applied to the analysis of biological samples. The low concentration of the target analytes in biota samples requires high sensitivity and selectivity. Therefore, mass spectrometric (MS) detection is the most suitable technique for determination of these compounds in such complex matrices. Relevant data on analytical methods are summarized in Table 2.

GC–MS

UV filters are, with very few exceptions (e.g., octyl triazone (OT) and BM-DBM), amenable to GC. Matrix effects are not critical for the ionization modes, e.g. electron impact (EI) or chemical ionization (CI), typically used in GC–MS. As a consequence, method detection limits (MDL) are usually quite low [21]. On the other hand, this technique can only be successfully applied to a limited number of non-polar and volatile compounds. For more polar or thermally unstable compounds an additional derivatization step is required; here differences in matrix components may result in quite different derivatization efficiencies which may affect both precision and accuracy of the analysis.

Analysis has always been performed in electron-impact mode (GC–EI-MS). Quantification is achieved by operating in selected ion monitoring mode (SIM). The fragment ions usually selected for the quantification and confirmation of the analytes are listed in Table 2.

BM-DBM, IDM, 4MBC, OD-PABA, HMS, EHMC, and BP3 have been analyzed by GC–MS on a SE-54-CB column (50 m×55 mm, 0.25-μm film), working in SIM mode [18]. Balmer et al. [14] analyzed 4MBC, BP3, EHMC, and OC in fish by GC–EI-MS using two different columns a BGB-5 (30 m×0.25 mm; 0.25 μm) and an SE54 (25 m×0.32 mm; 0.25 μm). In that study $^{13}C_{12}$-PCB 77 was used as surrogate standard. Under the same GC–EI-MS conditions Buser et al. [15] analyzed 4MBC and OC in fish tissue, using $^{15}N_3$-musk xylene as internal standard. Zenker et al. and Fent et al. [17, 19] analyzed nine UV filters with a large range of physicochemical properties (log K_{ow} from 0.9 to 5.7) in fish, macrozoobenthos, and bird tissue. Four out of the nine UV filters investigated, BP3, 3BC, 4MBC, and EHMC (the most lipophilic) were detected by GC–EI-MS on an Optima-5-MS (50 m×0.2 mm; 0.35 μm) column. In this study benzophenone-d_{10} was used as surrogate standard.

Mottaleb et al. [13] analyzed 4MBC and OC with 10 other personal care products in fish tissue by both GC–EI-MS and GC–EI-IT (with an ion trap mass spectrometer). The GC–EI-MS analysis was carried out with a XTI-5 capillary column (30 m×0.25 mm; 0.25 μm) operating in

Table 2 Analytical methodology and occurrence data for the UV filters addressed in this review

Matrix	Specie	Tissue	UV filter	Sample amount	Extraction	Purification	Technique
Fish	Bluegill (*Lepomis macrochirus*)	Muscle	4MBC, OC	1 g	Rotatory extraction with acetone	Silica	GC-EI-MS
	Sonora sucker (*Catostomus insignis*)	Muscle, belly flap and skin	4MBC, OC	1 g	Sonication with acetone	GPC Silica	GC-EI-IT
Fish	White fish (*Coregonus sp.*)	Muscle	4MBC, BP3, EHMC, OC	5 g	ASE extraction: Homogenized with diatomaceous earth 3 cycles DCM/ cyclohexane (1:1, v/v) at room temperature	GPC (EnviroSep-ABC or Biobeads S-X3) Silica	GC-EI-MS
	Roach (*Rutilus rutilus*) Perch (*Perca fluviatilis*)			20 g	Homogenized with sodium sulphate Column extracted with DCM/cyclohexane (1:1, v/v))		
Fish	Brown trout (*S.Trutta fario*)	Muscle plus adipose tissue under the skin	4MBC, OC	10–25 g	Homogenized in 100 ml water with hand blender Solvent Extraction using potassium oxalate (2 mL, 35 %), ethanol (100 mL), diethyl ether (50 mL) and n-pentane (70 mL)	GPC (EnviroSep-ABC or Biobeads S-X3) Silica	GC-EI-MS
Fish	Rainbow trout (*Oncorhynchus mykiss*)	Muscle	4MBC, BP3, EHMC, OC	10 g	Homogenized with sodium sulphate Soxhlet extracted with n-hexane/acetone (9/1, v/v)	GPC (Biobeads S-X3) Florisil	LC-ESI-MS/MS
Fish	Barb (*Barbus barbus*) and Chub (*Leucisus cephalus*)	Muscle plus adipose tissue under the skin	4MBC, 3BC, BP1, BP2, 4DHB, BP3, BP4, EHMC, Et-PABA	4 g	Solvent extraction using ethyl acetate, n-heptane and HPLC water (1:1:1, v/v/v) or Solvent extraction with MeOH:ACN (1:1, v/v)	RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 μm)	LC-ESI-MS/MS and GC-EI-MS
	Barb (*Barbus barbus*) and Chub (*Leucisus cephalus*)	Muscle plus adipose tissue under the skin	BP4, 4DHB, BP1, BP2, Et-PABA	1 g	Solvent extraction with MeOH/ACN (1:1, v/v)	Syringe filtration	LC-ESI-MS/MS
Fish	Roach (*Rutilus rutilus*) Perch (*Perca fluviatilis*)	Muscle, offal, rest and whole fish	IDM, BM-DBM, 4MBC, OD-PABA, HMS, EHMC, BP3		Homogenized with sodium sulphate Soxhlet extracted with petroleum ether:Etyl acetate (1:1, v/v)	GPC (Biobeads S-X3)	GC-EI-MS

Table 2 (continued)

Matrix	Specie	Tissue	UV filter	Sample amount	Extraction	Purification	Technique
macrozoobenthos	Mussels (*Dreissena polymorpha*)	Whole macroinvertebrate	BP1, BP2, BP3, BP4, 4DHB, Et-PABA, EHMC, 4MBC, 3BC	4 g (fraction 1) and 1 g (fraction 2)	Fraction 1: Solvent extraction using ethyl acetate, n-heptane and HPLC water (1:1:1, v.v:v) or Solvent extraction with MeOH: ACN (1:1, v/v)	Fraction 1: RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 μm) Fraction 2: Syringe filtration	LC-ESI-MS/MS and GC-EI-MS
	Gammarus sp				Fraction 2: Solvent extraction with MeOH: ACN (1:1, v/v)		
Fish	Chub (*Leuciscus cephalus*) Brown trout (*Salmo trutta*) Barb (*Barbus barbus*) Eel (*Anguilla anguilla*)	Muscle plus adipose tissue under the skin					
Bird	Cormorants (*Phalacrocorax sp*)	Muscle					
Mussel[a]	*Mytilus edulis* and *Mytilus galloprovincialis*	Soft tissue	EHMC OC OD-PABA	3 g	MAE extraction with acetone:heptane (1:1, v/v)	Filtered (0.2 μm)) through 10 g anhydrous sodium sulphate RP-HPLC (RP Spherisorb ODS2 column (4.6 mm × 150 mm, 5.0 μm)	GC-EI-IT

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
Fish	XTI-5 capillary column (30 m × 0.25 mm; 0.25 μm)	4MBC: 115, 211, 254 OC: 177, 249, 361	98–99	5.3–17 ng/g	nd	13
	VF-5 MS capillary column (30 m × 0.25 mm; 0.25 μm)	4MBC: 211>169,155 OC: 250>248, 221	57–79	36–120 ng/g	nd	
Fish	BGB-5 (30 m × 0.25 mm; 0.25 μm) or SE54 (25 m × 0.32 mm; 0.25 μm)	4MBC: 254, 239 BP3: 228, 229 EHMC: 178, 290	93–115	7–380 ng/g lipid	72 (OC)	14
		OC: 249, 361		3–37 ng/g lipid	44–94 (4MBC), 66–118 (BP3), 64 (EHMC)	
				10–56 ng/g lipid	166 (4MBC), 123 (BP3), 25(OC)	
Fish	BGB-5 (30 m × 0.25 mm; 0.25 μm) or SE54 (25 m × 0.32 mm; 0.25 μm)	4MBC: 254, 239 OC: 249, 361	No data	5–20 ng/g lipid	50–1800 (4MBC) 40–2400 (OC)	15
Fish	PerfectSil 120 ODS-2 (125 mm × 3 mm, 3.5 μm)	4MBC: 255>105 EHMC: 291>161	86–108	2.4 ng/g muscle	214 (4MBC), 193–525 (BP3), 414 (EHMC), 300 (OC)	16

Table 2 (continued)

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
		BP3: 229>151 OC: 362>250				
Fish	Zorbax SB-C18 (150 mm × 3.0 mm, 3.5 μm) and OPTIMA-5-MS (50 m × 0.2 mm; 3.5 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PABA: 166>138 EHMC: 178, 290 3BC: 240, 197 4MBC: 254, 237 BP3 : 228, 227	76–99 (BP4 no extracted)	8–205 ng/g lipid	45–700 (EHMC)	17
	Zorbax SB-C18 (150 mm+3.0 mm, 3.5 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PAB: 166>138	80–99	1.8–10.7 ng/Kg body weigh		17
Fish	SE-54-CB (50 m × 0.55 mm; 0.25 μm)	IDM: 105, 147, 294 BM-DBM: 135, 161, 338 4MBC: 211, 239, 254 OD-PABA: 148, 165, 277 HMS: 109, 138, 262 EHMC: 161, 178,248 BP3: 165, 225, 242	89–106	50–90 ng/Kg body weigh	Muscle: 810 (4MBC), 310 (EHMC), 298 (BP3), 3100 (HMS); offal: 880 (4MBC), 283 (BP3), 185 (HMS); rest: 990 (4MBC), 50 (EHMC), 40 (BP3), 79 (HMS) whole fish: 930 (4MBC), 120 (EHMC), 150 (BP3), 791 (HMS); Muscle: 161 (4MBC), 41 (EHMC), 230 (BP3), 720 (HMS), 150(IDM); offal: 106 (4MBC), 270 (BP3), 970 (HMS), 210 (BM-DBM); rest: 60 (4MBC), 16 (EHMC), 22 (BP3), 41 (HMS), 9(IDM), 18 (TDM); whole fish: 78 (4MBC), 20 (EHMC), 78 (BP3), 237 (HMS), 29 (IDM), 44 (BM-DBM)	18
macrozoobenthos	Zorbax SB-C18 (150 mm+3.0 mm, 3.5 μm) and OPTIMA-5-MS (50 m × 0.2 mm; 0.35 μm)	BP1: 213>213 BP2: 245>245 BP4: 307>307 4DHB: 213>213 Et-PABA: 166>138 EHMC: 178, 290	70–105	6–50 ng/g lipid	22–150 (EHMC)	19

Table 2 (continued)

Matrix	Chromatographic Column	MS/MS transition or SIM ions	Recovery (%)	MLOD	Concentrations (ng/g lipid)	Reference
		3BC: 240, 197				
		4MBC: 254, 237				
		BP3: 228, 227				
Fish					91–133 (EHMC)	
					23–79 (EHMC)	
					91–151 (BP3), 11–173 (EHMC)	
					9–337 (EHMC)	
					<LOQ (BP3), 30 (EHMC)	
Bird					16–701 (EHMC)	
Mussel[a]	SGE-BPX5 capillary column (30 m × 0.25 mm, 0.25 μm)	EHMC: 178>121, 132, 161	89–116	2 ng/g dw	3–256 ng/g (EHMC)	20
		OC: 248>220, 219, 176			2–7112 ng/g (OC)	
		OD-PABA: 165>91, 118, 148				

[a] Concentrations expressed in ng/g (not ng/g lipid), *nd* not detected

SIM mode, and with benzophenone-d_{10} and $^{13}C_6$-*p*-*n*-nonylphenol as surrogates. Analysis performed on samples of bluegill (*Lepomis macrochirus*) muscle, with only 0.4 % lipid content, provided good results. When this method was applied to samples of sonora sucker (*Catostomus insignis*), with an average of 4.9 % lipid content, GC–EI-MS failed to provide acceptable results. Analysis of spiked sample extracts that had not previously been subjected to GPC purification resulted in substantially compromised chromatographic performance. In this case, addition of a GPC step was essential. This additional purification enabled continuous analysis of sonora sucker samples with no compromise in chromatographic performance compared with GC–EI-MS analysis of spiked bluegill tissue. However, dramatic increases in background signal and/or reduction in analyte sensitivity were observed for several analytes. As a consequence some compounds, including 4MBC, were indistinguishable from the background. To increase the sensitivity and reduce the background signal observed, the method was improved by the application of tandem mass spectrometric detection. GC–EI-IT analysis was performed with a VF-5 MS capillary column (30 m×0.25 mm; 0.25 μm) under the same chromatographic conditions and with detection in selected reaction monitoring mode (SRM). The optimized transitions used are listed in Table 2. Nevertheless, this approach only slightly improved detection of 4MBC and a few other compounds, but to much less an extent than expected (this aspect will be further discussed in the section "Limits of detection").

Similarly, GC–EI-IT with an ion-trap mass spectrometer was used by Bachelot et al. [20] for determination of EHMC, OC and OD-PABA. In this work an SGE-BPX5 capillary column (30 m×0.25 mm, 0.25 μm) was used for the separation. Data were acquired in SIM mode for the isotopically labeled internal standard chrysene-d_{12} and in SRM mode for the UV filters.

LC–MS

This technique enables analysis of a wide range of compounds and significantly increases the possibility of analysis of metabolites, which are usually more polar that the parent compounds, without the need for derivatization.

For ionization of the UV filters three different techniques have been used—electrospray ionization (ESI) (which is by far the most commonly used for trace analysis of these pollutants in environmental samples), atmospheric-pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). All the approaches used for LC–MS analysis of UV filters in biota use ESI mode, which achieves efficient ionization of the analytes even though ESI is assumed to be susceptible to signal suppression or signal enhancement by components of sample matrix, as shown

by previous studies on UV filters in complex matrices such as sewage sludge [8]. Isotopically labeled compounds should be used as internal standards to compensate for the matrix effect. Quantification of UV filters in biota samples by external standard calibration is not recommended.

Meinerling and Daniels [16] developed an LC–MS–MS method for analysis of 4MBC, OC, BP3, and EHMC. Chromatographic separation was performed on a PerfectSil 120 ODS-2 (125 mm×3 mm) column with MeOH and water (each containing 0.05 % acetic acid) as mobile phase. Analysis was performed with a triple-quadrupole mass spectrometer fitted with electrospray interface operated in positive mode under SRM conditions (LC–ESI(+)–MS–MS). In this study external standard calibration was used.

Zenker et al. [17] analyzed nine UV filters by LC–ESI–MS–MS. Chromatographic separation was achieved on a Zorbax SB-C18 column (150 mm×3.0 mm, 3.5 μm) with a binary gradient prepared from a mixture of 0.1 % (*v*/*v*) formic acid in HPLC-grade water and 0.1 % (*v*/*v*) formic acid in ACN. Data acquisition was performed in SRM mode. Benzoic-d_5 acid was used as internal standard.

Method performance

Method recovery

High recovery was achieved in all the methods reported, especially when the lipid content of the biological sample analyzed was low.

Nagtegaal et al. [18] achieved recovery from 89 % to 96 % when extracting compounds with a Soxhlet-based procedure. Most studies analyzing lipophilic UV filters used solvent extraction and further clean up by GPC, and usually achieved good recovery (86–115 %). Mottaleb et al. [13] analyzed 4MBC and OC in fish tissue with low lipid content, with recovery of 98 and 99 %. In analysis of samples with higher lipid content lower recovery (57–79 %) was achieved.

Zenker et al. [17] used a mixture of ethyl acetate and *n*-heptane as extraction solvent; this enabled efficient extraction of eight of the nine UV filters with a wide range of polarity. Average recovery ranged from 76 to 99 % (SD from 0.3 to 4 %). However, extraction of the polar filter BP4 was feasible only with ACN and MeOH. Bachelot et al. [20] achieved even higher recovery, from 89 to 116 %, in analysis of lipophilic UV filters in mussel soft tissue extracted by MAE then further RP-HPLC purification.

Limits of detection

Method limits of detection were calculated by extraction of samples of fish spiked with low concentrations of analytes which can be detected in the presence of possible matrix effect. For biota samples MLODs are in the sub-ng g^{-1} range, although authors normalize their results differently, depending on the matrix, and express them in ng g^{-1} lipid or simply ng g^{-1}. The presence of UV filters in blanks is eventually reflected by higher MLODs.

MLODs are highly dependent on the matrix analyzed. Biological matrices may be quite different depending on the organism selected, the species, and the tissue chosen; even so, there is still great variability. As an example, Balmer et al. [14], in analysis of four lipophilic UV filters, obtained three significantly different MLODs ranges, 3–37, 10–56, and 7–380 ng g^{-1} lipid, as a function of the fish species analyzed. To compare MLODs between different methods and for different matrices is complicated. MLODs are usually lower when analysis is performed by GC–MS because matrix effects are usually smaller. Table 2 summarizes the MLODs obtained in each study.

Zenker et al. [17] developed a method for analysis of nine UV filters by GC–EI–MS and LC–ESI–MS–MS. In the first of these the limits of detection ranged between 8 and 36 ng g^{-1} lipid. For UV filters analyzed by LC–MS–MS limits of detection were between 86 and 205 ng g^{-1} lipid. These different MLODs are because of the greater matrix effect in analysis using electrospray interfaces, which can affect analyte ionization.

Mottaleb et al. [13] analyzed bluegill tissue (with low lipid content, 0.4 %) by GC–EI-MS and sonora sucker tissue (high lipid content, 4.9 %) by GC–EI-IT, which is, a priori, a more sensitive and selective technique. MLODs for most compounds in the GC–EI-IT study were higher than those obtained for bluegill tissue by use of GC–EI-MS (especially for 4MBC, 23-fold higher). Differences in detectability between the two approaches cannot be explained solely by differences in extraction efficiency. The authors suggest inefficient fragmentation of precursor ions in the ion trap. Because all MS–MS precursor ions are produced by EI, a relative hard ionization technique, it is likely that generation of product ions via collision-induced dissociation may eventually be problematic (precursor ions may be sufficiently stable, which makes further fragmentation unlikely). The MLODs afforded by GC–EI-IT exceeded the corresponding environmentally relevant concentration range identified in the literature. OC and 4MBC were not detected in any sample in the study.

Critical aspects in the analysis of UV filters

Background contamination is a common problem in the determination of UV filters at environmentally relevant levels. Therefore, several measures must be taken to prevent this problem. All glassware should be carefully cleaned. A typical procedure consists in washing and heating at 380 °C, then sequentially rinsing with different high-purity organic

solvents. Furthermore, gloves should be worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies, and glassware should be used.

In addition, a set of at least two operational blanks should be processed together with each batch of samples. Because many of the compounds analyzed undergo photodegradation, stock standard solutions should always be covered with aluminium foil and stored in the dark.

The presence of matrix effects has the potential to lead to compromised results, so precautions should be taken to minimize this effect. Measurements are further hindered by the lack of appropriate commercially available reference standards. Currently, only BP3-d_5 and 4MBC-d_4 are commercially available; none of the studies reported herein used these. Other isotopically labeled compounds, namely benzophenone-d_{10}, $^{15}N_3$-musk xylene, and $^{13}C_6$-*p*-*n*-nonylphenol were used for quantification. Development and further marketing of a wider range of isotopically labeled compounds for use as surrogate and internal standards is an important need for analysis of sunscreen agents in complex matrices.

Biota levels

UV filters enter the aquatic environment directly, as a result of swimming and other recreational activities or indirectly via wastewater treatment plants (WWTPs). Thus, it is expected that, because of the lipophilic properties of these compounds, they can reach and accumulate in tissues of aquatic organisms. Several fish species have been investigated together with, although to a lesser extent, mollusks, crustaceans, and birds. Table 2 summarizes UV filter occurrence data in biota.

A study carried out by Nagtegaal et al. [18] provided the first data on the occurrence of UV filters in fish. Perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from Maarfelder Lake (Eifel, Germany) were analyzed and the presence of seven UV filters with total concentrations of approximately 2000 ng g^{-1} lipid and 500 ng g^{-1} lipid, respectively, in whole fish were reported. This early study, besides providing the first data on bioaccumulation of UV filters in fish, reported prevalence profiles in different fish tissues (muscle, offal, the rest, and whole fish). Results indicated that 4MBC and HMS can be selectively accumulated depending on the species; perch accumulates 4MBC in muscle and HMS in offal. In contrast, roach had higher levels of 4MBC in offal and of HMS in muscle. In contrast, EHMC and BP3 had similar bioaccumulation profiles in both species.

In Switzerland similar concentrations were found in lake fish. 4MBC, BP3, EHMC, and OC were detected in white fish (*Coregonus sp.*), roach and perch in the range 25–166 ng g^{-1} lipid, and from 45 to 700 ng g^{-1} lipid for EHMC in barb (*Barbus barbus*) and chub (*Leucisus cephalus*) [17]. Meinerling et al. [16] reported concentrations from 193 to 525 ng g^{-1} lipid in rainbow trout (*Oncorhynchus mykiss*).

Higher levels for 4MBC and OC (up to 1800 and 2400 ng g^{-1} lipid, respectively) were found in fish (brown trout, *S. Trutta fario*) from rivers downstream from a WWTP discharge [15], revealing its impact on the ecosystem. Buser et al. [28] demonstrated that the enantiomeric composition of 4MBC in perch was much different from that observed in the surrounding lake water. In contrast, the enantiomeric composition of 4MBC in roach was similar to that of the lake water indicating that bioconcentration or metabolism of a compound can be quite different from one species to another. The factors responsible for the differences in the enantiomeric composition of 4MBC found in fish remain unclear.

Concerning organisms other than fish, Fent et al. detected EHMC in crustaceans (*Dammarus sp.*) and mollusks (*Dreissena polymorpha*) at concentrations between 22 and 150 ng g^{-1} lipid. EHMC was also detected in different fish species at concentrations up to 337 ng g^{-1} lipid and in cormorants (*Phalacrocorax sp.*), at levels above 700 ng g^{-1}. BP3 was also detected, but at lower concentrations, in brown trout (*Salmo trutta*) and eel (*Anguilla anguilla*). These results suggest that biomagnification occurs through the food web; this aspect will be further discussed in the section "Bioaccumulation and biomagnification") [19].

Bachelot et al. proved the presence of UV filters residues in marine mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) of the Mediterranean French coast [20]. In that study all mussel samples contained EHMC, at concentrations up to 256 ng g^{-1} dw. In addition, 55 % of the samples contained OC also. In particular one of these samples had the highest concentration reported so far for an UV filter in biota, 7,112 ng g^{-1} dw.

Besides WWTP discharges, another important factor affecting accumulation in aquatic biota samples is the season of the year, with summer being the period when peak concentrations of sunscreens are observed. As an example, the concentrations reported for OC in fish in September were found to be three to five-fold higher than those corresponding to May, before the swimming period [15]. Similarly, a study carried out by Fent et al. showed that concentrations in freshwater mussels collected in a lake with recreational activity were higher after the summer [19].

UV filter 4MBC and other benzotriazole compounds have been analyzed in a great variety of aquatic species, for example tidal flat organisms, fish, coastal birds, and even hammerhead sharks of the Japan coast, by Nakata et al. [22]. 4MBC was not detected in any of the samples analyzed, even though this common sunscreen agent has been detected in samples in different studies in Europe. The authors of that

study suggested these results were evidence of the different production and usage profiles of UV filters among countries.

As shown in Table 2, of all the sunscreen agents investigated, EHMC is the most frequently found, but at lower concentrations that those usually observed for UV filters of similar log K_{ow}, e.g. HMS (log K_{ow} 6.16) and OC (log K_{ow} 4.95). On the other hand, other sunscreens with log K_{ow} in the same range, OD-PABA (log K_{ow} 6.15) and 3BC (log K_{ow} 4.49) were never detected, suggesting fast and effective metabolism.

Bioaccumulation and biomagnification

The net accumulation of a chemical by an organism from its combined exposure to water, food, and sediment is known as bioaccumulation. Species higher in the food web can be exposed to all the chemicals that lower-order species accumulate (biomagnification). Bioaccumulation models are useful tools for understanding the biomagnification of such substances [23]. The extent of biomagnification for a given contaminant is expressed in terms of the biomagnification factor (BMF), the ratio of the concentration of the contaminant in a predator to its concentration in prey. A BMF above 1 indicates biomagnification of the contaminant. However, the most conclusive evidence of the accumulation of chemicals by organisms and biomagnification in food webs is expressed by the trophic magnification factor (TMF) [23, 24], which is used to estimate the accumulation of contaminants through trophic levels of different food webs, for instance between fresh water and marine water systems.

Because of the lipophilic character (low water solubility) of most UV filters they may be expected to accumulate in biota and in humans and be stored rather faster than they are metabolized or excreted. A recent study by León-González et al. [25] revealed that the metabolites of OD-PABA were detected in human urine after 8 days of single cutaneous application of a cream, indicating slow metabolism. Despite this, only one field-based study has examined biomagnification through food webs [19]. EHMC bioconcentration was proven in macrozoobenthos, fish from different trophic levels, and cormorants (*Phalacrocorax sp.*) a species of fish-eating birds, in Switzerland. For example, estimated BCF for fish were far above unity, in the range 167–1500. Biomagnification was also assessed, but higher concentrations of EHMC in cormorants than in fish were not statistically significant. Despite this, estimated BMF, occasionally slightly higher than unity, were indicative of possible positive trophic magnification of EHMC. Nevertheless, a possible explanation of this finding may be found in feeding ecology; because birds and mammals are homeotherms their rates of feeding higher than for fish and invertebrates (poikilotherms) [26]. Higher BCFs were, however, reported by Balmer et al. [14] for 4-MBC in fish from Lake Zürich. In particular, BCFs for roach ranged from 2,300 to 9,700.

Biological and chemical factors, for example size, sex, age, life cycle, and metabolic activity, are important when assessing bioaccumulation and trophic transfer of contaminants in food webs. The lipid and protein content of tissue, which vary according to season, reproduction, migration, feeding rate, diet composition, growth rate, and food chain length, should be taken into account in analysis of UV filters [27].

The phenomenon of chirality exists in all biological systems. All proteins, enzymes, and carbohydrates are chiral. Because biological processes may be stereoselective (favor one structural form over the other), enantiomers of chiral compounds, both parent compounds and transformation products (metabolites, products of photodegradation, and disinfection byproducts) must be investigated separately to obtain reliable information about their bioavailability and biomagnification through the food web. It must be taken into account that metabolites of achiral compounds can also be chiral. Buser et al. [28] investigated the enantiomeric pattern of 4-MBC in lake fish. This compound exists as two stereoisomers, (*Z*) and (*E*), as a consequence of an exocyclic C=C bond. Both are chiral compounds with two stereocenters provided by the camphor moiety of their chemical structure. First, HPLC separation of the (*E*) and (*Z*) isomers of 4MBC was performed on a Nucleosil 120–5 C-18 reversed-phase column (250 mm×4 mm) with ACN–water 60:40 (*v*/*v*) as mobile phase. Enantiomeric separation of 4MBC was subsequently achieved by GC on a laboratory-prepared column containing 2,6-bis-(*tert*-butyldimethylsilyl)-γ-cyclodextrin in 70 % PS086. Detection was performed in full scan and SIM (GC–MS) or SRM (GC–EI-IT) modes.

Although the study did not reach a sound conclusion, the isomer ratio observed seemed to indicate preferential accumulation of the (*E*) isomer. With regard to potential enantioselective fish metabolism of 4-MBC, results were completely different for roach and perch; roach accumulates both enantiomers whereas perch is unable to metabolize the [1*R*,4*S*-(*E*)-4-MBC] stereoisomer only.

Ecotoxicological considerations

The ecotoxicological implications of exposure of biota to sunscreens have been addressed quite frequently. Despite this, the sparse studies available are conclusive [29]. Fish have long been regarded as tracers for assessing the extent of lipophilic contamination of aquatic ecosystems. As a consequence most ecotoxicological studies on the effect of UV filters have been conducted on different fish species for "in vivo" testing. Several sunscreens have been found to

have estrogenic hormonal activity, in particular, two which are extensively used 4-MBC and OMC; moreover, some have been shown to have multiple endocrine-disrupting activity, for example androgenicity or antiestrogenicity. Adverse effects on fecundity and reproduction have also been observed for BP3, benzophenone 2 (BP2), and 3 benzylidene camphor (3BC). Analysis of vitellogenin (VTG) in rainbow trout and Japanese medaka (*Oricias latipes*) after aqueous exposure to BP3 indicated, however, that high effective concentrations in the range 620–749 μg g^{-1} were needed to induce these effects [30]. These concentrations are greater than the reported level (19 ng L^{-1}) of BP3 in estrogenic fractions of effluent wastewater extracts [31]. When a similar test was conducted on 4MBC and EHMC [32], 4MBC had high estrogenic potency.

In male fathead minnows (*Pimephales promelas*), concentrations of BP2 of 1.2 mg L^{-1} and higher were found to induce VTG, modify gonad histology, and emasculate secondary sex characteristics; oocyte production in female fish was also inhibited significantly [33, 34]. In the same fish species, 3BC had high estrogenic potency, inducing VTG at doses of 435 μg L^{-1} and higher [35]; at concentrations near predicted environmental levels (3 μg L^{-1}) significant VTG induction, loss of secondary sexual characteristics, and inhibition of spermatogenesis were observed for male specimens. Loss of gender-specific mating behavior and cessation of milt production were, moreover, observed at 74 μg L^{-1} and 285 μg L^{-1}, respectively [34, 35]. In females, at the same concentrations, first oogenesis was inhibited, followed by cessation of egg production and release of mature oocytes.

Effects on algae have also been assessed. In a test on inhibition of reproduction of the green alga *Scenedesmus vacuolatus* OC and 4MBC had no activity whereas EHMC, BP3, and OD-PABA at predicted no-effects concentrations (PNEC; calculated from EC_{50} with a safety factor of 1000) in the range 0.17–0.76 μg L^{-1} were found to significantly inhibit algal growth [36]. In a similar study, exposure to BP3, 3BC, 4MBC, and EHMC resulted in inhibition of the growth of *Desmodesmus suspicatus*, with 72-h EC10 values in the range 0.21–0.56 mg L^{-1} [37]. Potential endocrine and toxic effects of BM-DBM, EHMC, and OC on infaunal and epibenthic invertebrates and zebra fish (*Danio rerio*) embryos was recently investigated by the same authors [38]. Test results revealed toxicity of these sunscreens was low (Table 3), with effect concentrations far higher than those reported in the environment. In particular, EHMC was found to have a toxic reproductive effect on *Potamopyrgus antipodarum* and on *Melanoides tuberculata* with no-observed effect concentrations (NOECs) of 0.08 mg kg^{-1} and 2 mg kg^{-1}, respectively; it also had sub-lethal effects on zebra fish with even higher NOEC, 100 mg kg^{-1}. Toxic effects on reproduction of the crustaceans *Daphnia magna* [19] and *Acartia tonsa* [39] have also been reported for BP1, BP3, and BP4.

In an early study by Donavaro and Corinaldesi [40] the affect of sunscreen agents on marine ecosystems was also demonstrated. These authors observed that UV filters increased virus production via prophage induction in marine bacterioplankton. Most recently the same authors also provided scientific evidence of the effect of these chemicals on hard corals and their symbiotic algae in the Celebes Sea, the Caribbean Sea, the Andaman Sea, and the Red Sea, by inducing rapid and complete coral bleaching even at extremely low concentrations [41]. Coral bleaching, the loss of intracellular endosymbionts (symbiodinium, also known as zooxanthellae, which impart specific colors, depending on the particular clade) as a result of expulsion under stress situations, has a negative effect on biodiversity and functioning of the great reef ecosystems of tropical seas.

Despite studies are mainly focused on solely one chemical, an organism is exposed not to single environmental chemicals but to mixtures of many. According to the literature, assessment of the effects of mixtures of chemicals has attracted increasing attention in recent decades. With regard to UV filters additive effects of mixtures are largely unknown, and are an important concern in environmental studies, because these substances are usually formulated as complex mixtures to achieve the high sun protection factors (SPF) currently demanded. Taking into account the large number of sunscreens used, and other endocrine-disrupting compounds, hormonally-active UV filters, may act additively. Indeed, cumulative interactions have been shown in a few studies [34, 42–44]; in particular, these papers report significant synergistic effects of combinations of UV filters mixed at NOECs of the individual compounds.

The lack of environmental occurrence and ecotoxicological data for most UV filters and matrices hinders reliable and integral environmental risk assessment for comprehensive protection of the environment. Moreover, for complete risk assessment metabolites produced by the organisms should also be considered and their prevalence and their ecotoxicity be. Nevertheless, preliminary ERA has recently been conducted by Fent et al. [34, 45] using the limited data available. According to calculated hazard quotients, a potential risk to aquatic ecosystems may be posed by 3BC, 4MBC, and EHMC.

Conclusions and future research perspectives

The biomonitoring data so far available have been provided by a limited number of research groups. Different sample characteristics, for example location, species, season, tissue, target analytes, and the analytical methods used, hinders comparison among studies, which in turn also hinders

Table 3 Summary of toxicity data available in the literature for UV filters

UV filter	Fish LOEC (mg L^{-1})	Aquatic invertebrates EC_{50} (mg L^{-1})	Algae EC_{50} (mg L^{-1})	Ref.
BP1	–	0.49–1.5[a] (AT)	–	[38]
	0.005[f]	–	–	[34]
BP2	0.001[f]	–	–	[34]
BP3	–	1.67 (DM)	–	[37]
	–	1.9 (DM)	–	[45]
	0.75[d]	–	–	[30]
	0.62[e]	–	–	[30]
	–	–	0.36 (EV)	[36]
BP4	–	50 (DM)	–	[45]
EHMC	–	0.57 (DM)	–	[37]
	–	–	0.19 (EV)	[36]
	–	0.29 (DM)	–	[45]
	9.87[e]	–	–	[32]
3BC	–	3.61 (DM)	–	[37]
	–	26.9–5.95[b,c] (LV)	–	[48]
	0.003[f]	–	–	[34]
4MBC	–	0.80 (DM)	–	[37]
	–	4.6[b] (PA)	–	[46]
	–	0.56 (DM)	–	[45]
	9.9[e]	–	–	[32]
IAMC	–	–	0.76 (EV)	[36]
OD-PABA	–	–	0.17 (EV)	[36]
Et-PABA	0.004[f]	–	–	[34]

[a]Under different experimental conditions

[b]μmol L^{-1}

[c]Different endpoints

[d]Vitellogenin induction in rainbow trout

[e]Vitellogenin induction in medaka

[f]Vitellogenin induction in fathead minnow

DM, *Daphnia magna*; AT, *Acartia tonsa*; EV, *Scenedesmus vacuolatus*; LV, *Lumbriculus variegatus*; PA, *Potamopyrgus antipodarum*; LOEC, lowest observed effect concentration

reliable assessment of the fate and effects of UV-absorbing compounds in aquatic ecosystems. Expression of the data in different units (body weight, lipids-basis) should be standardized, enabling comparison among similar studies. Researchers have used quite different analytical approaches, although most are based on solvent extraction, GPC clean-up, and analysis by GC–MS. The sensitivity and selectivity afforded are suitable for environmental trace analysis and recovery is also very good, with values close to 100 % when the lipid content of tissues is not high. Fish and, specifically, muscle has been the preferred sample for analysis, despite results which seem to indicate that individual compounds are selectively accumulated in muscle or offal, depending on the species.

Besides smart experimental design, for accurate comparison of contamination levels among different tissues, species, and locations, reporting of range and median values may be quite useful. Moreover, reporting of sunscreen concentrations in a specific organ, instead of a whole burden estimate approach, may lead to overestimation of BMFs and TMFs. Stable isotope analysis should be conducted to properly identify the trophic position of every species for further biomagnification considerations.

Ecotoxicological assessment of exposure to UV filters is a challenging task. Despite being scattered and limited, current ecotoxicological data indicate that the potential risk posed by these widely used chemicals requires further investigation. The estrogenic activity of most of the commonly used sunscreen agents is in the range of other well-characterized estrogenic chemicals. Findings indicate that some UV filters have endocrine-disrupting activity in, and/or affect reproduction of several species, although at concentrations higher than those measured in the environment. However, a propensity for rapid accumulation and temporal effects at environmentally relevant concentrations and the potential of mixture effects indicate the need for further studies to evaluate the effects of long-term exposure of biota to UV filters. Moreover, ecotoxicological studies indicate the need to consider multicomponent mixtures when evaluating hormonal activity of UV filters in aquatic organisms, for use in risk assessment to consider potential synergistic and/or antagonistic effects.

There should be greater emphasis on measurement of ecological, biological, and physicochemical variables in field studies conducted to analyze contaminants in species, and, more importantly, when comparing data between studies. In particular, the chemical characteristics of the UV filters should be considered on the basis of their different structural forms, including isomers and enantiomers. Preliminary findings indicate that stereochemical aspects of sunscreens should be included in future environmental and

toxicological research for proper characterization of their global prevalence in the environment and for elucidation of the processes of biodegradation of theses contaminants, because these properties may result in different potential for accumulation, as observed for other contaminants (perfluorinated compounds, halogenated flame retardants, etc).

Marine and, particularly, terrestrial environments should be more widely investigated in future studies to better understand the fate and effects of UV filters. The effect of climate change should also be considered, because seasonal changes in ice formation, temperature, drought–flood episodes, or food webs might have important effects on bioaccumulation and/or biomagnification of contaminants. For UV filters this is especially relevant, because higher levels of sunlight radiation, a consequence of increased depletion of the ozone layer, would increase the use of such chemicals.

Combining monitoring field studies with work on species biology, behavioral science, and exposure biomarkers, among others, would significantly contribute to improving our knowledge about these compounds.

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Sunscreens Cause Coral Bleaching by Promoting Viral Infections

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Background: Coral bleaching (i.e., the release of coral symbiotic zooxanthellae) has negative impacts on biodiversity and functioning of reef ecosystems and their production of goods and services. This increasing world-wide phenomenon is associated with temperature anomalies, high irradiance, pollution, and bacterial diseases. Recently, it has been demonstrated that personal care products, including sunscreens, have an impact on aquatic organisms similar to that of other contaminants.

Objectives: Our goal was to evaluate the potential impact of sunscreen ingredients on hard corals and their symbiotic algae.

Methods: *In situ* and laboratory experiments were conducted in several tropical regions (the Atlantic, Indian, and Pacific Oceans, and the Red Sea) by supplementing coral branches with aliquots of sunscreens and common ultraviolet filters contained in sunscreen formula. Zooxanthellae were checked for viral infection by epifluorescence and transmission electron microscopy analyses.

Results: Sunscreens cause the rapid and complete bleaching of hard corals, even at extremely low concentrations. The effect of sunscreens is due to organic ultraviolet filters, which are able to induce the lytic viral cycle in symbiotic zooxanthellae with latent infections.

Conclusions: We conclude that sunscreens, by promoting viral infection, potentially play an important role in coral bleaching in areas prone to high levels of recreational use by humans.

Key words: bleaching, corals, sunscreens, UV filters, viruses. *Environ Health Perspect* 116:441–447 (2008). doi:10.1289/ehp.10966 available via *http://dx.doi.org/* [Online 3 January 2008]

Coral reefs are among the most biologically productive and diverse ecosystems in the world, representing hot spots of marine biodiversity, and directly sustaining half a billion people (Moberg and Folke 1999; Wilkinson 2004). Approximately 60% of coral reefs are currently threatened by several natural and anthropogenic impacts (Hughes et al. 2003; Pandolfi et al. 2003). Over the last 20 years, massive coral bleaching (i.e., loss of symbiotic zooxanthellae hosted within scleractinian corals) has increased dramatically, both in frequency and spatial extent (Hoegh-Guldberg 1999; Hughes et al. 2003; Knowlton 2001). This phenomenon has been associated with positive temperature anomalies, excess ultraviolet (UV) radiation or altered available photosynthetic radiation, and presence of bacterial pathogens and pollutants (Brown et al. 2000; Bruno et al. 2007; Douglas 2003; Glynn 1996; Jones 2004).

Production and consumption of personal care and cosmetic sun products are increasing worldwide, reaching unexpected levels, with potentially important consequences on environmental contamination. The release of these products is also linked with the rapid expansion of tourism in marine coastal areas (Wilkinson 2004). Chemical compounds contained in sunscreens and other personal care products have been demonstrated to reach detectable levels in both fresh and seawater systems (Daughton and Ternes 1999; Giokas et al. 2007). These compounds are expected to be potentially harmful for the environment; hence, the use of sunscreen products is now banned in a few popular tourist destinations, for example, in marine ecoparks in Mexico, and in some semi-enclosed transitional systems (Xcaret 2007; Xel-ha 2007). Because sunscreens are lipophilic, their UV filters can bioaccumulate in aquatic animals (Giokas et al. 2007) and cause effects similar to those reported for other xenobiotic compounds (Balmer et al. 2005; Daughton and Ternes 1999). Paraben preservatives and some UV absorbers contained in sunscreens have estrogenic activity (Daughton and Ternes 1999; Schlumpf et al. 2004). In addition it has been demonstrated that several sunscreen agents may undergo photodegradation, resulting in the transformation of these agents into toxic by-products (Giokas et al. 2007, and literature therein).

Recently, it has also been demonstrated that sunscreens have an impact on marine bacterioplankton (Danovaro and Corinaldesi 2003), but there is no scientific evidence for their impact on coral reefs.

To evaluate the potential impact of sunscreen ingredients on hard corals and their symbiotic algae, we conducted several independent *in situ* studies with the addition of different concentrations of sunscreens to different species of *Acropora* (one of the most common hard-coral genus), *Stylophora pistillata,* and *Millepora complanata.* These studies were performed from 2003 to 2007 in different areas of the world, including the Celebes Sea (Pacific Ocean), the Caribbean Sea (Atlantic Ocean), and the Andaman Sea and the Red Sea (Indian Ocean).

Materials and Methods

Study areas and experimental design. *In situ* experiments were conducted in four coral reef areas: Siladen, Celebes Sea (Indonesia, Pacific Ocean); Akumal, Caribbean Sea (Mexico, Atlantic Ocean); Phuket, Andaman Sea (Thailand, Indian Ocean), and Ras Mohammed, Red Sea (Egypt, Indian Ocean). Nubbins of *Acropora* spp. (~ 3–6 cm) were collected, washed with virus-free seawater filtered onto 0.02-μm membranes (Anotop syringe filters; Whatman, Springfield Mill, UK), immersed in polyethylene Whirl-pack bags (Nasco, Fort Atkinson, WI, USA) filled with 2 L virus-free seawater, and incubated *in situ.* Additional experiments were also performed with other hard coral genera: *S. pistillata* and *M. complanata.* Replicate sets containing nubbins from different colonies (n = 3, including more than 300 polyps each) were supplemented with aliquots of sunscreens (at final quantities of 10, 33, 50, and 100 μL/L seawater) and compared with untreated systems (used as controls). Corals were incubated at the same depth of donor colonies at *in situ* temperature (Table 1). During two experiments conducted in the Red Sea and in the Andaman Sea, we tested the effects on coral bleaching of the same chemical filters and preservatives contained in the sunscreen formula of different brands (Tables 1 and 2). Subsamples (50 mL) of seawater surrounding coral nubbins were collected at 12-hr intervals and fixed in 3% glutaraldehyde for subsequent

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analyses (i.e., zooxanthellae counts and transmission electron microscopy, TEM). Additional seawater samples were immediately processed without any preservation for viruslike particles counts. At the end of the experiments, samples of coral tissue were fixed in 3% glutaraldehyde and stored at 4°C for zooxanthellae count and TEM.

Quantification of bleaching. To quantify the levels of coral bleaching (Siebeck et al. 2006), we performed a colorimetric analysis on digital photographs of corals taken at the beginning of the experiments and after various times of treatment with sunscreen and organic UV filters. Photographs were taken under identical illumination with a Canon PowerShot A620 digital camera (Canon Inc., Tokyo, Japan) with a scale meter on the background. The photographs were successively analyzed with a photo-editing software for color composition [cyan, magenta, yellow, black (CMYK)]. Levels of bleaching were measured as the difference between the coral's color at the beginning of the experiments and after treatments. Variations in the percentage of the different color components (CMYK) were analyzed with one-way analysis of variance (ANOVA; Table 3). To rank the bleaching effect due to the different ingredients tested, we obtained Bray–Curtis similarity matrix and multidimensional scaling analysis of the shifts in CMYK color composition of treated corals using Primer 5.0 software (Primer-E Ltd., Plymouth, UK). Bleaching rates were measured as the dissimilarity percentage in CMYK color composition between treated and control corals using the SIMPER tool of Primer 5.0 software (Primer-E Ltd).

Analysis of zooxanthellae. Zooxanthellae were extracted from coral nubbins using a jet of artificial seawater with a WaterPick (Braun, Germany) and centrifuged (4,000 × *g*, for 10 min) to separate the algae from the host tissue. Replicate suspensions (200–500 µL) of zooxanthellae extracted from coral tissue and those released during the experiment were filtered through 2.0-µm polycarbonate filters and mounted on glass slides. Zooxanthellae were counted under a Zeiss Axioplan epifluorescence microscope (Carl Zeiss Inc., Jena, Germany; ×400 and ×1,000), and the number of cells was normalized to nubbins' area. Based on the autofluorescence and gross cell structure, zooxanthellae released or extracted from nubbins were classified as *a*) healthy (H, brown/bright yellow color, intact zooxanthellae); *b*) pale (P, pale yellow color, vacuolated, partially degraded zooxanthellae); transparent (T, lacking pigmentations, mostly empty zooxanthellae; Mise and Hidaka 2003). Cell integrity was also examined by TEM (see below).

Standard sunscreen UV filters for the experiments. The UV filters ethylhexylmethoxycinnamate (OMC), octocrylene (OCT), benzophenone-3 (BZ), ethylhexylsalicylate (EHS), and the solvent propylene glycol (PG) (Table 2) were purchased from Sigma-Aldrich Co. (Milan, Italy); 4-*tert*-butyl-4-methoxydibenzoylmethane was obtained in the form of Eusolex 9020 from Merck (Darmstadt, Germany). 4-Methylbenzylidene camphor was synthesized according to Saito et al. (2004). Specifically, a mixture of *d*-camphor (10 mmol), *p*-tolualdehyde (12 mmol), and potassium *t*-butoxide (15 mmol) was refluxed in *t*-butyl alcohol (12 mL) for 5 hr. The reaction course was monitored by thin-layer chromatography using cyclohexane–ethyl acetate 8:2 as the eluant. The reaction mixture was neutralized with 5% HCl and extracted with ethyl acetate (10 mL × 3); the combined organic extracts were washed with saturated NaCl solution and dried over Na_2SO_4. Evaporation of the solvent and column chromatography of the crude residue on silica gel

Table 1. Experiments on hard-coral species treated with different sunscreens and sunscreen ingredients.

Ocean	Reef area	Reef water temperature (°C)	Treatments	Sun protecting factor	Quantity [µl/L (%)][a]	Species	No. of experimental sets	Bleaching initiation (hr)	Bleaching rate [hr (%)][b]	Zooxanthellae released (%)
Pacific	Celebes Sea, Indonesia	28, 30[c]	Sunscreen brand 1	15	100	*Acropora divaricata*	6	ND	24 (81, 95)	ND
			Sunscreen brand 1	15	10	*A.divaricata*	6	ND	36 (ND)	ND
			Nutrients		100[d]	*A. divaricata*	6	No bleaching	No bleaching	ND
			Controls			*A. divaricata*	6	No bleaching	No bleaching	ND
Atlantic	Caribbean Sea, Mexico	28	Sunscreen brand 2	8	10	*Acropora cervicornis*	3	18	36 (84)	87
			Controls			*A. cervicornis*	3	No bleaching	No bleaching	3
			Sunscreen brand 2	8	10	*Millepora complanata*	3	24	36 (35)	10
			Controls			*M. complanata*	3	No bleaching	No bleaching	2
Indian	Red Sea, Egypt	24	Sunscreen brand 1	8	33	*Acropora* sp.	3	24	48 (81)	44
			Sunscreen brand 1	15	33	*Acropora* sp.	3	24	48 (89)	30
			Controls			*Acropora* sp.	3	No bleaching	No bleaching	1
			Sunscreen brand 1	15	33	*Stylophora pistillata*	3	nd	48 (65)	ND
			Controls			*S. pistillata*	3	No bleaching	No bleaching	ND
			BMDBM		33 (2)	*Acropora* sp.	3	No bleaching	No bleaching	13
			MBC		33 (3)	*Acropora* sp.	3	24	48 (63)	10
			OCT		33 (6)	*Acropora* sp.	3	No bleaching	No bleaching	3
			EHS		33 (5)	*Acropora* sp.	3	No bleaching	No bleaching	3
			OMC		33 (6)	*Acropora* sp.	3	2	24 (91)	86
			BZ		33 (6)	*Acropora* sp.	3	24	48 (86)	83
			BP		33 (0.5)	*Acropora* sp.	3	24	48 (84)	90
			PG (solvent)		33	*Acropora* sp.	3	No bleaching	No bleaching	16
Indian	Andaman Sea, Thailand	25[e]	Sunscreen brand 3	8	50	*Acropora pulchra, Acropora aspera, Acropora intermedia, Acropora sp.*	15	24	48–62 (74–88)	88–95
			Controls			*A. pulchra, A. aspera, A. intermedia, Acropora sp.*	15	No bleaching	No bleaching	1–2
			MBC		50 (3)	*A. pulchra*	3	48	62 (95)	95
			OMC		50 (6)	*A. pulchra*	3	48	96 (91)	90
			BZ		50 (6)	*A. pulchra*	3	48	96 (93)	84
			BP		50 (0.5)	*A. pulchra*	3	48	96 (90)	79

Abbreviations: BMDBM, 4-*tert*-butyl-4-methoxydibenzoylmethane; BP, butyl paraben; BZ, benzophenone-3; EHS, ethylhexylsalicylate; MBC, 4-methylbenzylidene camphor; ND, not detected; OCT, octocrylene; OMC, ethylhexylmethoxycinnamate; PG, propylene glycol.

[a]Percentage concentrations of the filters allowed in sunscreen formulations in both American and European markets. [b]Bleaching rates measured as percentage chromatic dissimilarity with the coral used as a control (CMYK) at different experiment times (hr). [c]Temperature in outdoor aquarium. [d]Concentrations of nutrients relative to added sunscreen are calculated on the ratio of organic carbon to total nitrogen and phosphorous (wt:wt) of 31:2:1. [e]Local temperature during the experiment was below average season values.

eluting with cyclohexane–ethyl acetate 8:2 gave 4-methylbenzylidene camphor as a white solid which was crystallized from hexane (70% yield). ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.8 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 1.48–1.60 (m, 2H), 1.70–1.85 (m, 1H), 2.12–2.20 (m, 1H), 2.37 (s, 3H), 3.10 (d, 1H, *J* = 4.1 Hz), 7.19 (d, 2H, *J* = 8.0 Hz). 7.21 (s, 1H), 7.38 (d, 2H, *J* = 8.0 Hz) ppm. The preservative BP (butyl paraben) was obtained through esterification of 4-hydroxybenzoic acid with butyl alcohol: 20 mmol 4-hydroxybenzoic acid was dissolved in 25 mL butyl alcohol in the presence of a catalytic amount of *p*-toluensulfonic acid (~ 2 mmol) and refluxed for 7 hr. The reaction mixture was washed with $NaHCO_3$ 0.5 M and extracted with diethyl ether (25 mL × 3). The organic layer was dried over Na_2SO_4 and the solvent evaporated under reduced pressure. Butyl paraben was obtained with a 75% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.97 (t, 3H, *J* = 7.1 Hz), 1.38–1.65 (m, 2H), 1.70–1.76 (m, sH), 4.30 (t, 2H, *J* = 6.5 Hz), 6.89 (d, 2H, *J* = 8.88 Hz), 7.95 (d, 2H, *J* = 8.8 Hz) ppm. The amounts of UV filters and preservatives used in the sunscreen addition experiments were calculated on the basis of the percentage concentrations of the respective filters allowed in sunscreen formulations in both American and European markets. Hence, concentrations below the more restricted limits imposed by American regulations were used: BMDBM (2%), BZ (6%), OMC (6%), OCT (6%), EHS (5%), MBC (3%), BP (0.5%).

Quantification of sunscreen release in seawater. To estimate the amount of UV filters and preservatives released from sunscreen formulae, 2 mg sunscreen/cm^2 [dose recommended by the U.S. Food and Drug Administration (FDA); Poiger et al. 2004] was applied to the hands of two volunteers. The hands were then immersed in 2 L of 0.45-µm filtered seawater at 24°C for 20 min. Hands without sunscreen applications were used as controls. All experiments were repeated 3 times. The percentage of sunscreen released into the seawater was estimated by high performance liquid chromatography (HPLC) analyses on the sunscreen and seawater samples.

Some investigators suggest that the sunscreen dose recommended by the U.S. FDA is much lower than the amount actually used by tourists (Giokas et al. 2007, and literature therein); thus, the quantity of sunscreen released during a usual bath could be far higher than that estimated in this study.

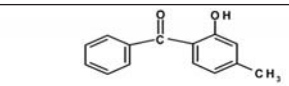
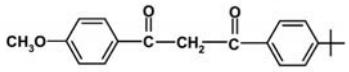
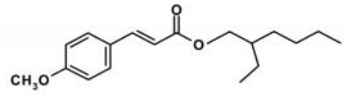
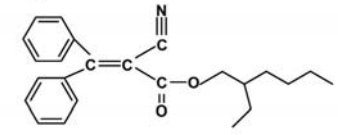
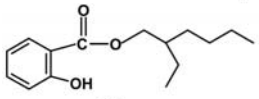
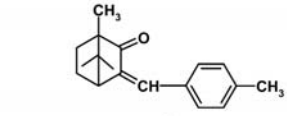
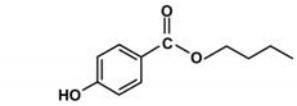
HPLC analysis of sunscreens. UV filters were extracted from 1 L seawater obtained from the sunscreen release experiment by solid-phase extraction (SPE) (C_{18} Bakerbound

Table 3. Shifts in the percentage contribution of the different coral color components [cyan, magenta, yellow, black (CMYK)] that occurred during the experiments (addition of sunscreen and sunscreen ingredients).

Treatments	Coral color shift[a] C	M	Y	K	Bleaching	Significance[b]
Control	0	2	3	0	NV	NS
Sunscreen	19	25	17	33	Visible	****
BMDBM	6	22	12	33	NV	**
BZ	6	24	7	43	NV	**
OMC	13	37	23	53	Visible	***
OCT	7	23	18	39	NV	**
EHS	6	20	7	38	NV	NS
MBC	8	17	5	37	NV	**
BP	9	32	33	29	Visible	***

Abbreviations: NS, none of the four variables is significant; NV, nonvisible bleaching. For acroynm definitions under "Treatment," see Table 1.
[a]Shift estimated as the average of 20 measurement points of the four colorimetric variables (CMYK). [b]Significance ($p < 0.05$) of each variable calculated by ANOVA; number of asterisks indicate the number of significant variables.

Table 2. Physicochemical properties of the UV filters.

Chemical name (INCI name)	Key[a]	Chemical structure	Molecular weight (g/mol)	Water solubility (mg L^{-1}) at 25°C	Log K_{ow}[b]	λ_{max}
2-Hydroxyl-4-methoxybenzophenone (*benzophenone-3*)	BZ		228.25	68.56	3.52	286
4-*tert*-Butyl-4′-Methoxydibenzoyl methane (*butyl methoxydibenzoylmethane*)	BMDBM		310.39	1.52	2.41	355
2-Ethylhexyl-4-methoxycinnamate (*ethylhexylmethoxycinnamate*)	OMC		290.41	0.15	5.80	305
2-Ethylhexyl 2-cyano-3,3-diphenylacrylate (*octocrylene*)	OCT		361.49	1.3	6.88	303
2-Ethylhexyl salicylate (*ethylhexyl salicylate*)	EHS		250.37	NA	6.02	305
3-(4′-Methylbenzylidene) camphor (*4-methylbenzylidene camphor*)	MBC		240.35	0.57	5.47	300
Butyl *p*-hydroxybenzoate[c] (*butylparaben*)	BP		194.23	207	3.57	253

Abbreviations: INCI, International Nomenclature for Cosmetic Ingredients; NA, not available.
[a]Key abbreviations adopted in this paper. For acronym definitions," see Table 1. [b]Octanol/water partition coefficient. [c]This is a preservative, not a UV filter.

SPE column, 500 mg/6 mL; J.T. Baker, Phillipsburg, NJ, USA). Before extraction an internal standard, butyl-cinnamate (BC, Sigma-Aldrich Co.) was added to the seawater sample. The SPE column was conditioned with 10% methanol, and the sample was passed through the column at approximately 20 mL/min. The ingredients were recovered from the column using 1 mL acetonitrile. Analyses were performed on an HPLC apparatus consisting of a Varian RP-C18 column (5 µm, 250 × 4.60 mm), a 20-µL injection loop, a Varian Pro Star solvent delivery module, a Varian Star 5.0 Workstation and Varian 9050 variable wavelength UV-VIS detector (Varian Inc., Palo Alto, CA, USA). The analytes injected into the chromatograph eluted in 18 min (1 mL/min) using a linear gradient starting from solution A (methanol:acetonitrile:water:acetic acid, 55:20:24:1, vol/vol) and ending with solution B (methanol:acetonitrile:water:acetic acid, 55:40:4:1, vol/vol). UV detection was carried out at λ = 255 nm for BP and λ = 300 nm for MBC, OMC and BC. Chromatograms were analyzed with the Varian Interactive Graphics Program.

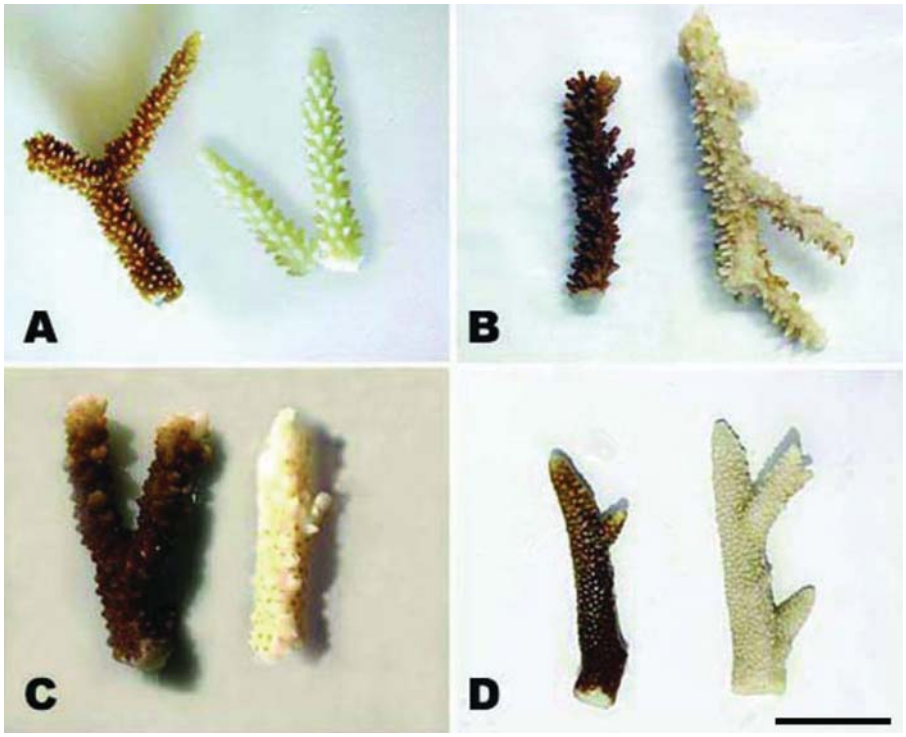


Figure 1. Impact of sunscreen addition on nubbins of *Acropora*. Untreated (brown) and treated (bleached) nubbins of (*A*) *Acropora cervicornis* (Caribbean Sea, Mexico); (*B*) *Acropora divaricata* (Celebes Sea, Indonesia); (*C*) *Acropora* sp. (Red Sea, Egypt); and (*D*) *Acropora intermedia* (Andaman Sea, Thailand). Images were taken within 62 hr of the start of sunscreen incubations. Scale bar = 2 cm.

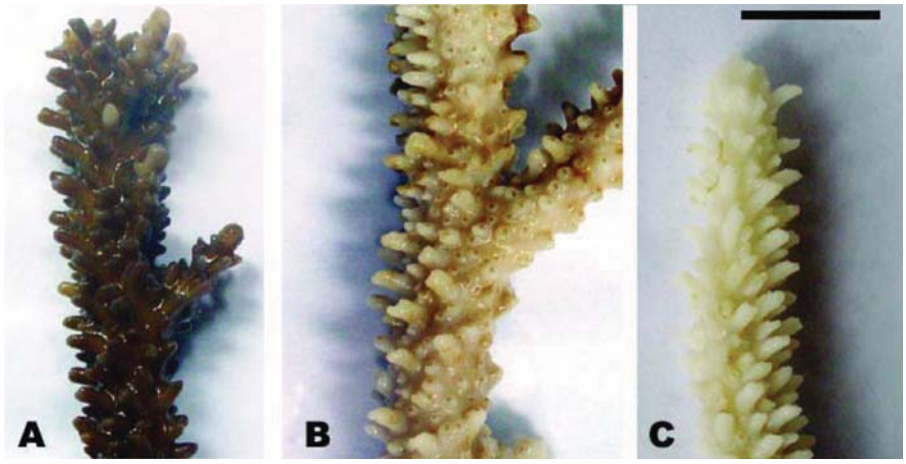


Figure 2. Effect of 100-µL sunscreens on *Acropora divaricata* nubbins after 24-hr incubation at various temperatures. (*A*) control; (*B*) nubbins incubated at 28°C; and (*C*) nubbins incubated at 30°C. Scale bar = 1 cm.

Viral counts and infection of zooxanthellae and TEM analysis. Water samples for viral counts were processed immediately without any fixative with SYBR green and SYBR Gold staining (Shibata et al. 2006). Immediately after collection, subsamples (200 µL) of seawater surrounding coral nubbins were diluted 1:10 in prefiltered MilliQ, filtered through a 0.02-µm pore-size Anodisc filter (25-mm diameter, Al_2O_3; Whatman) and immediately stained with 20 µL SYBR Green I and SYBR Gold (stock solution diluted 1:20 and 1:5,000 respectively; Invitrogen, Carlsbad, CA, USA). Filters were incubated in the dark for 15 min and mounted on glass slides with a drop of 50% phosphate buffer (6.7 mM, pH 7.8) and 50% glycerol containing 0.25% ascorbic acid (Shibata et al. 2006; Helton et al. 2006; Wen et al. 2004). Slides were stored at 20°C until analysis. Counts were obtained by epifluorescence microscopy (magnification, ×1,000; Zeiss Axioplan) by examining at least 10 fields, that is, at least 200 cells or particles per replicate.

TEM analyses were conducted on decalcified corals (2% vol/vol formic acid, 4°C, 8 days. *Acropora* tissue and pellets of zooxanthellae released during the experiment were post-fixed in 1% osmium tetroxide (Sigma-Aldrich Co.), dehydrated through an increasing acetone series (25%, 50%, 75%, 100%) and embedded in an Epon–Araldite mixture (Multilab Supplies, Fetcham, UK). Ultrathin resin sections (50–70 nm) were cut with a Reichert Ultracut E microtome (Reichert, Wien, Austria). Before analysis, sections were stained with saturated uranyl acetate and 1% lead citrate and collected on 200-mesh copper/rhodium grids (Multilab Supplies).

Estimates of release of sunscreen in reef areas. The global release of sunscreens in areas harboring coral reefs can be roughly estimated from their average daily use and the number of tourists. An average dose application of 2 mg/cm^2 of sunscreen (dose suggested by the U.S. FDA) for a full body surface of 1.0 m^2 results in an average usage of 20 g per application (Poiger et al. 2004). We consider a conservative measure of two daily applications per tourist traveling on a 5-day average tourist package, and a rough estimate of 78 million of tourists per year in areas hosting reefs [10% of world tourists registered in 2004; United Nations World Trade Organization (UNWTO) 2007]. Based on this calculation and on annual production of UV filters, between 16,000 and 25,000 tons of sunscreens are expected to be used in tropical countries. According to our experiment, it is estimated that at least 25% of the amount applied is washed off during swimming and bathing, accounting for a potential release of 4,000–6,000 tons/year in reef areas. Because 90% of tourists are expected to be concentrated in approximately 10% of the total reef areas,

we estimated that up to 10% of the world reefs is potentially threatened by sunscreen-induced coral bleaching.

Results and Discussion

Coral bleaching caused by sunscreens and UV filters. In all replicates and at all sampling sites, sunscreen addition even in very low quantities (i.e., 10 µL/L) resulted in the release of large amounts of coral mucous (composed of zooxanthellae and coral tissue) within 18–48 hr, and complete bleaching of hard corals within 96 hr (Figure 1; Table 1). Different sunscreen brands, protective factors, and concentrations were compared, and all treatments caused bleaching of hard corals, although the rates of bleaching were faster when larger quantities were used (Table 1). Untreated nubbins (coral branches of 3–6 cm) used as controls did not show any change during the entire duration of the experiments (Table 1). Bleaching was faster in systems subjected to higher temperature, suggesting synergistic effects with this variable (Table 1; Figure 2). TEM and epifluorescence microscopy analyses revealed a loss of photosynthetic pigments and membrane integrity in the zooxanthellae released from treated corals (30–98% of zooxanthellae released from *Acropora* nubbins were partially or totally damaged, appearing pale and transparent), whereas zooxanthellae membranes from untreated corals were intact (37–100% of the zooxanthellae released showed a defined shape and red fluorescing color; Figures 3 and 4). All these results indicate that sunscreens have a rapid effect on hard corals and cause bleaching by damaging the symbiotic zooxanthellae.

We tested sunscreen (10 µL/L) containing concentrations of UV filters higher than those reported in most natural environments. At the same time, the coral response to sunscreen exposure was not dose dependent, as the same effects were observed at low and high sunscreen concentrations. Therefore, we hypothesize that UV filters can have potentially negative impacts even at concentrations lower than those used in the present study.

Sunscreens typically comprise up to 20 or more chemical compounds. To identify the organic UV filters or preservatives possibly responsible for coral bleaching, seven compounds typically present in sunscreens were selected (Table 2), and additional experiments were carried out in which each single ingredient was tested on *Acropora* spp. Among the ingredients tested, butylparaben, ethylhexylmethoxycinnamate, benzophenone-3 and 4-methylbenzylidene camphor caused complete bleaching even at very low concentrations (parabens account for 0.5% of sunscreen ingredients). Conversely, all other compounds tested (i.e., octocrylene, ethylhexylsalicylate, and 4-*tert*-butyl-4-methoxydibenzoylmethane) and the solvent propylene glycol, which is also present in sunscreen formulations, had a minor effect or no effects when compared with controls (Table 1). These results suggest that sunscreens containing parabens, cinnamates, benzophenones, and camphor derivatives can contribute to hard-coral bleaching if released into natural systems.

Amounts of sunscreen released into tropical environments and their impacts. Sunscreen product sales exceed half a billion dollars (Shaath and Shaath 2005), and it is estimated that 10,000 tons of UV filters are produced annually for the global market. According to official data of the UNWTO, it can be estimated that 10% of sunscreens produced are used in tropical areas with coral reefs (Wilkinson 2004). We estimated that, on average, about 25% of the sunscreen ingredients applied to skin are released in the water over the course of a 20-min submersion. According to these estimates, we believe that up to 10% of the world's coral reefs would be threatened by sunscreen-induced coral bleaching.

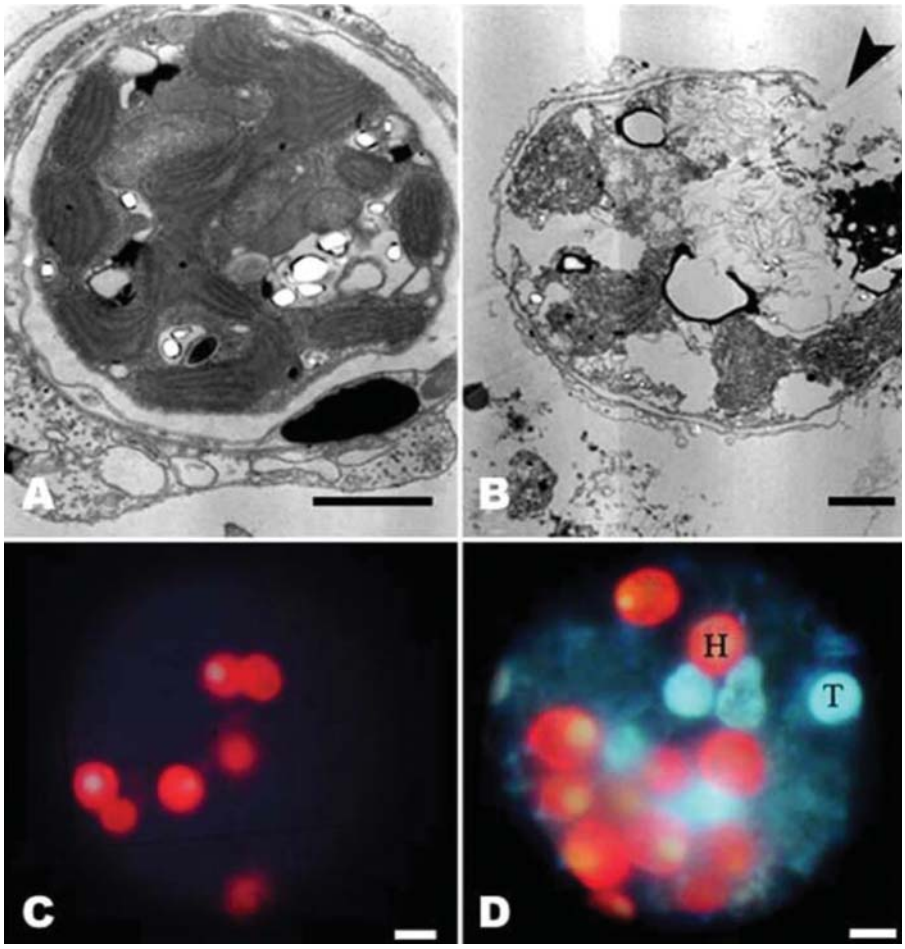


Figure 3. Zooxanthellae release from hard corals in control and sunscreen addition samples. (*A*) TEM images of healthy zooxanthellae (intact cell structure and membrane) in control untreated *Acropora* nubbin, and (*B*) zooxanthellae damaged by sunscreen treatment: cells appear swollen and vacuolated, without chloroplasts and double the size of the controls; the thylakoids are unpacked and dispersed inside the cells, and cell-membrane integrity is lost (arrowhead). (*C*) Autofluorescence images showing healthy (red) zooxanthellae in control sample and (*B*) some healthy (H) and damaged and partially damaged (T, transparent and pale) zooxanthellae released after sunscreen treatment. Scale bars = 2 µm (*A*, *B*) and 5 µm (*C*, *D*).

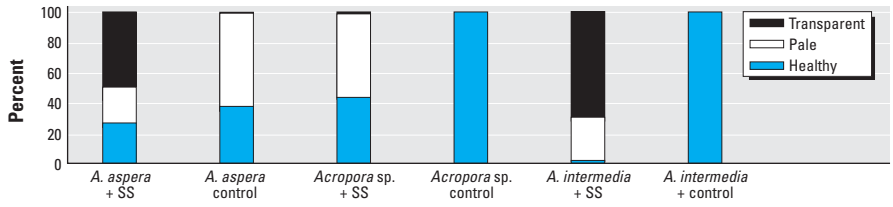


Figure 4. Epifluorescence microscopy analysis of the level of damage in zooxanthellae released after sunscreen (SS) addition.

The impact of sunscreens would be expected to be crucial in atolls and coastal coral reefs with low water renewal and strong tourist vocation. Our results provide strong scientific evidence of the potential impact of these products in tropical habitats and represent a pointer for outlining specific regulations for protecting coral reefs.

Effect of sunscreen ingredients on viral infections. Previous studies have demonstrated that sunscreens can significantly enhance viral production in seawater by inducing the lytic cycle in prokaryotes with lysogenic infection (equivalent to the latent infection of eukaryotes; Danovaro and Corinaldesi 2003). Here, we demonstrate that a similar phenomenon occurs also in hard corals. After the addition of sunscreens, viral abundance in seawater surrounding coral branches increased significantly, reaching values greater by a factor of 15 than in controls (Figure 5A). Because, prior to any treatment, the hard corals were carefully washed with and incubated in virus-free seawater, we conclude that the viruses encountered were released from the corals or their symbionts. Moreover, addition of organic nutrients without UV filters or preservatives did not result in coral bleaching or in a significant increase in the number of viruses in the ambient seawater (Figure 5A). Hard-coral bleaching and the increase in viral abundance in seawater were also seen after coral treatment with mitomycin C, an antibiotic commonly used to induce the lytic cycle in latent viral infections (Figure 5B). TEM analysis of sunscreen-treated corals showed the presence of virus-like particles (VLPs) around and inside the zooxanthellae. The VLPs were round-hycosahedral in shape and 50–130 nm in size (Figure 6). No viruses were encountered either inside or outside the zooxanthellae in control samples. All these results indicate that sunscreens caused coral bleaching by inducing the lytic cycle in symbiotic zooxanthellae with latent viral infections.

Causative agents (mostly bacteria and fungi; Rosenberg et al. 2007) have been isolated and characterized for only 6 of more than 20 coral diseases described in natural environments. To date, viruses have been found in cells of about 50 algal species, representing nearly all major algal classes. This suggests that viruses have a significant role in algal ecology (Brussard 2004). There are, however, only a few studies on viruses infecting zooxanthellae: viruses were encountered in heat-shocked or UV-treated zooxanthellae of *Pavona danai*, *Acropora formosa,* and *S. pistillata*, suggesting the presence of latent viral infections (Davy et al. 2006; Lohr et al. 2007). All our samples from different areas of the world showed viral lytic cycles after treatment with sunscreens and other inducing factors. The results of the present study and these data from the literature indicate that latent infections are common in symbiotic zooxanthellae.

Viruses have a key role in population dynamics and in community composition and diversity of marine bacterioplankton and phytoplankton (Brussard 2004; Suttle 2005) Viruses also contribute significantly to horizontal gene transfer, and can influence the pathways of energy and material flow in aquatic ecosystems, with important implications for global biogeochemical cycles (Fuhrman 1999). The results presented here provide new insights into the functional and ecological role of aquatic viruses and indicate that induction of the lytic cycle in zooxanthellae with latent infection represents an important factor contributing to coral bleaching.

Recent studies have reported that pesticides, hydrocarbons, and other contaminants can cause coral bleaching (Brown 2000; Douglas 2003). We suggest that these factors, which also have the potential to induce the viral lytic cycle in microorganisms or algae with latent infections (Cochran et al. 1998; Danovaro and Corinaldesi 2003; Davy et al. 2006; Jang and Paul 1996) could act synergistically with suncare products, thereby increasing the frequency and extent of coral bleaching.

Our results indicate that sunscreens promoting lytic cycle in viruses can cause coral bleaching. Because human use of tropical ecosystems and coral reef areas is progressively increasing, we predict that the impact of sunscreens on coral bleaching will grow considerably in the future on a global scale. Actions are therefore needed to stimulate the research and utilization of UV filters that do not threaten the survival of these endangered tropical ecosystems.

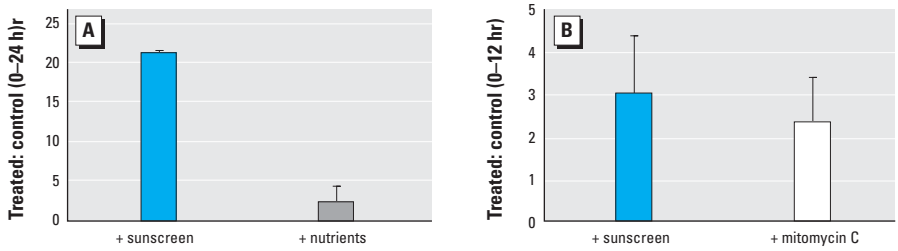


Figure 5. Viral enrichment factors of ambient seawater (as the ratios of viral density in treated and control samples) after the addition of sunscreen, nutrient, and mitomycin C. (*A*) Viral enrichment factor of ambient seawater within 24 hr after sunscreen and organic nutrients addition. (*B*) Viral enrichment factor of ambient seawater within 12 hr after sunscreen and mitomycin C addition. Organic nutrients (lipids, proteins, and carbohydrates) were added at concentrations equivalent to those contained in sunscreens according to Danovaro and Corinaldesi (2003). Values are ± SE.

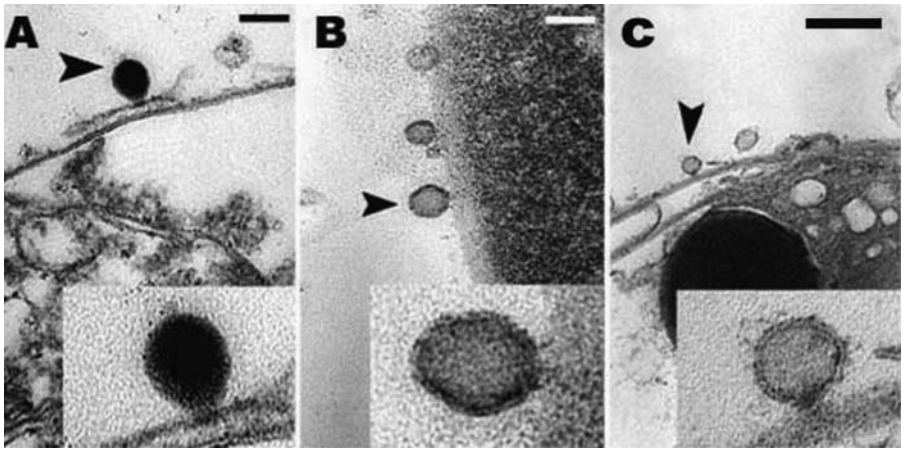


Figure 6. TEM images of viruslike particles (VLPs) associated with zooxanthellae released from nubbins after sunscreen treatment. (*A, B*) VLPs attached to zooxanthellae membranes. (*C*) Viruses attached to outer part of zooxanthellae with visible tail penetrating cell membrane. Scale bars = 100 nm (*A, B*); 200 nm (*C*). Arrowheads indicate sections magnified in insets.

Correction

In Table 2, the log K_{ow} value for 2-ethylhexyl salicylate has been corrected from "NA" in the original version published online to "6.02."

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Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: Correlation of UV filters with use of cosmetics

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ABSTRACT

In order to assess potential risks of exposure to environmental chemicals, more information on concomitant exposure to different chemicals is needed. We present data on chemicals in human milk of a cohort study (2004, 2005, 2006) of 54 mother/child pairs, where for the first time, cosmetic UV filters, synthetic musks, parabens and phthalate metabolites were analyzed in the same sample along with persistent organochlor pollutants (POPs), i.e., organochlor pesticides and metabolites, polybrominated diphenylethers and polychlorinated biphenyls (PCBs). The two groups of chemicals exhibited different exposure patterns. Six out of seven PCB congeners and a majority of pesticides were present in all milk samples, with significant correlations between certain PCB congener and pesticide levels, whereas the cosmetic-derived compounds, UV filters, parabens and synthetic musks, exhibited a more variable exposure pattern with inter-individual differences. UV filters were present in 85.2% of milk samples, in the range of PCB levels. Comparison with a questionnaire revealed a significant correlation between use of products containing UV filters and their presence in milk for two frequently used and detected UV filters, 4-methylbenzylidene camphor and octocrylene, and for the whole group of UV filters. Concentrations of PCBs and organochlor pesticides were within ranges seen in Western and Southern European countries. For several POPs, mean and/or maximum daily intake calculated from individual concentrations was above recent US EPA reference dose values. Our data emphasize the need for analyses of complex mixtures to obtain more information on inter-individual and temporal variability of human exposure to different types of chemicals.

1. Introduction

Humans are exposed to a mixture of many different contaminants. However, there are few comparative studies on the simultaneous presence of different types of chemicals in the same human sample that would allow for cross-comparisons of exposure. This is particularly important in the case of endocrine disrupting chemicals (EDCs), which belong to a large range of different chemicals. EDCs present in our environment, in food and consumer products cause growing concern because of their potential to interfere with homoeostatic control and reproduction (Andersson et al., 2008; Diamanti-Kandarakis et al., 2009). A central issue in this context is the age of exposure. Early life stages are particularly vulnerable and disturbance of developmental processes can lead to persisting alterations in structure and function that sometimes becomes manifest only later in life. Information on early exposure to multiple chemicals can be obtained from analyses of human milk, which reflect exposure of the infant and, for chemicals with a longer half life, also provides information on prenatal exposure. We conducted an investigation of human milk in 2004, 2005 and 2006. Our goal was to compare exposure to classical persistent organic pollutants (POPs) whose levels are considered to be generally declining, with exposure to chemicals that were more recently identified as environmental pollutants and exhibit increasing production rates. The

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latter differ from classical POPs also with regard to kinetics and can be expected to exhibit different exposure patterns. We determined seven indicator polychlorinated biphenyls (PCBs), six polybrominated diphenylethers (PBDEs), 32 organochlor pesticides (including DDT) and metabolites, four parabens, 11 phthalate metabolites, 16 synthetic musk fragrances, and eight UV filters.

The present investigation represents the first analysis of exposure of a human population to cosmetic UV filters. It was combined with a detailed questionnaire on the use of different types of cosmetic products and their content of UV filters. All eight UV filters analyzed in the present study have been found to exhibit estrogenic, some of them also anti-androgenic activity and interactions with the thyroid axis (reviewed by Schlumpf et al., 2008a,b; octocrylene: Matsumoto et al., 2005). Two compounds, 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) were found to interfere with development of reproductive organs, brain and behavior (Schlumpf et al., 2008a,b; Faass et al., 2009). For ethylhexyl-methoxy cinnamate (EHMC), long-term effects have been described in adult ovariectomized rats (Seidlová-Wuttke et al., 2006).

Personal care products such as UV filters spread into the ecosphere and are detected in surface water and fish (Balmer et al., 2005; Fent et al., 2010). The present investigation revealed a widespread internal exposure of humans to UV filters. The exposure pattern differed from that of classical environmental pollutants. The presence of UV filters in human milk was significantly correlated with individual use of products containing these filters, but not with levels of POPs.

2. Materials and methods

2.1. Recruitment of nursing women

Human milk samples were collected from mothers of three different cohorts in 2004, 2005 and 2006, who gave birth to a singleton child at the University Women's Hospital Basel. Birth dates of infants were: cohort 2004 ($n = 13$) August 8–November 9, 2004, cohort 2005 ($n = 21$) August 1–October 29, 2005 (except for one child born on June 6), cohort 2006 ($n = 20$) August 4–October 29, 2006. We chose to recruit human milk samples from summer to late fall, when sunscreens were used in addition to other cosmetics. Participating mothers were asked to fill out a questionnaire (see Section 2.2). The study was approved by the Ethic's Committee of the University Hospital Basel, and the participating women gave their informed consent.

2.2. Questionnaire

Fifty-four women returned the questionnaire (Appendix A); 53 completed the section on use of cosmetics. The women were assisted by the study nurse. If necessary, answers were further clarified by subsequent telephone interviews. The questionnaire contained the following sections.

2.2.1. Maternal and child data

Maternal data: birth date, size and body weight before pregnancy and body weight and BMI shortly before parturition. Child data: date of birth, sex, first or second child, body weight at birth and at milk sampling, birth dates and duration of lactation of siblings. We chose to also include mothers giving birth to a second child in order to increase the range of the study.

2.2.2. Nutritional habits

General type of nutrition (mixed, vegetarian, vegan, others), consumption of fish and other seafood (frequency), of milk and milk products without cheese (frequency, fat content, milk volume), of cheese (frequency, fat content), and of red meat (frequency) were recorded (see Section 2.5 and Appendix A).

2.2.3. Smoking, drugs, profession and domicile

Non-smokers, women having stopped smoking (year, month), smokers (duration of smoking, products). Medicinal drugs taken during milk sampling. Present and previous professions of the mother (with dates), present domicile and domicile during the last 5 years.

2.2.4. Use of cosmetics

Mothers were asked to list defined products (brands) for seven different product categories, skin care, perfumes, deodorants, sunscreens, lip sticks, make ups and hair tinting lotions, which they had been using in pregnancy and during the nursing period. We also asked the mothers to quantify their product consumption describing their use as "daily", "several times per week" or" several times per month". The answers on cosmetic use were analyzed based on the ingredient declarations of the cosmetic products. The use of chemicals contained in cosmetic products was correlated with chemical analysis of milk samples. For certain cosmetics, especially perfumes, no declaration or no product description were available.

2.3. Human milk sampling

For most participants, milk collection for the study started at home. Milk sampling was done with the instruction and assistance by the study nurse Monika Birchler and under medical supervision by Dr. Cora Vökt. Instruction was on how to avoid contamination of breast milk and how to clean and care for breast and nipples. No use of cream soapes or nipple crèmes was allowed. Hands and breast/nipples were rinsed with warm water before breast feeding. Mothers were instructed how to use pumps and milk containers in order to avoid contamination. A freshly hot water rinsed, BPA-free manual breast pump (Type Harmony, Medela AG, Baar, Switzerland) was used to obtain breast milk that was collected into a clean sterilized glass bottle (Schott, Duran ISO 4796) with a Teflon lined screw cap. The bottle was immediately stored at −20 °C. The study nurse collected all milk samples. The bottles were sent to the laboratory of the State Institute for Chemical and Veterinary Analysis of Food in Freiburg, Germany.

Mothers were asked to collect 5–30 mL milk per day on 7–10 consecutive days. The total amount of milk per mother ranged between 60 and 150 mL, pooled from an average of eight samples per participant. Milk sampling took place before 30 d post partum in 49 mothers and after this date in five mothers (Table 7). Twenty-nine samples (53.7%) contained "mature" milk ($\geqslant$10 d after delivery), 23 samples (42.6%) were a mixture between transitional milk (4–10 d post partum) and mature milk. In two cases, only transitional milk was collected. Colostrum was not represented. Duration of sampling was similar in all cases, lipid content did not differ significantly between groups. Details of milk sampling were listed in the questionnaire.

2.4. Chemical analyses

2.4.1. Analysis of chemicals extracted from milk lipids

All chemicals except parabens and phthalate metabolites were extracted from milk lipids and analyzed at the Institute for Chemical and Veterinary Analysis of Food, Freiburg im Breisgau (Karin Kypke and coworkers). The analytical procedure followed the principles of the European standardized methods, Fatty food – Determination of pesticides and PCBs, EN 1528 part 1–4, 1996-10 (confirmed 2001) (Hui et al., 2008; Malisch and Moy, 2006).

2.4.1.1. Lipid extraction. Homogenized samples were centrifuged at 3000 rpm, 4 °C and the supernatant cream (lipid) was separated from the aqueous phase (defatted milk). Defatted milk was kept at −20 °C for analysis of parabens. The cream was melted and grinded with sodium sulfate to yield a dry powder. The powder was extracted with *n*-hexane for analysis of all chemicals except UV filters. For extraction of UV filters, *n*-hexane/acetone (1:1) was used, followed by dichloromethane/acetone (1:1). The extracts were filtered and the solvents were evaporated to get a dry crude lipid extract.

2.4.1.2. Clean-up procedures.

2.4.1.2.1. Gel permeation chromatography. A defined amount of lipid was dissolved in cyclohexane/ethyl acetate (1:1 v/v) and a selection of the internal standards, 2,4,5-trichlorobiphenyl (TCB), mirex, heptachlor, for GC determinations as well as Diuron D6 for LC determinations was added according to the chemical groups to be determined. To remove the lipids, gel permeation chromatography was performed on a chromatography column (length 580 mm, 25 mm i.d., filling level 330 mm) using Bio-Beads S-X3 with cyclohexane/ethyl acetate (1:1 v/v) as eluting solvent at a flow rate of 5,0 mL min^{-1}. For UV filters, the eluate was evaporated to about 5 μL. Then 250 μL ethyl acetate was added and the solution transferred into two sampler vials for GC and LC determination. Internal standards were TCB + Mirex + Diuron D6.

2.4.1.2.2. Silica gel mini-column chromatography. Halogenated pesticides, PCB's, PBDE's, nitromusks. The eluate was concentrated after adding iso-octane and evaporated to about 1 mL. Chromatography on a column of partially deactivated silica gel was performed as the final clean-up step. Silica gel (70–230 mesh) was heated overnight at 130 °C and cooled in a desiccator. After adding 1.5% of water, it was shaken for 30 min and then stored in a tightly sealed container. The chromatographic tube was packed with 1 g of deactivated silica gel and rinsed with 10 mL *n*-hexane. The compounds were eluted with 10 mL toluene in three portions. The eluate was evaporated to about 5 μL. 150 or 250 μL cyclohexane was added and the solution transferred into a sampler vial for GC determination.

Polycyclic and macrocyclic musks. The eluate was evaporated to about 5 μL and then 2000 μL toluene was added. Chromatography on a column of activated silica gel was performed as the final clean-up step. The silica gel (70–230 mesh) was heated at 200 °C for at least 8 h and cooled in a dessicator. The chromatographic tube was packed with 1.5 g of silica gel and rinsed with 5 mL toluene before use. The compounds were eluted three times with 28 mL toluene. The more polar macrocyclic musk compounds were eluted with 30 mL toluene + acetone 95 + 5 (v/v) in three portions. The eluates were evaporated to about 5 μL, 250 μL cyclohexane was added and the solution transferred into sampler vials for the GC determination.

2.4.1.3. GC/MS and LC/MS determination.

2.4.1.3.1. Determination of UV filters by GC/MS. Benzophenone-3 (Bp-3), ethylhexyl-methoxy cinnamate (EHMC), homosalate (HMS), 4-methylbenzylidene camphor (4-MBC), 3-benzylidene camphor (3-BC), octocrylene (OCT), octyldimethyl-PABA (OD-PABA) (Table 1a) were determined by GC–LRMS (GC: HP6890; MS: HP5973). The HP6890 is equipped with a HP5-MS. Analytes were separated on a 30 m by 0.25 mm i.d. Supelcowax column with 0.25 μm film thickness and a 2.5 m pre-column and detected by a quadrupole mass spectrometer or mass-selective detector (MSD) in MSD-EI detection mode, using selected ion monitoring (SIM mode) selecting one target and three qualifier-ions as characteristic mass ions (Table 1a). To compensate for matrix effects, matrix-matched calibration was used. The purity of reference chemicals was as follows: Bp-3 98%, 3-BC > 97%, 4-MBC > 99.7%, EHMC 98%, HMS > 98%, OCT 98%, OD-PABA > 98.5%.

Determination of Bp-2 by LC/MS. Benzophenone-2 (Bp-2) (Table 1a) was the only UV filter analyzed by LC–MS. The liquid chromatograph (LC) HP1100 (Agilent) was coupled with MS Quattro LC (Micromass). The LC–MSD was equipped with a Luna C18(2) column, 50 × 2 mm, 5 μm (Phenomenex), and a mass-selective detector using the ESI + (Electrospray Ionisation) detection mode and a quadrupole mass analyzer for ion mass detection. Eluent A was 1 mM ammonium acetate, pH 4.75 and eluent B methanol. The injection volume was 5 μL and the oven temperature was set at 40 °C.

Time (min)	0.00	10.00	16.50	16.60	19.00	19.01	26.00	26.01
Solvent B (%)	5	85	85	100	100	5	5	5
Flow (mL)	0.2	0.2	0.2	0.6	0.6	0.6	0.6	0.2

Identification and quantification of Bp-2 was done by measuring of two daughter ions generated by the precursor ion M+H. (see below). Internal standard solution: Diuron D6 in ethyl acetate.

	M+H	Cone V	Daughter 1/eV	Daughter 2/eV
Bp-2	247	25	137/18	81/37
Diuron-D6	239	27	78/20	

Concentrations of UV filter substances are reported as ng g^{-1} of milk lipid (ng g^{-1} lipid). The limit of quantification (LOQ) and the limit of determination (LOD) for the UV filters HMS, 3-BC, Bp-3, 4-MBC and OCT was 4.0 ng g^{-1} lipid for LOQ and 2.0 ng g^{-1} lipid for LOD. For OD-PABA, EHMC and Bp-2, LOQ was 2.0 ng g^{-1} lipid and LOD 1.0 ng g^{-1} lipid. The mean level for each residue was calculated with the assumption of zero level for undetected value and half LOQ for levels determined between LOD and LOQ. The level was stated as "nd" (undetected) if it was below LOD.

2.4.1.3.2. Halogenated pesticides, PCBs, PBDEs, synthetic musks: GC/ECD and GC/MSD. Determination was performed with GC/ECD using a GC (Fisons Mega 2) with two columns of different polarity in parallel: (1) fused silica No. 1: 30 m PS-088 [97.5%dimethyl-2.5% diphenylsiloxane copolymer], 0.32 mm i.d., 0.3 μm film thickness and (2) fused silica No. 2: 30 m OV-1701-OH, 0.32 mm i.d., 0.25 μm film thickness. Results were confirmed by GC–LRMS (GC: HP 6890/MS: HP 5973; 30 m HP 5-MS, 0.25 mm i.d., 0.25 μm film thickness, +2.5 m pre-column; ionisation mode: EI, registration mode: SIM (Tables 1a and 1b). Concentrations of DDT and its metabolites were reported as ng g^{-1} milk lipid, the limit of quantification (LOQ) was 0.5 ng g^{-1} lipid. For halogenated pesticides, PCBs, PBDEs and nitromusks, the limit of quantification (LOQ) was 0.5 ng g^{-1} lipid and the limit of determination (LOD) 0.1 ng g^{-1} lipid. For polycyclic musks, LOQ was 5.0 ng g^{-1} lipid, for macrocyclic musks 20.0 ng g^{-1} lipid, with LOD at half LOQ values.

2.4.1.4. Quality control. Quality control procedures followed the guidelines for the Monitoring of pesticide residues in the European Union (Document No. SANCO/10476/2003 of 5 February 2004, updated by Document No. SANCO/10232/2006 of 24 March 2006). The following criteria were checked for each batch: (1) Recovery rates of internal standards and analyses were in the range of 70–120% which met the requirements of the guidelines. (2) Reagent blanks obtained by conduction of a complete analysis procedure using solvents and reagents only in the absence of any sample, were added. (3) Quality control samples of certified reference

Table 1a
Determination of UV filters, synthetic musks, PCBs, and PBDEs.

Substance (INCI name)	Chemical name	Mass of target-ion	Mass of qualifier-ions
UV filters			
Benzophenone-2 (Bp-2)[a]	2,2′,4,4′-Tetrahydroxy-benzophenone		
Benzophenone-3 (Bp-3)	2-Hydroxy-4-methoxy-benzophenone	151	228, 227, 77
Ethyl-hexyl cinnamate (EHMC)[b]	2-Ethylhexyl-4-methoxy cinnamate	178	161, 290, 179
Homosalate (HMS)	3,3,5-Trimethyl-cylclohexyl-salicylate	138	120, 109, 262
4-Methylbenzylidene camphor (4-MBC)	3-(4′-Methylbenzylidene) bornane-2-on	254	211, 171, 128
3-Benzylidene camphor (3-BC)	3-Benzylidene bornane-2-on	240	225, 197, 157
Octrocrylene (OC)	2-Cyano-3,3′diphenyl acrylic acid 2′-ethyl-hexyl ester	249	360, 232, 204
Octyl-dimethyl PABA (OD-PABA)	4-Dimethylamino-benzoic acid-2-ethyl-hexyl ester	165	148, 277, 164
Synthetic musks			
Musk xylene	5-Tert-butyl-2,4,6,-trinitro-m-xylene	282	283, 297, 252
Musk ketone	1-(4-(1,1-Dimethylethyl)-2,6-dimethyl-3,5-dinitrophenyl)-ethanon	279	280, 294, 264
DPMI, Cashmeran	6,7-Dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone	191	207, 163, 192
ADBI, Celestolide	1-(6-Tert-Butyl-1,1-dimethyl-2,3-dihydro-1H-inden-4-yl)ethanone	229	244, 173, 230
AHI, Phantolide	1-(1,1,2,3,3,6-Hexamethyl-indan-5-YL)-ethanone	229	244, 187, 230
ATII, Traseolide	5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane	215	216, 258, 173
HHCB, Galaxolide	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta [G]-2-benzopyran	243	213, 244, 258
AHTN, Tonalide	1-(3,5,5,6,8,8-Hexamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)ethanone	243	258, 244, 187
Musk MC-4	1,4-Dioxacyclohexadecane-5,16-dione	213	197, 173, 98
Musk NN	1,4-Dioxacycloheptadecane-5,17-dione	227	211, 187, 98
Exalton	Cyclopentadecanone	224	125, 96, 71
Habanolid	Oxacyclohexadecen-2-one	238	95, 81, 68
Exaltolid	Oxacyclohexadecan-2-one	240	222, 97, 55
Muscon	3-Methylcyclopentadecanone	238	209, 125, 85
Ambrettolid	Oxacycloheptadec-8-en-2-one	252	109, 96, 82
Civetton	9-Cycloheptadecen-1-one	250	251, 81, 55
PCBs			
PCB 28	2,4,4′-Trichloro-1,1′-biphenyl	256	258, 260, 186
PCB 52	2,2′,5,5′-Tetrachloro-1,1′-biphenyl	292	290, 294, 220
PCB 101	2,2′,4,5,5′-Pentachloro-1-1′-biphenyl	326	328, 324, 254
PCB 118	2,3′,4,4′,5-Pentachloro-1,1′-biphenyl	326	324, 328, 254
PCB 138	2,2′,3,4,4′,5′-Hexachloro-1,1′-biphenyl	360	362, 290, 358
PCB 153	2,2′,4,4′,5,5′-Hexachloro-1,1′-biphenyl	360	362, 358, 290
PCB 180	2,2′,3,4,4′,5,5′-Heptachloro-1,1′-biphenyl	396	394, 324,398
PBDEs			
BDE 28	2,4,4′Tribromodiphenyl ether	406	408, 246, 248
BDE 47	2,2′,4,4′Tetrabromodiphenyl ether	486	484, 488, 326
BDE 99	2,2′,4,4′,5-Pentabromodiphenyl ether	406	402, 404, 408
BDE 100	2,2′,4,4′,6-Pentabromodiphenyl ether	564	566, 568, 562
BDE 153	2,2′,4,4′,5,5′-Hexabromodiphenyl ether	484	642, 644, 646
BDE 154	2,2′,4,4′,5,6′-Hexabromodiphenyl ether	484	482, 486, 480

[a] Bp-2 see under LC–MS determination of Bp-2.
[b] Previous name octyl-methoxy cinnamate (OMC).

material as well as spiked lipid samples were analyzed in each batch. (4) Matrix-matched calibration, based on a multilevel (3 or 4 levels) calibration curve, was used to compensate for matrix effects for determination of UV filters.

2.4.2. Analysis of chemicals extracted from defatted milk: Parabens

2.4.2.1. Sample extraction. Frozen samples of defatted milk of cohorts 2005 and 2006 were analyzed by pharm-analyt, Baden (Austria) for analysis of methyl-paraben (methyl-4-hydroxybenzoate), ethyl-paraben (ethyl-4-hydroxybenzoate), propyl-paraben (propyl-4-hydroxybenzoate) and butyl-paraben (butyl-4-hydroxybenzoate) (Mascher et al., 2008). Frozen defatted human milk samples were thawed at 20–25 °C (water bath), 0.2 mL of each sample was transferred into a conical vial of approx. 5 mL size. Only total parabens were analyzed as the conjugated species had been found to be stable and most prevalent in human milk (Ye et al., 2008, 2009). The samples were shaken on a vortex apparatus for 2 × 10 s and then 0.2 mL of acetonitrile (Merck, gradient grade for Liquid chromatography, Reag. Ph.Eur) was added and the mixture was centrifuged at 4000 rpm for 2 min (separation of precipitated proteins). Three hundred and fifty microlitres of the samples was transferred into conical auto sampler vials and the organic part of the sample was evaporated in a rotation vacuum centrifuge during 20 min, 12 min thereof at 45 °C at 100 mbar pressure (security pressure 110 mbar). After sealing with aluminium caps the vials were kept at 4 °C.

2.4.2.2. Detection of parabens. Twenty microlitres of the sample was injected into the HPLC–MS/MS system in MRM mode. The column (Luna C18, 50 × 2 mm, 5 μm particle size) was kept at 45 °C and the flow rate was 1.0 mL min^{-1}. The mobile phase consisted of 2 solutions: solvent A (20 mM ammonia (ammonium hydroxide solution, puriss., from 25% NH_3 in H_2O (Fluka)) and solvent B (20 mM ammonia in acetonitrile). The gradient steps were: 0.0–0.3 min isocratic:1% B; 0.3–2.8 min linear: 1% B → 75% B; 2.8–2.9 min isocratic: 75% B; 2.9–3.7 min isocratic: 1% B. The column effluent was directed into mass spectrometer, the temperature at the ion source was 550 °C, the ionisation voltage at 4.5 kV and gas pressures of gas 1 and 2 was at 70 psi.

The analytes were detected in the ESI negative mode by multiple reaction monitoring (MRM). Parent-ion/fragment-ion and retention time were as follows: Methyl-paraben 151.1/92.1 (0.55 min), ethyl-paraben 165.1/137.1 (1.15–1.2 min), propyl-paraben 179.1/137.1 (1.35 min), butyl-paraben 193.1/137.1 (1.6 min). Reference chemicals were purchased from Sigma–Aldrich Chemie GmbH, D-89552 Steinheim (Germany), with 99% purity. Calibration was done at

Table 1b
Determination of pesticides and internal standards.

Substance Pesticide	Mass of target-ion	Mass of qualifier-ions	Substance Pesticide	Mass of target-ion	Mass of qualifier-ions
p,p′ DDT	235	237, 212, 165	Oxychlordane	389	378, 391, 185
op′ DDT	235	237, 212, 165	Endrine	263	279, 317, 345
pp′ DDE	318	316, 246, 248	Endrine Ketone	317	319, 315, 345
op′ DDE			4 4-Methoxychlor	227	228, 274, 344
pp′ DDD	235	237, 165, 199	Bromocyclene	357	359, 361, 394
op′ DDD	235	237, 165, 199	2,4,6-Tribromoanisol	344	346, 329, 301
HCB	284	282, 286, 288	2,4,6-Tribromoanilin	329	331, 327, 333
Alpha HCH	219	181, 217, 221	Parlar 26	331	305, 231, 233
Lindane, gamma HCH	219	217, 221, 118	Parlar 50	375	279, 339, 377
Dieldrin	181	217, 221, 219	Parlar 62	339	279, 375, 413
Heptachlor	272	274, 337, 237	Nitrofen	283	285, 202, 253
Cisheptachlorepoxid	353	351, 355, 357	Triclosan-methyl	304	302, 306, 252
Alpha endosulfan	339	241, 195, 267	Octachlorodipropyl ether (S-421)	109	79, 130, 181
Beta endosulfan	339	195, 239, 267	Chlorpyrifos-ethyl	197	199, 314, 316
Endosulfan sulphate	387	272, 229, 424	*Internal standards*		
Trans nonachlor	409	407, 411, 405	TCB, ISTD	256	258, 260, 186
Alpha chlordane	373	375, 377, 371	Mirex, ISTD	270	272, 274, 237
Gamma chlordane	373	375, 377, 371	Heptachlor	276	270, 274, 272

concentrations of 10/100/1000 ng mL^{-1}. The 1:1dilution of defatted human milk (200 μL) with 200 μL acetonitril did not change the normal recovery rate of 100% as only proteins precipitate. As concentrations of 10 ng compound mL^{-1} revealed a rather large peak, peak values at concentrations down to 1 ng mL^{-1} were extrapolated (peak area about 2.000–3.000).

2.4.3. Analysis of phthalate metabolites in whole human milk

Phthalate metabolites were analyzed in portions of whole milk samples of the 2006 cohort at the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg (Matthias Wittassek, Jürgen Angerer) following procedures described by Koch et al. (2003), with small modifications. The following phthalate metabolites were analyzed: Five metabolites of **DEHP** (di(2-ethylhexyl) phthalate): MEHP, mono-(2-ethylhexyl) phthalate; 5OH-MEHP, mono-(2-ethyl-5-hydroxyhexyl)phthalate; 5oxo-MEHP, mono-(2-ethyl-5-oxohexyl) phthalate; 5cx-MEPP, mono-(2-ethyl-5-carboxypentyl)phthalate, 2cx-MMHP, mono-[2-(carboxymethyl)hexyl]phthalate. One metabolite of **DnBP** (di-*n*-butyl phthalate): MnBP, mono-(*n*-butyl)phthalate; one metabolite of **DiBP** (di-isobutyl phthalate): MiBP, mono-(isobutyl) phthalate; one metabolite of **BBzP** (butyl-benzyl phthalate): MBzP, mono-(butylbenzyl)phthalate; three metabolites of **DiNP** (di-isononyl phthalate): 7OH-MMeOP, mono-(4-methyl-7-hydroxy-octyl)phthalate, 7oxo-MMeOP, mono-(4-methyl-7-oxo-octyl) phthalate, 7cx-MMeHP, mono-(4-methyl-7-carboxy-heptyl) phthalate.

The LC/LC–MS/MS analysis was done according to the protocol used for the analysis of phthalates in urine samples (Koch et al., 2003, 2007; Preuss et al., 2005) with some modifications. Milk samples stored at −25 °C were thawed, and 75 μL of Internal standard solution (deuterium-labeled analytes) and 190 μL of (1 M) phosphoric acid was added to 1.5 mL of human milk sample. Phosphoric acid eliminates esterase activity and prevents formation of monoesters from the ubiquitously present phthalate diesters (Calafat et al., 2004). Then the sample was vortexed, sonicated and centrifuged for removal of milk lipids. To 0.5 mL of the defatted human milk sample, 0.5 mL ammoniumacetate buffer (pH 6.0) and 10 μL of glucuronidase was added for enzymatic hydrolysis of the metabolites (37 °C, 2 h). Liquid chromatography was carried out on a Hewlet–Packard HP1100 Series HPLC apparatus. The analytes were on-line extracted from milk-matrix using a restricted access (pre) column (LiChrospherRP-8, ADS, Merck, Darmstadt, Germany), transferred in backflush-mode and resolved by reversed phased HPLC (Synergi Fusion-RP column (4 μm, 250 mm × 3 mm, Phenomonex). HPLC solvent consisted of 1% aqueous solution of acetic acid and methanol (95:5 (v/v)).

Eluted metabolites were detected by ESI–tandem mass spectrometry in negative ionisation mode and quantified by isotope dilution. MS–MS detection was performed on a Sciex API 2000 LC–MS–MS system. For each analyte at least two specific parent-daughter ion combinations were monitored with one combination being used for quantifications and the other(s) for verification. MS operating conditions were in the MRM mode. Analyte specific parameters are given in Koch et al., 2003). Detection limits for all analytes were in the low ppb range (LOD 0.5–1.0 μg L^{-1} milk).

2.5. Data analysis

Concentrations of chemicals analyzed in milk fat are given as ng g^{-1} lipid, concentrations of chemicals analyzed in defatted milk or whole milk as ng mL^{-1}. Mean, SD, median, percentiles and range are based on those samples where a given chemical was detected (above LOD), with the number of positive samples (*N* pos.). Since concentrations of UV filters and other chemicals exhibited skewed frequency distributions (data not shown), nonparametric tests were used. The possible relationship between the presence of an individual UV filter in milk (yes/no) and the reported use of this UV filter (yes/no) was analyzed by contingency tables using Fisher's exact test. Three separate analyses were performed: comparison of the presence of a UV filter in milk with use of the UV filter in sunscreen products, with use in other types of cosmetics, and with use in any cosmetic product. Levels of all chemicals were compared with maternal parameters (age, BMI, number of children, domicile, smoking) by Mann–Whitney tests. Maternal age and BMI were dichotomized at approximately their respective median (for age, <34 years, >34 years, for BMI, <26, >26). Age and BMI were also analyzed by Spearman correlation. Information on workplace was not sufficiently detailed for analysis of possible influences of profession.

Spearman analysis was further used for the comparison of chemical levels with nutritional factors, and for the comparison of levels of one chemical with the levels of all other chemicals. For analysis of nutritional influences, scores were calculated as follows: Milk products without cheese, total score = frequency score × quantity score × lipid content score (frequency score: 0 = seldom, 1 = 2× $week^{-1}$, 2 = >2× $week^{-1}$, 3 = daily. Quantity score: 1 = <250 g d^{-1}, 2 = 250–499 g d^{-1}, 3 = >500 g d^{-1}. Lipid content score: 1 = 0.5–1.9%, 2 = 2–2.9%, 3 = >3%). Cheese, total

score = frequency score × lipid content score (Frequency score: 0 = seldom, 1 = 2× week^{-1}, 2 = > 2× week^{-1}, 3 = daily. Lipid content score: 1 = low, 2 = high). Fish consumption score = frequency score: 0 = seldom to <1× week^{-1}, 1 = 1× week^{-1}, 2 = 2× week^{-1} and more. Red meat score = frequency score: 0 = seldom to <1× week^{-1}, 1 = 1× week^{-1}, 2 = 2× week^{-1}, 3 = >2× week^{-1}. The free statistical software R (version 2.5.1) and GraphPad Prism (version 5.00, GraphPad Software, San Diego California USA) was used. Statistical analyses were performed jointly by Valentin Rousson (University of Lausanne) and our laboratory.

For calculation of daily intake of chemicals by individual infants, the quantity (g) of milk consumed per day was extrapolated from Table 2 in Neville et al. (1988) according to the milk sampling period of individual mothers: Day 7–14 post partum 615 g d^{-1} (n = 26), day 15–28 689 g d^{-1} (n = 22), day 30–59 707 g d^{-1} (n = 4), ⩾day 60 753 g d^{-1} (n = 2). The intake of chemicals analyzed in milk fat was calculated according to the formula: Dose = Cf × F × Im, Cf = contaminant concentration as ng g^{-1} lipid, F = lipid content in breast milk as g lipid g^{-1} milk, Im = intake of breast milk as g milk kg^{-1} body weight of infant at lactation d^{-1} (Hoover, 1999). The intake of parabens and phthalates was calculated according to the formula: Dose = Cvol × Im/Density of milk (1.03), Cvol = contaminant concentration as ng mL^{-1} milk, Im = intake of breast milk as g milk kg^{-1} body weight of infant at lactation d^{-1}.

3. Results and discussion

3.1. Maternal and child data

The age of the mothers across all three study cohorts was similar with a mean of 32.3 years (Table 2). Body weights at parturition were also comparable throughout the cohorts. Only mothers giving birth to singleton children were enrolled in the study. For 34 out of 54 mothers (63%) it was the first, for 20 mothers the second child. Mothers were working in a wide range of professions including office work, sales departments, hairdressing, teaching, laboratory technicians, nursing, human and veterinary medicine, chemistry, biology; two were housewives without profession. There was no statistically significant correlation between profession and chemicals in breast milk. 72.2 percent of the mothers lived in the city, considerably more than in suburban (9.3%) or rural (18.5%) areas. Data on maternal nutritional habits are given in Appendix B. Of the total of 54 children, 27 were boys and 27 girls, yielding a sex ratio of 0.50 (Table 2). Birth dates in all three cohorts were within the months August–November, except for two children in cohort two born in June and July.

Milk samples were taken in August/September, October and November/December. When all three cohorts are taken together, the three periods are similarly represented (Table 2). In 49 women, sampling started at 9.5 ± 4.5 d post partum, in five women, the onset of sampling was delayed to 64.2 ± 36.6 d post partum (Table 7). The duration of the sampling period was 9.7 ± 3.4 d (n = 49) and 9.4 ± 3.7 d (n = 5), respectively. Levels of chemicals in human milk did not differ significantly between these two groups.

3.2. Use of cosmetic products containing UV filters

The questionnaire on use of cosmetics was completed by 53 out of 54 women. Analysis of the three cohorts disclosed that 41 out of 53 women or 77.36% reported use of cosmetics containing at least one of the eight analyzed UV filters during pregnancy and/or lactation (Table 3). 54.72% of the study participants used sunscreens

Table 2
Mother and Child Data.

	Cohort 2004	Cohort 2005	Cohort 2006	All cohorts			
	Mean ± SD (N) or count	Mean ± SD (N) or count	Mean ± SD (N) or count	Mean ± SD (N) or count	Median	95 Percentile	Range
Mother							
Age at parturition (years)	32.92 ± 6.63 (13)	32.19 ± 4.34 (21)	32.05 ± 5.10 (20)	32.31 ± 5.15 (54)	33.00	40.00	22–43
Body weight at parturition (kg)	78.46 ± 11.84 (13)	72.71 ± 5.34 (21)	79.65 ± 14.36 (20)	76.67 ± 11.26 (54)	74.00	101.70	59–111
Body mass index (BMI)	27.76	25.8	28.35	27.21	26.85	32.48	21.2–37.5
Domicile: city	9	16	14	39			
Domicile: suburb	0	3	2	5			
Domicile: countryside	4	2	4	10			
Child							
Number of children[a]	1.23 ± 0.44 (13)	1.43 ± 0.51 (21)	1.40 ± 0.50 (20)	1.37 ± 0.49 (54)	1.0	2.0	1–2
Newborn = first child	10	12	12	34			
Newborn = second child	3	9	8	20			
Sex ratio: males/females	5/8	11/10	11/9	27/27			
Birth weight (kg)	3.55 ± 0.47 (13)	3.31 ± 0.36 (21)	3.44 ± 0.53 (20)	3.42 ± 0.46 (54)	3.37	4.10	2.28–4.50
Male birth weight (kg)	3.62 ± 0.55 (5)	3.36 ± 0.33 (11)	3.66 ± 0.46 (11)	3.53 ± 0.44 (27)	3.46	4.28	2.65–4.50
Female birth weight (kg)	3.51 ± 0.44 (8)	3.25 ± 0.41 (10)	3.17 ± 0.49 (9)	3.30 ± 0.45 (27)	3.22	3.98	2.28–4.10
Weight at lactation[b] (kg)	3.77 ± 1.03 (13)	3.41 ± 0.76 (21)	3.70 ± 0.63 (20)	3.61 ± 0.79 (54)	3.44	4.74	2.35–6.80
Milk sampling							
Onset (days post partum)[c]				14.6 ± 19.4 (54)	9.00	38.5	See[c]
Duration of sampling (d)				9.7 ± 3.4 (54)	9.0	16.0	3–23
Lipid content (g lipid g^{-1} milk)				0.035 ± 0.009 (54)	0.035	0.052	0.015–0.054
Sampling period	Number of women	Number of women	Number of women	Number of women			
August–September	0	16	8	24			
October	0	5	8	13			
November–December	13	0	4	17			

[a] Including newborn.
[b] Body weight at onset of milk sampling.
[c] Subgroup A (N = 49) sampling period before 30 d post partum, onset of sampling between postnatal days 4 and 21, mean duration 9.7 d. Subgroup B (N = 5) sampling after 30 d post partum, onset of sampling between postnatal days 34 and 108, mean duration 9.7 d. No significant differences between A and B in levels of chemicals.

Table 3
Reported use of UV filters in cosmetic products and detection in human milk.

	Reported use (n = 53)[a]						Detection in milk (n = 54)[a]		Correlation of reported use and detection (n = 53)
	Sunscreens		Other cosmetics		All products				
	Number of women reporting use	% of total	Number of women reporting use	% of total	Number of women reporting use	% of total	Number of positive milk samples	% of total	
All UV filters[b]	29	54.72	32	60.38	41	77.36	46	85.19	p = 0.0104
Ethylhexyl-methoxycinnamate (EHMC)	25	47.17	26	49.06	35	66.04	42	77.78	p = 0.0799
Octocrylene (OCT)	21	39.62	2	3.77	23	43.40	36	66.67	p = 0.0401
4-Methylbenzylidene camphor (4-MBC)	14	26.42	0	0	14	26.42	11	20.37	p = 0.0488
Homosalate (HMS)	8	15.09	0	0	8	15.09	3	5.56	p = 0.3943
Benzophenone-3 (Bp-3)	1	1.89	6	11.32	7	13.21	7	12.96	p = 1.0000
Benzophenone-2 (Bp-2)	0	0	10	18.87	10	18.87	0	0	nca
Octyl-dimethyl-PABA (OD-PABA)	0	0	1	1.89	1	1.89	1	1.85	nca
3-Benzylidene camphor (3-BC)	0	0	0	0	0	0	0	0	nca

[a] One woman did not complete the questionnaire. Her milk data were excluded from correlation statistics.
[b] Reported use (questionnaire) or detection (chemical analysis), respectively, of any of the eight UV filters. nca: no correlation analysis.

and 60.38% used other cosmetic products containing UV filters. Ethylhexyl-methoxy cinnamate (EHMC, also known as octyl-methoxy cinnamate, OMC) and octocrylene (OCT) were the most frequently used UV filters (66% and 43% of women), followed by 4-methylbenzylidene camphor (4-MBC, 26% of women).

The 53 questionnaires contained a total of 410 reports of use of a cosmetic product (data not shown). 47.3% (194) of the reports concerned skin care products, 12% (49) deodorants, 11.7% (48) make ups, 10.2% (42) lip sticks, 9.5% (39) perfumes, and 9.3% (38) sunscreens. 4-MBC (14) and homosalate (8) were present exclusively in sunscreens. For OCT, the great majority of reports of use were related to sunscreen products (91.6%, 22), one report (4.2%) concerned a lip stick, another one a skin care product. In contrast, reports on use of EHMC included a larger range of products (sunscreens 38.8% (26), lip sticks 32.8% (22), make up 10.4% (7), perfumes 9% (6), skin care products 9% (6)). Reports on benzophenone-3 (Bp-3) use concerned perfumes in 57.1% (4), but also skin care products (1), sunscreens (1) and lip sticks (1), those on benzophenone-2 (Bp-2) almost exclusively perfumes (90.9%, 10/11). 3-Benzylidene camphor (3-BC) was not declared in any of the products used. Deodorants contained none of the eight UV filters.

3.3. UV filters in human milk: trends and correlations

We analyzed eight frequently used UV filters out of the 29 UV filters admitted for use in cosmetics and sunscreens in Switzerland (Swiss Ordinance for Cosmetics (Vkos), 2009). UV filters were detected in 46 out of 54 or 85.19% of breast milk samples (Tables 3 and 4), with the rank order of frequency of detection corresponding to that of reported use of these filters (Table 3). Use of cosmetics containing the UV filters and presence in the milk sample of individual mothers was positively correlated for 4-MBC and OCT (Table 3). Use of a product containing any one of the eight UV filters was also correlated with the presence of any one of these compounds in human milk. For EHMC, the correlation did not reach statistical significance, possibly because some sources of this compound with more widespread use had been missed in the questionnaire. For the remaining UV filters, sample sizes were too small for correlation statistics. The reason for the failure to detect Bp-2 in spite of reported use, is presently unknown. 3-BC was neither present in products nor detected in milk samples. A correlation between use of products containing certain UV filters and their presence in human milk would be compatible with the reported short half life of 4-MBC (20 h in females) following dermal application in humans (Schauer et al., 2006). Whether the UV filter levels in the pooled milk samples from about 10 consecutive days (9.7 ± 3.4 d, Table 2) exclusively represent exposure during this period, or whether there may have been a contribution from hitherto unidentified tissue stores, remains to be further elucidated.

In contrast to the significant positive correlation with use of cosmetics, no correlation was found between UV filters in human milk and mother's age, body weight, body mass index, domicile (city, suburban, countryside), or nutritional parameters (fish and red meat consumption, frequency, quantity of use and fat content of milk and cheese). There was *no positive* correlation with levels of any of the other chemicals determined in the same milk sample. In contrast, *inverse* correlations were observed between levels of the most frequently used UV filters and those of PCB congeners: between EHMC and PCB 28 (p = 0.018), PCB 52 (p = 0.000) and PCB 101 (p = 0.000), between OCT and PCB 101 (p = 0.000) and between 4-MBC and PCB 101 (p = 0.045).

These data point to a basic difference in exposure patterns of UV filters as compared to classical POP contaminants. UV filters are widely present in aquatic ecosystems and exhibit bioaccumulation in invertebrates and fish (Fent et al., 2010), but their presence in human tissues appears to correlate with consumer habits rather than with environmental exposure. This leads to a greater inter-individual variability of human exposure patterns to UV filters (see numbers of positive samples in Tables 4–6). Individual milk samples contained all or almost all PCBs and a majority of the pesticides analyzed. In contrast, one single milk sample contained between one (20.4% of all samples), two (35.2%), three (24.1%) or four (5.5%) of the six UV filters detected in milk; 14.8% of the samples were negative. DDT and its metabolites and phthalate metabolites exhibited distributions similar to PCBs, whereas PBDEs and parabens occupied intermediate positions suggesting a greater degree of inter-individual exposure variability. In the case of synthetic musks, three of the six compounds detected, musk xylene, musk ketone and HHCB, were predominating.

A comparison of samples collected in August + September and in November + December did not show marked seasonal differences, even in the case of two UV filters used exclusively (4-MBC) or almost exclusively (OCT) in sunscreens. For 4-MBC, the percentage of positive milk samples was 25.0% (6/24) in

Table 4
Concentrations of UV filters, musk fragrances, parabens and phthalate metabolites in human milk.

	Cohort 2004[a] Total $N = 13$		Cohort 2005[a] Total $N = 21$		Cohort 2006[a] Total $N = 20$		All Cohorts[a] Total $N = 54$				
	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Median	95%	Range
UV filters	ng g^{-1} lipid		ng g^{-1} lipid		ng g^{-1} lipid		ng g^{-1} lipid				
Ethylhexyl-methoxy cinnamate	35.31 ± 17.22	13	19.60 ± 25.05	9	25.98 ± 23.09	20	27.50 ± 22.15	42	18.41	73.31	2.10–79.85
Octocrylene	22.57 ± 25.46	7	15.08 ± 10.08	9	39.63 ± 25.21	20	30.18 ± 24.51	36	28.32	70.64	4.70–134.95
4-Methyl-benzylidene camphor	19.00	1	14.50 ± 6.76	3	25.84 ± 14.53	7	22.12 ± 12.80	11	18.70	43.15	6.70–48.37
3- Benzylidene camphor	0	0	0	0	0	0	0	0			
Homosalate	0	0	0	0	29.37 ± 27.64	3	29.37 ± 27.64	3	15.50	56.63	11.40–61.20
Benzophenone-3	117.00	1	35.48 ± 48.63	5	71.18	1	52.23 ± 50.69	7	26.70	120.08	7.30–121.40
Benzophenone-2	0	0	0	0	0	0	0.00	0			
Octyldimethyl-PABA	49.00	1	0	0	0	0	49.00	1			
Musk fragrances[b]	ng g^{-1} lipid		ng g^{-1} lipid		ng g^{-1} lipid		ng g^{-1} lipid				
Musk xylene	4.01 ± 8.42	13	1.67 ± 1.34	20	3.72 ± 7.89	14	2.93 ± 6.17	47	1.34	5.67	0.25–31.60
Musk ketone	2.26 ± 3.25	12	0.83 ± 1.00	16	2.34 ± 4.54	6	1.60 ± 2.77	34	0.64	6.47	0.25–12.00
Galaxolide (HHCB)	35.83 ± 24.51	10	55.40 ± 67.33	21	68.15 ± 58.94	14	55.02 ± 57.87	45	36.13	165.57	6.09–309.66
Tonalide (AHTN)	16.25 ± 16.19	2	14.09 ± 9.96	4	8.96	1	13.97 ± 9.96	7	10.23	28.47	4.80–28.80
Phantolide (AHDI)	15.30	1	0	0	0	0	15.30	1			
Habanolide					15.00 ± 0.00	2	15.00 ± 0.00	2	15.00	15.00	15.00–15.00
Parabens[c]			ng mL^{-1}		ng mL^{-1}		ng mL^{-1}				
Methyl-paraben			2.69 ± 2.52	8	1.50 ± 0.84	6	2.18 ± 2.02	14	1.00	5.40	1.00–8.00
Ethyl-paraben			1.33 ± 0.24	4	1.20 ± 0.24	4	1.26 ± 0.23	8	1,30	1.50	1.00–1.50
Propyl-paraben			1.50 ± 0.41	4	1.25 ± 0.35	2	1.42 ± 0.38	6	1.50	1.88	1.00–2.00
Butyl-paraben			0	0	0	0	0	0			
Phthalate metabolites[d]					ng mL^{-1}		ng mL^{-1}				
MEHP (DEHP)					34.05 ± 26.00	20	34.05 ± 26.00	20	26.20	74.98	9.60–122.00
MnBP (DnBP)					7.88 ± 6.21	20	7.88 ± 6.21	20	5.95	15.27	1.20–29.80
MiBP (DiBP)					26.61 ± 18.03	20	26.61 ± 18.03	20	24.25	55.28	2.60–66.20
7OH-MMeOP (DiNP)					1.12 ± 0.30	6	1.12 ± 0.30	6	1.20	1.43	0.60–1.50

[a] Mean, standard deviation (SD), median, 95 percentile (95%) and range represent values from positive samples, with number (*N* pos.) of positive samples. The total number of samples analyzed (*N*) is shown on top of each cohort. Values < LOD = 0.

[b] Synthetic musks analyzed but <LOD: (a) In all cohorts: polycyclic musks: Cashmeran (DPMI), Celestolide (ADBI), Traseolide (ATII). (b) In cohort 2006: macrocyclic musks: musk MC4, musk NN, Exalton, Exaltolid, Muscon, Ambrettolid, Civetton.

[c] Total $N = 41$ (cohort 2005 + cohort 2006).

[d] Total $N = 20$ (cohort 2006). Phthalate metabolites analyzed but <LOD: 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, 2cx-MMHP, MBzP (BBzP), 7oxo-MMeOP (DiNP), 7cx-MMeHP (DiNP).

Table 5
Concentrations of polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs) in human milk (ng g^{-1} lipid).

	Cohort 2004[a] Total $N = 13$		Cohort 2005[a] Total $N = 21$		Cohort 2006[a] Total $N = 20$		All cohorts[a] Total $N = 54$				
	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Median	95%	Range
PCBs											
PCB 28	1.49 ± 1.42	13	1.86 ± 1.67	21	1.03 ± 0.42	18	1.48 ± 1.33	52	1.10	4.38	0.25–7.70
PCB 52	0.25 ± 0.00	4	0.38 ± 0.43	19	0.29 ± 0.15	16	0.33 ± 0.32	39	0.25	0.61	0.25–2.13
PCB 101	0.80 ± 0.65	12	1.22 ± 1.73	21	0.37 ± 0.22	19	0.80 ± 1.19	52	0.60	8.67	0.25–8.67
PCB 118	10.28 ± 4.03	13	12.04 ± 6.48	21	10.98 ± 4.78	20	11.22 ± 5.31	54	10.45	18.97	4.10–35.41
PCB 138	56.45 ± 26.02	13	48.23 ± 17.96	21	49.88 ± 25.85	20	50.82 ± 22.92	54	49.95	97.56	10.70–115.95
PCB 153	76.19 ± 36.79	13	68.85 ± 29.78	21	69.77 ± 42.18	20	70.96 ± 35.89	54	61.40	132.35	11.05–180.20
PCB 180	42.00 ± 25.87	13	37.66 ± 18.85	2	40.09 ± 29.19	20	39.61 ± 24.37	54	36.48	81.85	4.50–130.80
Σ PCB	187.23 ± 90.34	13	170.20 ± 68.12	21	172.23 ± 100.41	20	175.05 ± 85.22	54	154.46	321.92	32.83–435.72
PBDEs											
BDE 28	0	0	0.44 ± 0.21	6	0.25	1	0.41 ± 0.21	7	0.25	0.68	0.25–0.70
BDE 47	2.39 ± 1.74	13	2.45 ± 3.40	21	1.36 ± 1.16	20	2.03 ± 2.41	54	1.20	5.55	0.25–15.93
BDE 99	0.66 ± 0.54	11	0.80 ± 1.43	20	0.50 ± 0.31	11	0.68 ± 1.03	42	0.47	1.49	0.25–6.76
BDE 100	0.55 ± 0.37	7	0.70 ± 1.30	19	0.27 ± 0.06	15	0.51 ± 0.91	41	0.25	1.27	0.25–5.73
BDE 153	1.04 ± 1.12	13	4.22 ± 15.87	21	0.69 ± 0.24	20	2.15 ± 9.91	54	0.68	1.60	0.25–73.45
BDE 154	0	0	0.25 ± 0.00	5	0	0	0.25 ± 0.00	5	0.25	0.25	0.25–0.25

[a] Mean, standard deviation (SD), median, 95 percentile (95%) and range represent values from positive samples, with number (*N* pos.) of positive samples. The total number of samples analyzed (*N*) is shown on top of each cohort. Values < LOD = 0.

Table 6
Concentration of pesticides in human milk (ng g^{-1} lipid).

Pesticide or metabolite	Cohort 2004[a] Total $N = 13$		Cohort 2005[a] Total $N = 21$		Cohort 2006[a] Total $N = 20$		All cohorts[a] Total $N = 54$				
	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Mean ± SD	*N* pos.	Median	95%	Range
o,p′-DDT	0	0	0.86 ± 0.75	21	0.91 ± 0.49	13	0.88 ± 0.66	34	0.64	2.18	0.25–3.08
p,p′-DDT	5.20 ± 2.68	13	6.14 ± 7.47	21	6.19 ± 4.31	20	5.93 ± 5.43	54	4.3	12.72	37.34–170
o,p′-DDE	0	0	0	0	0	0	0	0			
p,p′-DDE	112.46 ± 61.68	13	155.78 ± 101.52	21	141.51 ± 194.52	20	140.07 ± 136.38	54	93.88	365.59	39.45–892.70
o,p′-DDD	0	0	0	0	0	0	0	0			
p,p′-DDD	0.78 ± 0.32	13	0.75 ± 0.72	7	0	0	0.77 ± 0.48	20	0.7	2.32	0.25–2.32
Σ DDT	131.28 ± 71.40	13	181.00 ± 119.96	21	164.57 ± 221.02	20	162.95 ± 156.44	54	110.86	421.78	47.07–1017.49
Alpha HCH	0	0	0.50 ± 0.43	3	0	0	0.50 ± 0.43	3	0.25	0.92	0.25–0.99
Beta HCH	13.54 ± 6.64	13	15.12 ± 13.68	21	13.89 ± 9.85	20	14.28 ± 10.76	54	10.68	29.74	4.51–66.82
Gamma HCH	0.29 ± 0.12	8	0.73 ± 1.58	20	0.39 ± 0.31	14	0.54 ± 1.11	42	0.25	0.95	0.25–7.39
Trans nonachlor	3.68 ± 1.61	13	4.21 ± 4.26	21	2.88 ± 1.08	20	3.59 ± 2.86	54	2.94	5.71	1.29–21.83
Cis heptachlor	1.38 ± 0.67	13	1.57 ± 1.10	21	1.72 ± 1.73	19	1.58 ± 1.27	53	1.2	2.88	0.70–8.40
Σ heptachlor	1.34 ± 0.62	13	1.51 ± 1.05	21	1.65 ± 1.66	19	1.52 ± 1.22	53	1.2	2.71	0.60–8.06
Alpha chlordane	0.25	1	0	0	0	0	0.25	1			
Oxychlordane	4.40 ± 1.17	13	5.67 ± 4.75	21	4.49 ± 1.39	19	4.84 ± 3.21	53	4.26	7.06	2.15–21.14
Σ Chlordane	4.29 ± 1.14	13	5.49 ± 4.59	21	4.34 ± 1.35	19	4.78 ± 3.06	53	4.19	6.83	2.07–20.45
Hexachlorobenzene	16.72 ± 4.79	13	18.99 ± 8.27	21	17.00 ± 5.33	20	17.71 ± 6.50	54	16.67	28.51	10.20–45.00
Dieldrin	3.24 ± 1.64	13	3.37 ± 1.36	21	2.88 ± 1.16	20	3.16 ± 1.36	54	2.79	5.64	1.20–7.60
Parlar 26 toxaphen	0.62 ± 0.43	9	0.88 ± 0.33	9	0.67 ± 0.12	8	0.73 ± 0.33	26	0.69	1.25	0.25–1.52
Parlar 50 toxaphen	1.18 ± 0.78	8	1.91 ± 0.74	9	1.16 ± 0.37	13	1.39 ± 0.69	30	1.18	2.7	0.25–3.10
Σ Parlar	1.67 ± 1.24	9	2.79 ± 1.02	9	1.58 ± 0.68	13	1.96 ± 1.08	31	1.73	3.95	0.25–4.39
Chlorpyrifos-ethyl	4.00 ± 2.16	3	6.20 ± 14.30	12	1.16 ± 1.54	5	4.61 ± 11.14	20	1.06	17.06	0.25–49.59

Analyzed but <LOD: Bromocyclene, gamma chlordane, alpha-endosulfane, beta-endosulfane, endosulfane sulfate, endrine, endrine keton, heptachlor, methoxychlor, nitrofen, parlar 62 toxaphen, triclosan methyl, 2,4,6 tribromoanisol, 2,4,6 tribromoanilin.

[a] Mean, standard deviation (SD), median, 95 percentile (95%) and range represent values from positive samples, with number (*N* pos.) of positive samples. The total number of samples analyzed (*N*) is shown on top of each cohort. Values < LOD = 0.

August + September and 23.5% (4/17) in November + December, for OCT, the corresponding values were 66.7% (16/24) and 64.7% (11/17). In line with these data, use of 4-MBC was reported by 25% (6/24) of the women in August + September and by 23.5% (4/17) in November + December. For OCT, the corresponding values were 45.8% (11/24) and 29.4% (5/17). On the other hand, a time-trend across the 3 years was visible for OCT with detection rates of 54% in 2004, 43% in 2005, and 100% in 2006, and for 4-MBC with detection rates of 7.7% in 2004, 14.3% in 2005 and 35% in 2006 (Table 4).

3.4. Musk fragrances: nitro musks, polycylic and macrocyclic musks

The nitro musks, musk xylene (MX) and musk ketone (MK), and the polycyclic musks, galaxolide (HHCB), tonalide (AHTN) and phantolide (AHDI) were analyzed in all cohorts; macrocyclic or benzenoid musks were included in the 2006 cohort (Table 4). Concentrations of nitro musks and polycyclic musks were close to those of a previous study done in Basel (Zehringer and Herrmann, 2001). MX was more frequently detected than MK and present at higher levels. Even though studies in Switzerland and Germany

Table 7
Daily intake of chemicals in human milk by infants (ng kg^{-1} body weight (bw) per day).

		Mean ± SD[a]	*N* positive/*N* total[a]	Median	Range	RfD, MRL ng kg^{-1} bw d^{-1}[b]	Source	Mean as % of RfD, MRL	Maximum as % of RfD, MRL
UV filters	EHMC	178.9 ± 153.0	42/54	127.1	14.8–635.7				
	Octocrylene	195.7 ± 142.6	35/54	182.0	23.2–563.7				
	4-MBC	132.7 ± 88.8	11/54	103.6	54.8–293.5				
Musks	Musk xylene	20.6 ± 50.1	47/54	7.9	1.4–261.9				
	Musk ketone	11.1 ± 22.3	34/54	4.2	0.9–99.1				
	HHCB	370.4 ± 484.5	45/54	211.1	34.8–2456.8				
Parabens	Methyl-paraben	422.4 ± 376.8	14/41	192.5	161.4–1314.9	Total parabens			
	Ethyl-paraben	223.7 ± 76.7	8/41	219.4	137.3–381.1	0–10,000000	EFSA 2004	0.02	0.04
	Propyl-paraben	277.6 ± 91.8	6/41	301.3	132.7–381.1				
Phthalate metabolites	MEHP (DEHP)	5780 ± 4219	20/20	5158	1450–20 381	5800	US Cons. Prod. Safety Comm. 2010		
	MnBP (DnBP)	1351 ± 1041	20/20	1079	237.7–4978				
	MiBP (DiBP)	4533 ± 3017	20/20	3508	515.1–9999				
PCBs	PCB 138	325.2 ± 164.2	54/54	304.5	104.9–802.2				
	PCB 153	451.5 ± 245.4	54/54	409.6	108.3–1109.8				
	PCB 180	250.0 ± 161.6	54/54	215.4	44.1–737.4				
	Sum PCB	1114.4 ± 585.4	54/54	999.4	321.8–2732.9	20	RfD US EPA 1996	5572	13 664
						20	MRL ATSDR 2004	5572	13 664
PBDEs	BDE 47	12.8 ± 14.9	54/54	8.9	1.0–100.0	100	RfD US EPA 2008a	12.8	100
	BDE 99	4.2 ± 6.4	42/54	2.7	1.0–42.5	100	RfD US EPA 2008b	4.2	42.5
	BDE 153	15.9 ± 79.3	54/54	4.3	1.4–587.2	200	RfD US EPA 2008c	7.95	293.6
Pesticides and metabolites	Sum DDT	1028.0 ± 889.8	54/54	770.8	213.0–5007	500	RfD US EPA 1996	205.6	1001
	p,p′-DDE	882.9 ± 771.6	54/54	661.7	181.2–4393				
	Dieldrin	20.6 ± 9.7	54/54	18.16	4.43–49.5	50	RfD US EPA 1990	41.1	99.1
	HCB	115.2 ± 49.5	54/54	109.0	40.1–256.0	20	MRL ATSDR 2004	576.0	1280
	β-HCH	91.6 ± 71.3	54/54	69.6	21.1–452.8	600	MRL ATSDR 2005	15.3	75.5
	γ-HCH	4.36 ± 13.1	42/54	1.73	0.73–86.6	10	MRL ATSDR 2005	43.6	865.9
	Oxychlordane	32.2 ± 23.8	53/54	28.3	7.8–143.3	500	RfD US EPA 1998	6.44	28.7

Source of RfD or MRL

Ethylparaben	EFSA, 2004. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a Request from the Commission related to para hydroxybenzoates (E 214–219). Question number EFSA-Q-2004-063. EFSA J. 83, 1–26
DEHP	US Consumer Product Safety Commission, 2010. Memorandum. Toxicity review of di(2-ethylhexyl) phthalate (DEHP). http://www.cpsc.gov/ABOUT/Cpsia/toxicityDEHP.pdf
Sum PCB	1. US EPA, Integrated Risk Information System (1996). Aroclor 1254 (CASRN 11097-69-1). http://www.epa.gov/ncea/iris/subst/0389.htm 2. ATSDR (Agency for Toxic Substances and Disease Registry), 2004. Interaction profile for: Persistent chemicals found in breast milk (chlorinated dibenz-p-dioxins, hexachlorobenzene, p,p′-DDE, methylmercury, and polychlorinated biphenyls). http://www.atsdr.cdc.gov/interactionprofiles/IP-breastmilk/ip03.pdf
BDE 47	US EPA, Integrated Risk Information System (2008a). 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47) (CASRN 5436-43-1). http://www.epa.gov/IRIS/subst/1010.htm
BDE 99	US EPA (2008b). Toxicological review of 2,2′,4,4′,5-pentabromodiphenyl ether (BDE 99) (CAS No. 60348-60-9). http://www.epa.gov/IRIS/toxreviews/1008tr.pdf
BDE 153	US EPA, Integrated Risk Information System (2008c). 2,2′,4,4′,5,5′-Hexabromodiphenyl ether (BDE 153) (CASRN 68631-49-2). http://www.epa.gov/IRIS/subst/1009.htm
DDT	US EPA, Integrated Risk Information System (1996). p,p′Dichlorodiphenyltrichloroethane (DDT). http://www.epa.gov/ncea/iris/subst/0147.htm
Dieldrin	US EPA, Integrated Risk Information System (1990). Dieldrin (CASRN 60-57-1). http://www.epa.gov/iris/subst/0225.htm
HCB	ATSDR (Agency for Toxic Substances and Disease Registry), 2004. Interaction profile for: Persistent chemicals found in breast milk (chlorinated dibenz-p-dioxins, hexachlorobenzene, p,p′-DDE, methylmercury, and polychlorinated biphenyls). http://www.atsdr.cdc.gov/interactionprofiles/IP-breastmilk/ip03.pdf
β,HCH- γHCH	ATSDR (Agency for Toxic Substances and Disease Registry), 2005. Toxicological profile for alpha-, beta-, gamma- and delta-hexachlorocyclohexane. http://www.atsdr.cdc.gov/toxprofiles/tp43.pdf
Oxychlordane	US EPA, Integrated Risk Information System (1996). Chlordane (Technical) (CASRN 12789-03-6). http://www.epa.gov/ncea/iris/subst/0142.htm

[a] Mean, SD, median and range of positive samples. Number of positive samples (*N* positive) and total sample number (*N* total). Daily milk intake (g) calculated according to Neville et al., 1988.
[b] RfD: reference dose, MRL: minimum risk level (ATSDR). Values refer to the parent compound.

have shown a decline of nitro musks in human milk (Eschle et al., 1995), MX remained the most frequent musk in the present analysis (87% of milk samples). In line with the increased use of polycyclic musks (Reiner et al., 2007), HHCB was found in 81% of the samples. In agreement with recent European and US studies (Duedahl-Olesen et al., 2005; Reiner et al., 2007; Lignell et al., 2008), levels of polycyclic musks were about one order of magnitude higher than those of nitro musks (Table 4). No macrocyclic musks could be detected except for Habanolide. The relationship between cosmetic use and presence of musk fragrances in human milk could not be investigated because individual fragrances need not to be declared on cosmetic products. No correlations were found between levels of fragrances and other chemicals.

3.5. Parabens

Parabens, esters of p-hydroxibenzoic acid, are widely used as antimicrobial preservatives, and human exposure to these compounds, used in over 13,200 formulations, is widespread (Ye et al., 2008). The *p*-hydroxibenzoic acid esters are of interest because of their estrogenic and anti-androgenic activities (Routledge et al., 1998; Darbre et al., 2003; Chen et al., 2007). Few data exist so far for parabens in breast milk. Analysis of free methyl-paraben, ethyl-paraben and propyl-paraben revealed their presence in 15–34% of the defatted milk samples (Table 4). Butyl-paraben was not detectable. Ye et al. (2008) concluded from their studies on free and total (free + conjugated) parabens in human serum and milk that the free form was predominating in human milk and that conjugated species of parabens do not appear to hydrolize appreciably to their free forms in serum (Ye et al., 2009). Only free parabens were analyzed in the present study.

Frequency of detection and level in defatted human milk were highest for the most hydrophilic compound, methyl-paraben, and decreased with increasing lipophilicity. Lack of detection of the more lipophilic butyl-paraben therefore may not indicate its absence from human milk but rather, its low potential to appear in the defatted matrix. More lipophilic parabens might be found in milk fat. Concentrations were in the range of nitro musks, about 10 times lower than those of UV filters or polycyclic musks. Levels of ethyl-paraben, but not of methyl- or propyl-paraben, increased significantly with maternal age ($p = 0.027$). No correlations were observed with diet.

3.6. Phthalate metabolites

Phthalates (phthalate diesters) are ubiquitously present in our environment. Some compounds are known reproductive toxicants, but little information exists on exposure of the human neonate and infant through breast milk. Human exposure may occur via ingestion, inhalation and dermal routes. Upon exposure phthalates are rapidly hydrolyzed to their monoesters that can be further metabolized by oxidation and/or glucuronidation and excreted in urine and feces. Because of the high background exposure, levels of rapidly formed metabolites in the organism, the monoesters, are usually determined (Koch et al., 2003, 2007; Calafat et al., 2004; Mortensen et al., 2005; Main et al., 2006; Högberg et al.,2008; Latini et al., 2009).

Phthalate metabolites were analyzed in cohort 2006 (Table 4). The monoesters mono-(2-ethylhexyl) phthalate (MEHP), monoisobutyl phthalate (MiBP) and mono-n-butyl phthalate (MnBP) were detected in all samples. Mono-benzyl phthalate was not present in detectable amounts. Monophthalate ester concentrations in milk samples were comparable to levels detected in human milk of other European countries like Italy (Latini et al., 2009), Denmark, Finland and Sweden (Mortensen et al., 2005; Main et al., 2006; Högberg et al., 2008). In US human milk, the monoesters MnBP and MEHP were present at considerably lower levels than in Europe (Calafat et al., 2004). Values of (secondary) DEHP metabolites such as mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP) and mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP), as well as the 2- and 5-carboxy metabolites of DEHP were below LOD. Among the postulated major metabolites of di-iso-nonyl phthalate (DiNP) (Koch et al., 2007), only small amounts of mono-(4-methyl-7-hydroxy-octyl)phthalate (7OH-MMeOP) were found in 6 out of 20 samples. The DiNP mono-metabolites mono-(4-methyl-7-oxo-octyl)phthalate (7oxo-MMeOP) and mono-(4-methyl-7-carboxy-heptyl)phthalate (7carboxy-MMeHP) were below LOD (Table 4). Because of the small number of samples with phthalate metabolite determinations no correlation statistics were performed.

3.7. Polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs)

PCBs and PBDEs as well as halogenated pesticides (see Section 3.8) were determined in human milk as classical POPs for comparison of exposure patterns (detection frequency and level) with cosmetic constituents (UV filters, musks, parabens) (Table 5). Levels of indicator PCBs, the non-dioxin-like (ndl) congeners PCB 28, 52, 101, 138, 153, 180, the mono-ortho PCB 118 and the sum of these congeners were similar to recent reports from other European regions such as Northern Germany (Zietz et al., 2008), Norwegia (Polder et al., 2009), Belgium (Colles et al., 2008), or Italy (Abballe et al., 2008). The sum of PCBs was correlated with maternal age ($p < 0.0001$), in line with earlier data (Rogan et al., 1986), while no correlation was found with weight, BMI or domicile. Increased fish consumption was positively correlated with PCB 52 ($p = 0.035$); similar tendencies were observed with PCBs 101 and 153 ($p = 0.077$, $p = 0.081$). Total PCB levels were positively correlated with a number of pesticides (see Section 3.8).

Levels of PBDEs (Table 5) were within the range of values recently reported for European countries (Colles et al., 2008; Fromme et al., 2009) and Australia (Toms et al., 2009), but lower than those found in the US, where BDE 47 levels were about one magnitude higher (Schecter et al., 2003) There were no significant correlations between PBDE levels and maternal parameters (age, weight), but levels of BDE 28 were found to be positively correlated with red meat consumption ($p = 0.017$), and levels of BDE 47 and BDE 99 with milk intake ($p = 0.014$ for both congeners).

3.8. Halogenated pesticides and metabolites

Nineteen out of 32 pesticides or pesticide metabolites were detected in human milk of the Basel cohorts (Table 6). Highest levels were observed for p,p′-DDE and the sum of DDT and DDT metabolites. Levels of hexachlorobenzene (HCB) and β-hexachlorcyclohexane (β-HCH) were one order of magnitude lower. Values are in the range of recent European studies in Belgium (Colles et al., 2008), Germany (Zietz et al., 2008), Norwegia (Polder et al., 2009) and Italy (with somewhat higher DDT and DDE levels, Abballe et al., 2008). Maternal age was significantly correlated with levels of Parlar (total Parlar $p = 0.029$, Parlar 50 $p = 0.042$) and nonachlor ($p = 0.001$), but not with any of the DDT compounds. There were no correlations with maternal weight. Parlar levels were also positively correlated with the frequency of fish consumption (Parlar 50 $p = 0.039$, sum Parlar $p = 0.048$), but a systematic relationship between pesticide levels and nutritional factors did not become apparent, possibly because the questionnaire did not address nutrition in the same detail as cosmetic use. Significant positive correlations were observed between the sum of DDT and DDT metabolites on the one hand and total chlordane ($p = 0.004$), oxychlordane ($p = 0.003$) beta-HCH ($p = 0.007$) and nonachlor ($p = 0.002$) on the other hand, as well as between the sum of DDT

and several PCB congeners (total PCB, PCB 101, 118, 138, 153, $p < 0.01$, PCB 28 $p = 0.016$, PCB 180 $p = 0.049$), supporting the idea of a common exposure pattern.

3.9. Daily intake of chemicals

Individual daily intake of all chemicals was calculated from their concentrations in human milk using data of Neville et al. (1988) for quantities of milk consumed by suckling infants at defined postnatal stages. Table 7 shows values of selected compounds. Highest intake values in the range of several micrograms per kg body weight per day are reached by some phthalate metabolites, followed by the sum of indicator PCBs, p,p′-DDE and the sum of DDT and metabolites, UV filters, parabens and the polycyclic musk HHCB. When intake values are compared with recent reference doses or minimal risk levels, it becomes evident that mean and/or maximum concentrations of several chemicals are considerably above these levels (Table 7). This includes the pesticides DDT (mainly as p,p′-DDE), HCB and γ-HCH, the sum of PCB, and the flame retardant BDE 153. So far, no reference values exist for UV filters, synthetic musks, and parabens. In the case of the estrogenic UV filter, 4-MBC, the level in rat milk was 208.6 ng g^{-1} lipid (Schlumpf et al., 2008b) at the LOAEL (lowest observed adverse effect level) for reproductive toxicity endpoints (7 mg kg^{-1} body weight d^{-1}, Durrer et al., 2007; Faass et al., 2009). This is 9.4 times the mean level or 4.3 times the maximum level in human milk observed in the present study (Table 4), which would not be considered to yield a sufficient margin of safety.

3.10. Conclusions

This is the first comprehensive analysis of the simultaneous presence of lipophilic POP chemicals and less lipophilic, less persistent cosmetic constituents in human milk, such as cosmetic UV filters, synthetic musk fragrances, parabens and phthalate metabolites. Frequently used UV filters were found in a large proportion of milk samples, at concentrations comparable to PCBs. Comparison with a detailed questionnaire revealed that the presence of UV filters in human milk was closely linked with the use of cosmetics containing these chemicals, indicating that internal exposure resulted from repeated application of cosmetics rather than from general environmental exposure. This exposure pattern differs completely from the general exposure pattern of classical POPs. It is more variable with respect to products used and types of UV filters present, and can also vary within shorter time periods as a result of changes in the products used. When long-term toxicology of contaminant mixtures is discussed, it would be important to consider that real life mixtures consist of chemicals with comparatively little variation on the one hand, and other compounds that can vary considerably between individuals and over time on the other hand. This is particularly important with respect to effects of endocrine active chemicals, which are present in both groups of chemicals.

Possible sources of the new type of contaminants in human milk include a diversity of products. This is illustrated by the questionnaire data on UV filters, showing that 54.7% of the women used sunscreens and 60.4% used other cosmetics containing UV filters (Table 3). Additional uses of UV filters as UV absorbers in tools and clothings would not have been identified by the questionnaire. Sources of synthetic musks cannot be identified because they need not be declared in the products. Parabens and phthalates are used in many different products in addition to cosmetics. The continuous remodeling of cosmetic products makes it difficult for the consumer to gain access to "stable" informations on product ingredients.

Levels of PCBs, PBDEs, DDT and other organochlor pesticides compared with levels reported in human milk of Western European countries (Campoy et al., 2002; Abballe et al., 2008; Raab et al., 2008; Zietz et al., 2008; Polder et al., 2009). In the late nineties and around the turn of the century, a downward trend of levels of these contaminants in human milk has been reported. However, this should not lead to the conclusion that the problem of contamination of human milk is being solved. On the one hand, levels of POPs are still high, exceeding ADI values in parts of Asia, Africa and Eastern Europe (Sudaryanto et al., 2006; Tsydenova et al., 2007; Zhao et al., 2007; Hui et al., 2008; Ennaceur et al., 2008). The development of E-waste recycling industry appears to play a devastating role (Tue et al., 2009). In these regions, levels of POPs sometimes are markedly above allowed daily intake levels, but levels of some of these chemicals also remain high in relation to more recent standards (US EPA) in countries such as Switzerland, as demonstrated by our study.

On the other hand, an exclusive focus on POPs can obstruct the view on more recently developed and/or detected contaminants in human milk, such as UV filters, synthetic fragrances, parabens or phthalates, whose production and use is increasing. As demonstrated by the present investigation, some of these chemicals reach levels in the range of classical POPs. Compounds with a potential to act at low concentrations, e.g., endocrine active chemicals, are present among classical as well as new contaminants. It would therefore seem to be of outmost importance to monitor the entire set of contaminants, and to study toxicities of complex mixtures in vivo, in particular with respect to variable effects at different life stages.

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Appendix A, B

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Toxic heritage: Maternal transfer of pyrethroid insecticides and sunscreen agents in dolphins from Brazil

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ABSTRACT

Pyrethroids (PYR) and UV filters (UVF) were investigated in tissues of paired mother-fetus dolphins from Brazilian coast in order to investigate the possibility of maternal transfer of these emerging contaminants. Comparison of PYR and UVF concentrations in maternal and fetal blubber revealed Franciscana transferred efficiently both contaminants to fetuses (F/M > 1) and Guiana dolphin transferred efficiently PYR to fetuses (F/M > 1) different than UVF (F/M < 1). PYR and UVF concentrations in fetuses were the highest-ever reported in biota (up to 6640 and 11,530 ng/g lw, respectively). Muscle was the organ with the highest PYR and UVF concentrations ($p < 0.001$), suggesting that these two classes of emerging contaminants may have more affinity for proteins than for lipids. The high PYR and UVF concentrations found in fetuses demonstrate these compounds are efficiently transferred through placenta. This study is the first to report maternal transfer of pyrethroids and UV filters in marine mammals.

1. Introduction

The number of organic contaminants found in the environment is constantly increasing. Among those that have emerged recently are synthetic pyrethroid insecticides and active ingredients in sunscreen products. Along with the emerging POPs (persistent organic pollutants, e.g. emerging flame retardants), these both classes of chemicals have been detected in biota (Alonso et al., 2012a, 2012b; Gago-Ferrero et al., 2012), in human matrices (Corcellas et al., 2012; Kunisue et al., 2012) as well as in other environmental samples (Feo et al., 2010b; Gago-Ferrero et al., 2011). The main concern for these emerging contaminants is endocrine disrupting activity in non-target organisms (Schreurs et al., 2005; Weybridge, 2012).

Synthetic pyrethroids (PYR) are hydrophobic, particle reactive, and are found in low concentrations in water (e.g. up to 70 ng/L in California creeks and 40 ng/L in Ebro river) (Amweg et al., 2006; Feo et al., 2010b). They are applied to land and/or around man-made structures for the control of arthropod-borne diseases, and are also used in agricultural, garden and veterinary products (Feo et al., 2010a; Santos et al., 2007). This group of insecticides has a high degree of toxicity in standard laboratory studies with fish and arthropods (Maund et al., 2002; Woudneh and Oros, 2006; You et al., 2008). Studies suggested carcinogenic, neurotoxic, immunosuppressive, allergenic and reproductive potential toxicity in mammals (Jin et al., 2012; Scollon et al., 2011; Shafer et al., 2008). Newborn rats were reported as 4 to 17 times more vulnerable to acute

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toxicity of PYR than adults (Ostrea et al., 2013; Shafer et al., 2004). Recent studies have reported their accumulation in biotic matrices, such as human breast milk (Corcellas et al., 2012; Feo et al., 2012) and in liver of marine mammals (Alonso et al., 2012b).

Ultra-violet filters (UVFs) are sunscreen agents that reduce the intensity of UV light incidence on cells. UVFs can be found in many products, including cosmetics (e.g. perfumes, shampoos, creams and make up products), as well as in industrial and/or commercial products as an agent to minimize photodegradation. UVFs produced estrogenic and androgenic activity in *in vivo* and *in vitro* studies (Díaz-Cruz and Barceló, 2009; Schreurs et al., 2005). Oral administration of UVF to mammals during pre and postnatal life, has shown that the central nervous system and reproductive organs are targets for the damaging effects on the development of the offspring; resulting in changes in gene expression in organs and regions of the brain related to sexual dimorphism (Schlumpf et al., 2004). Ethylhexyl methoxycinnamate (EHMC) can alter the gene expression in zebrafish (*Danio rerio*) and showed multiple hormonal activities at environmentally relevant concentrations (Zucchi et al., 2011). Recent studies showed that these chemicals are detected in the environment (Gago-Ferrero et al., 2011; Jurado et al., 2014), in biota (Buser et al., 2006; Gago-ferrero et al., 2013) and in human breast milk, placenta and semen (Jiménez-Díaz et al., 2011; León et al., 2010; Schlumpf et al., 2004).

Long-lived marine mammals such as cetaceans are exposed to multiple persistent bioaccumulative toxicants compounds throughout their life history. Dolphins in particular serve as excellent sentinel species for contamination in the marine environment (Alonso et al., 2014; Bossart, 2011; Kucklick et al., 2011). When concentrations reach high enough levels, these sentinels may experience neurotoxic, immunologic and multiple endocrine effects (McDonald, 2002; Zhou et al., 2002). The two primary exposure routes are via the diet and through maternal transfer that in turn occurs via lactation and gestation (Bossart, 2011; Kajiwara et al., 2008). Thus, the exposure of a pregnant female cetacean to toxicants may pose a health threat to the developing fetus, resulting in an increased susceptibility to disease in adulthood (Desforges et al., 2012; Park et al., 2010).

Few works have focused on maternal transfer of organohalogenated compounds in marine mammals. Most data are derived from studies of lactational transfer of contaminants in seals and sea lions (Debier et al., 2012; Vanden Berghe et al., 2012; Wang et al., 2013). Prenatal exposure of cetaceans to organohalogen contaminants was slightly recorded, mainly due to difficulty in obtaining such samples. Some reports recorded the transplacental transfer of organochlorines and organobrominated compounds in cetaceans (Desforges et al., 2012; Dorneles et al., 2010; Hoguet et al., 2013; Kajiwara et al., 2008; Weijs et al., 2013), and rare researches on perfluorinated compounds were conducted (Dorneles et al., 2008a; Van De Vijver et al., 2007), however with a very limited number of samples from mother-fetus pairs.

Our previous studies reported on residues of PYR and UVFs in free-ranging dolphins, including in placenta and milk samples (Alonso et al., 2012b; Gago-Ferrero et al., 2013). However, no direct evidence of maternal transfer was found in these studies. In this paper, we investigated for the first time the likelihood of maternal transfer of pyrethroids and UV filters in cetaceans by analyzing paired samples of mother-fetus of Franciscana and Guiana dolphins from the Southwestern Atlantic Ocean.

2. Materials and methods

2.1. Target species

The Franciscana dolphin (*Pontoporia blainvillei)* is a small odontocete that occurs exclusively in the southwestern Atlantic where it is considered to be among the most threatened cetacean species along the east coast of Latin America (Secchi and Wang, 2002). It has a small coastal home range due to its limited movement patterns (Wells et al., 2012). The site fidelity exhibited by *P. blainvillei* makes this species a particularly useful sentinel for regional contamination (Alonso et al., 2012a, 2012b). The Guiana dolphin (*Sotalia guianensis)* is another exclusively coastal species, ranging from south Brazil northward into Central America. This small delphinid inhabits shallow waters and is often found year-round in bays and estuaries (Batista et al., 2014). Studies have shown *S. guianensis* to reside in Guanabara and Sepetiba bays in Rio de Janeiro State, Brazil (de Freitas Azevedo et al., 2004; Flach et al., 2008). Their largely near-shore distribution render *S. guianensis* particularly vulnerable to impacts due to human activities, such as fishing by-catch and exposure to organohalogenated contaminants (Alonso et al., 2010; Dorneles et al., 2010; Lailson-Brito et al., 2010).

2.2. Sample collection

Fig. S1 (Supplementary Information) shows the coastal areas of Brazil where dolphins were sampled for this study. The southeast region of Brazil, where the states of São Paulo (SP) and Rio de Janeiro (RJ) are located, is one of the most developed areas of South America. The coastal ecosystems near these urban centers historically receive discharges of industrial, domestic and agricultural effluents that contain chemical contaminants (Buruaem et al., 2013), as reflected in residue analysis of biota (Lailson-Brito et al., 2012). In contrast, the land use and economy associated with Ceará state (CE), located in northeastern Brazil, specially where the samples where collected (C3 region, following Santos-Neto et al. (2014)), is based on agriculture, fishing and tourism and the population density is very low (44 hab./Km^2) (CEARA, 2014).

Tissue samples from five mother-fetus pairs of Franciscana dolphins from the São Paulo coast (Baixada Santista) in southeastern Brazil were collected, along with samples from three mother-fetus pairs of Guiana dolphins (two pairs from Sepetiba Bay, Rio de Janeiro coast and one pair from Canoa Quebrada Beach, Ceará coast) from 2004 to 2011 (Fig. S1). Pregnant female dolphins as by-catch were brought to research labs, where they were classified as carcasses in early decomposition stage. After dissection, biological samples were stored in aluminum foil and kept frozen (−20 °C) until processing for analysis. Total length of females and fetuses were measured and pregnancy stages were estimated assuming Franciscana dolphins average 10.2 months of gestation and length at birth is 72 cm (Bertozzi, 2009); for Guiana dolphins, gestation averages 11.4 months and length at birth is 106 cm (Ramos et al., 2000). Blubber and muscle from both mother and fetus were taken from both species. Maternal blubber (n = 7), fetal blubber (n = 5), maternal muscle (n = 4) and fetal muscle (n = 4), as well as umbilical cord (n = 3), placenta (n = 4) and milk (n = 3) from Franciscana dolphins; and maternal blubber (n = 3), fetal blubber (n = 3), maternal muscle (n = 3) and fetal muscle (n = 3) from Guiana dolphins.

2.3. Standards and reagents

A standard mixture of six PYRs containing cyfluthrin, cypermethrin, deltamethrin, fenvalerate, permethrin and tetramethrin, and single analytical standards of bifenthrin, lambda-cyhalothrin, esfenvalerate, tau-fluvalinate, tralomethrin. d_6-*trans*-permethrin and d_6-*trans*-cypermethrin, used as internal standard, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The certified UVF standards 4-methylbenzylidene camphor (4MBC), 2-ethylhexyl-4-trimethoxycinnamate (EHMC), 2-ethyl-hexyl-4-dimethyl-

aminobenzoate (OD-PABA) and octocrylene (OCT) were supplied by Dr Ehrenstorfer and Sigma–Aldrich (Steinheim, Germany). Isotopically labeled 3-(4-methylbenzylidene-d_4)camphor (4MBC-d_4) used as internal standard was obtained from CDN isotopes (Quebec, Canada). Pesticide grade hexane, dichloromethane, ethyl acetate and acetonitrile were obtained from Sigma–Aldrich (St. Louis, MO, USA). Standard solutions were prepared in ethyl acetate for instrumental analysis. In order to check the linearity of the method, calibration curves were prepared at different concentrations ranging between 0.15 and 40 pg/μL for PYR and from 0.10 to 200 pg/μL for UVF. The solutions were stored in the dark at − 20 °C.

In order to prevent contamination and photodegradation of samples and standard solutions all glassware used was previously washed and heated overnight at 380 °C, and further sequentially rinsed with different organic solvents and HPLC grade water. Separate solvents and only previously unopened packages of solvents, chemicals and other supplies were used. In addition, a set of at least two operational blanks were processed together with each batch of samples. Standard solutions and samples were always covered with aluminum foil and stored in the dark. Furthermore, gloves were worn during the sample preparation process.

2.4. Sample preparation

The sample analyses were performed using previously described methods (Alonso et al., 2012b; Feo et al., 2012; Gago-Ferrero et al., 2013). Biological samples (0.1 g dry weight) were fortified with d_6-*t*-PERME (4 ng), d_6-*t*-CYPE (2 ng) and 4MBC-d_4 (4 ng) as surrogate standards and extracted by sonication with 20 mL hexane: dichloromethane (2:1) in a Raypa, UCI-200 bath for 15 min. Samples were centrifuged at 3500 rpm (20 min) and the organic phase was transferred to a vial and evaporated under a nitrogen stream. This extraction step was repeated twice and all the solvent residues were collected together. The lipid content was determined gravimetrically for each sample. Extracts were cleaned up by elution through C18 (2 g/15 mL) coupled to basic alumina cartridges (5 g/25 mL) and conditioned with 25 mL of acetonitrile. Samples were eluted with 30 mL of acetonitrile and evaporated under a nitrogen stream. The residue was dissolved in 100 μL of ethyl acetate for GC and LC analysis.

2.5. Instrumental analysis

The extracts were injected on a GC–NCI-MS/MS for pyrethroid analysis and on an HPLC-(ESI)-MS/MS for UV filters analysis. GC–MS–MS analysis were performed in negative chemical ionization mode on an Agilent Technologies 7890A GC system coupled to 7000A GC/MS Triple Quad. A DB-5 ms capillary column (15 m × 0.25 mm i.d., 0.1 μm film thickness) containing 5% phenyl methyl siloxane was used with helium as carrier gas at constant flow of 1 mL/min. The temperature program was from 100 °C (held for 1 min) to 230 °C at 15°Cmin−1, then from 230 to 310 °C (held for 2 min) at 10°Cmin−1, using the splitless injection mode during 0.8 min. Inject volume was 3 μL. The inlet temperature was set at 275 °C and ion source temperature at 250 °C. Ammonia was used as reagent gas at 2×10^{-4} Torr. More details on MS/MS and selected transitions were reported in Feo et al. (2011, 2010a). HPLC-MS/MS analysis were performed in positive electrospray ionization mode on an Agilent Technologies HP 1100 pump connected to a 4000 Q TRAP™ MS/MS system from Applied Biosystems-Sciex (Foster City, California, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) from Merck, preceeded by a guard column of the same packaging material. A gradient using a mixture of HPLC grade water and ACN, both 0.15% formic acid was applied. The elution gradient started with 5% of ACN, increasing to 75% in 7 min, and then to 100% in 3 min. The mobile phase flow-rate was 0.3 mL/min. A detailed description of methods for both groups of pollutants can be found in Gago-Ferrero et al. (2013 a; 2013 b), Alonso et al. (2012b) and Feo et al. (2010a).

MS/MS analyses of both, PYRs and UVFs, were performed using selected reaction monitoring (SRM). Two major characteristic fragments of the protonated molecular ion $[M+H]^+$ were monitored per analyte to enhance method sensitivity and selectivity. The most abundant transition was used for quantification, whereas the second most abundant was used for confirmation. The quantification for both classes of contaminants was performed using internal standards. MS/MS identification was based on the following criteria: (i) simultaneous responses for the two monitored transitions (SRM1 and SRM2) must be obtained at the same retention time than those of available standards; (ii) signal-to-noise ratios must be > 3; and (iii) relative peak intensity ratio must be within ±20% of the theoretical values obtained with standard solutions.

2.6. Quality assurance

Blank tests were carried out to rule out possible contamination from the sampling, storage or instrumentation. In order to comply with internal quality control procedures, two control spiked samples, two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behavior chart during the entire duration of the study to establish if the analysis was in a state of statistical control. The quality parameters were performed in the liver samples from individuals of the same population of Franciscana dolphins (SP coast) and were previously published in Alonso et. (2012b) and Gago-Ferrero et al. (2013). Briefly, procedural recovery was assessed by addition of each target contaminant to biological samples. These samples were previously analyzed in order to determine the compounds present before spiking (blank). Three replicates were prepared for the evaluation of the reproducibility of the method. The limits of detection of the method (mLOD) (3 times the signal to noise ratio level), and the limit of quantification of the method (mLOQ) (10 times the signal to noise ratio level), were calculated. To determine linearity, calibration curves were produced for all compounds. The linear calibration range studied was from 0.15 to 40 pg/μL for PYR and from 0.10 to 200 pg/μL for UVF at six concentration levels per compound class. Good correlations were obtained within the interval studied with correlation coefficients ranging between 0.998 and 0.999. Recoveries of spiked PYR ranged between 53 and 116%, with a relative standard deviation (RSD) < 20%; mLODs ranged between 0.02 and 0.71 ng/g lipid weight (lw), and mLOQs ranged between 0.08 and 2.38 ng/g lw. Recoveries of spiked UVFs ranged between 60% and 115% (RSD < 20%); mLODs ranged between 1.50 and 25 ng/g lw and the mLOQs ranged between 1.90 and 75 ng/g lw.

2.7. Statistical analyses

Statistical analyses were conducted using the R Core Team (Vienna, Austria, 2014) statistical package. The level of statistical significance was defined at $p < 0.05$. Non-parametric statistical tests were used since the data were found to have a non-normal distribution (Shapiro Wilk's W test). Kolmogorov–Smirnov tests were used to determine which concentrations were significantly different between the tissues in mother-fetus pairs according to each species and between species. Spearman's coefficients were calculated to understand the correlation between PYR and UVF concentrations and lipid content for both species.

Table 1
Insecticide pyrethroid concentrations (ng/g lw) in Franciscana and Guiana dolphins from Brazilian coast.

Mother/Fetus	Organs	Lipid (%)	Tetramethrin	Bifenthrin	*l*-Cyhalothrin	Delta/Tralomethrin	Fluvalinate	Es/Fenvalerate	Permethrin	Cyfluthrin	Cypermethrin	ΣPYR	References
Franciscana dolphin													
Pair 1													
Mother	*Blubber*	88	1.80	6.00	nd	nd	nd	nd	85.5	nd	7.60	101	this study
	Muscle	11	6.90	13.5	2.15	nd	nd	nd	113	nd	75.5	211	this study
	Placenta	1	37.0	38.0	21.5	nd	20.0	6.95	260	14.5	460	855	(Alonso et al., 2012b)
	Cord	4	47.0	nd	15.0	nd	nd	12.0	460	nd	325	860	this study
	Milk	58	0.60	0.70	nq	0.35	nd	0.35	2.30	0.20	0.30	4.80	(Alonso et al., 2012b)
Fetus	*Blubber*	9	18.5	55.0	29.0	nd	nd	40.5	1120	nd	700	1965	this study
	Muscle	8	77.0	55.0	35.5	nd	nd	nd	5280	nd	890	6340	this study
Pair 2													
Mother	*Blubber*	86	3.40	nd	nd	nd	nd	nd	24.5	nd	3.30	31.5	this study
	Muscle	2	41.0	nd	12.0	nd	nd	nd	355	nd	455	860	this study
	Placenta	12	25.0	51.0	9.25	13.0	7.15	12.5	105	15.0	95.5	331	(Alonso et al., 2012b)
	Cord	3	22.0	nd	29.5	nd	nd	nd	725	nd	420	1195	this study
Fetus	*Blubber*	44	1.45	nd	0.30	3.70	nd	nd	99.5	nd	30.0	130	this study
	Muscle	15	11.5	nd	7.65	nd	nd	2.15	73.5	nd	145	240	this study
Pair 3													
Mother	*Blubber*	82	0.35	2.55	nd	1.25	nd	nd	4.65	nd	3.65	12.5	this study
	Placenta	5	7.70	5.00	nd	22.0	nd	20.0	105	9.45	56.0	225	this study
Fetus	*Blubber*	81	6.55	nd	2.30	nd	nd	0.15	15.0	4.15	37.0	65.0	this study
	Muscle	28	1.30	nd	1.45	2.20	nd	0.90	21.5	0.00	5.85	33.5	this study
Pair 4													
Mother	*Placenta*	3	96.5	86.0	48.5	38.5	nd	10.1	385	41.5	1105	1810	(Alonso et al., 2012b)
	Cord	2	10.5	nd	33.5	12.5	nd	nd	530	15.5	475	1080	this study
Fetus	*Blubber*	71	4.65	1.50	0.45	nd	nd	nd	15.0	nd	32.0	54.0	this study
	Muscle	4	6.50	nd	8.75	nd	nd	nd	148	nd	350	511	this study
Mother 5													
Mother	*Blubber*	89	1.80	nd	nd	nd	nd	nd	nd	nd	15.0	16.5	this study
	Muscle	6	3.85	nd	2.45	nd	nd	10.1	225	42.5	117	400	this study
Mother 6													
Mother	*Blubber*	79	0.40	1.05	nd	nd	1.15	nd	3.95	nd	1.40	8.00	this study
Mother 7													
Mother	*Blubber*	82	0.70	1.15	nq	nd	nd	nd	5.15	nd	2.00	9.00	this study
	Milk	70	0.15	0.60	nq	0.55	nd	0.20	0.80	0.10	0.30	2.80	(Alonso et al., 2012b)
Mother 8													
	Milk	38	0.25	0.40	nq	0.45	nd	0.25	0.90	0.15	0.20	2.60	(Alonso et al., 2012b)
Calf-Mother-Fetus 9													
Calf	*Blubber*	80	0.55	20.0	1.50	nd	nd	nd	17.5	nd	20.5	60.0	this study
	Muscle	6	8.20	6.70	nd	nd	nd	nd	115	nd	33.5	165	this study

Mother	*Blubber*	80	0.65	14.0	1.35	4.50	nd	nd	17.0	nd	37.5	75.0	this study
	Muscle	2	8.20	6.70	nd	nd	nd	nd	115	nd	33.5	165	this study
Fetus	*Blubber*	22	1.15	nd	1.00	25.0	nd	nd	13.5	nd	44.5	90.0	this study
Guiana dolphin													
Pair 1													
Mother	*Blubber*	33	3.60	2.75	0.65	nd	5.45	nd	16.5	nd	9.8	40.0	this study
	Muscle	8	1.55	27.0	nd	nd	nd	17.0	99.5	nd	120	265	this study
Fetus	*Blubber*	14	1.50	4.05	nd	2.65	nd	2.95	25.5	8.60	12.5	58.0	this study
	Muscle	26	1.85	10.5	0.70	3.65	nd	0.00	41.5	nd	32.5	91.0	this study
Pair 2													
Mother	*Blubber*	25	4.35	12.0	0.20	12.0	0.60	0.90	82.0	nd	23.5	135	this study
	Muscle	5	4.10	17.5	41.0	nd	nd	nd	245	nd	315	620	this study
Fetus	*Blubber*	8	2.15	7.60	nd	nd	nd	nd	27.0	nd	19.5	57.0	this study
	Muscle	31	1.05	11.0	nd	nd	nd	nd	33.0	nd	10.5	55.5	this study
Pair 3													
Mother	*Blubber*	38	0.45	1.65	nd	0.35	nd	0.55	9.75	1.05	2.5	16.0	this study
	Muscle	4	2.00	nd	nd	nd	nd	nd	265	nd	300	570	this study
Fetus	*Blubber*	24	0.55	2.50	nd	nd	nd	nd	9.80	nd	9.5	22.5	this study
	Muscle	20	0.65	nd	nd	nd	nd	nd	43.0	nd	24.0	67.5	this study

nd – below mLOD.
nq – below mLOQ. mLOD and mLOQ were published in Alonso et al. (2012b).

3. Results and discussion

3.1. Biological parameters in females and fetus

Table S1 presents the biological data for individual animals, body length of females and fetus, collection date, fetus sex, estimated pregnancy stage for each species according to birth length and gestation period in the literature (Bertozzi, 2009; Ramos et al., 2000), number of corpus luteum and albicans to Franciscana (Bertozzi, 2009) and Guiana dolphins (Santos-Neto personal comm.) and age. The number of corpus albicans is correlated with the number of pregnancies in *Sotalia guianensis* (Rosas and Monteiro-Filho, 2002), however the presence of corpus albicans is temporary in *Pontoporia blainvillei* (Bertozzi, 2009). Lipid content in tissues of Franciscana and Guiana dolphin mother-fetus pairs were displayed in Tables 1 and 2.

3.2. Maternal transfer of pyrethroid insecticides

3.2.1. Concentration levels: fetuses vs. mothers

Pyrethroids were detected in all 44 samples analyzed, which included blubber and muscle of female and fetus, as well as placenta, milk and umbilical cord (Table 1). Mean ΣPYR concentrations found in Franciscana maternal blubber was 36.0 ng/g lw (SD ± 36.3); in maternal muscle 410 ng/g lw (SD ± 315); in fetal blubber 460 ng/g lw (SD ± 840); and in fetal muscle 1780 ng/g lw (SD ± 3045) (Fig. 1a). ΣPYR concentrations in Guiana dolphin in maternal and fetal tissues from Rio de Janeiro state (RJ) and Ceará state (CE) are in Table 1 and represented in Fig. 1b and c, respectively.

Fetus-mother ratios (F/M) ΣPYR concentrations in Franciscanas were 1.43, 2.67, 4.13 and to 19.5 (n = 4 pairs) in blubber tissue, and 0.28 and 30 (n = 2 pairs) in muscle. A significant difference (p = 0.04) was observed in blubber concentrations of PYR in Franciscana fetuses compared with their mothers (Fig. 1a). A higher proportion of F/M ratios >1 indicated that the fetuses contained a higher offload of PYR than their respective mothers in the blubber tissue (Fig. 1a). The same pattern (F/M > 1) was observed in relation to HCB in mother-fetus pairs of long-finned pilot whales (*Globicephala melas*) from Australia and in beluga whales (*Delphinapterus leucas*) from Alaska, demonstrating a strong offload tendency where a potential for bioaccumulation in fetuses is higher compared to their respective mothers (Hoguet et al., 2013; Weijs et al., 2013). Cetacean studies have shown a tendency to transfer from mother to fetus the less lipophilic organohalogens (lower chlorinated contaminants and with lower log K_{ow}), such as HCB that have six chlorines and log K_{ow} 5.24 (Desforges et al., 2012; Hoguet et al., 2013). The two predominant pyrethroids in fetal blubber in this study were permethrin and cypermethrin (log K_{ow} 6.5 and 6.6, respectively), which contain two chlorines in their molecular formulas, leading to suggest that these two pyrethroids may have a similar tendency to lower chlorinated pesticides, in relation to their transplacental transfers in cetaceans.

Fetus-mother ratios of blubber ΣPYR concentrations were 0.42 and 1.47 in Guiana dolphins from RJ and 1.39 in Guiana from CE, and muscle ΣPYR concentrations were 0.09 and 0.35 in Guiana dolphin pairs from RJ and 0.12 in a Guiana dolphin pair from CE (Table 1, Fig. 1b, c). Two different patterns of transference were observed in Guiana dolphins, accordingly to the tissue. F/M > 1 was similar to Franciscana and to PFOS (perfluorooctane sulfonate) in the same species from a close area (Guanabara Bay, F/M = 2.75 and 2.62) (Dorneles et al., 2008b), which characterizes an efficiently transfer of PYR in the blubber from mother to fetuses. In the other hand, F/M < 1 in all *Sotalia guianensis* muscles, indicated that mothers retained a higher load of PYR in muscle than their respective

Table 2
U.V. filter concentrations (ng/g lw) in Franciscana and Guiana dolphins from Brazilian coast.

Mother/Fetus	Organs	Lipid (%)	EHMC[a]	4MBC[b]	OD-PABA[c]	OCT[d]	ΣUVF
Franciscana dolphin							
Pair 1 (BP 106)							
Mother	*Blubber*	88	60.5	nd	nd	nd	60.5
	Muscle	11	43.0	nd	nd	nd	43.0
	Milk	58	120	20.0	nd	nd	140
Fetus	*Blubber*	9	66.5	nd	50.0	nd	115
	Muscle	8	250	110	45.0	11130	11,530
Pair 2 (BP 62)							
Mother	*Blubber*	86	35.5	47.5	nd	nd	83.0
	Muscle	2	42.5	355	nd	nd	400
Fetus	*Blubber*	44	nd	nd	5.90	nd	6.0
	Muscle	15	245	86.0	60.0	1780	2175
Pair 3 (BP 161)							
Mother	*Blubber*	82	nd	8.65	nd	nd	8.5
	Placenta	5	nd	nd	1385	nd	1385
Fetus	*Blubber*	81	68.5	nd	2.50	nd	71.0
	Muscle	28	69.0	nd	nd	nd	70.0
Pair 4 (BP 71)							
Fetus	*Blubber*	71	117	35.0	4.00	50.0	205
	Muscle	4	250	170	155	2090	2660
Mother 5 (BP 151)							
Mother	*Blubber*	89	43	13	nd	nd	56.5
	Muscle	6	54	110	nd	nd	165
Mother 6 (BP 152)							
Mother	*Blubber*	79	nd	nd	nd	nd	nd
Mother 7 (BP 153)							
Mother	*Blubber*	82	74.5	nd	nd	nd	74.5
	Milk	70	nd	17.5	8.50	nd	25.5
Mother 8 (BP 132)							
	Blubber	38	77.5	28.5	nd	113	219
Calf-Mother-Fetus 9 (BP 182)							
Calf	*Blubber*	80	67.0	nd	nd	nd	67.0
	Muscle	6	133	250	36.5	925	1345
Mother	*Blubber*	80	85.0	20.0	3.15	nd	110
	Muscle	2	67.5	855	nd	nd	920
Fetus	*Blubber*	22	70.0	97.0	67.5	nd	235
Guiana dolphin							
Pair 1 (RJ)							
Mother	*Blubber*	33	nd	nd	nd	nd	nd
	Muscle	8	155	230	50.0	970	1405
Fetus	*Blubber*	14	nd	nd	nd	nd	nd
	Muscle	26	83.5	80.0	26.0	165	355
Pair 2 (RJ)							
Mother	*Blubber*	25	205	48.0	34.0	220	505
	Muscle	5	545	570	1050	8310	10,475
Fetus	*Blubber*	8	nd	33.0	nd	nd	32.5
	Muscle	31	85.0	61.0	17.0	115	280
Pair 3 (CE)							
Mother	*Blubber*	38	48.0	18.5	nd	nd	67.0
	Muscle	4	70.0	395	nd	1350	1810
Fetus	*Blubber*	24	nd	34.0	nd	nd	34.0
	Muscle	20	40.0	60.0	25.0	240	365

nd – below mLOD.
[a] EHMC – ethylhexyl methoxycinnamate.
[b] 4MBC - 4-methylbenzylidene camphor.
[c] OD-PABA - 2-ethylhexyl-4-dimethyl-aminobenzoate.
[d] OCT – octocrylene.

fetuses, pointing to an opposite pattern than observed in blubber. F/M < 1 characterizes a lower input of the contaminants from mother to fetus in muscle tissue, but it is necessary to point out that ΣPYR concentrations in maternal muscle were up to 35 times higher than in maternal blubber. More studies are necessary to understand the exposition of fetuses to pyrethroids and their transplacental transport, however, is evident that PYR traverse placental membrane and bioaccumulate in fetal tissues in critical stages of development. The transplacental transfer of pyrethroids insecticides in dolphins was characterized by the fact that the pyrethroids were present in all fetus samples.

ΣPYR concentrations in a Franciscana fetus in muscle and blubber tissues (6340 ng/g lw and 1965 ng/g lw, respectively, Pair 1 in Table 2) and the placenta and cord (1810 ng/g/lw and 1080 ng/g lw, respectively, Pair 4 in Table 2) were the highest-ever reported in biota. These animals were taken from São Paulo coastal waters in which persistent organic pollutants in dolphins from the same population (Baixada Santista) show this to be one of the most contaminated coastal regions in Brazil (Alonso et al., 2012a). Pyrethroids are largely used in Brazil and their use is enforced by the Government Health Agency as a control of insect-borne diseases (Santos et al., 2007).

Concentrations of PYR in the maternal blubber of Franciscanas were between 1 and 2 orders of magnitude lower than DDTs and PCBs and similar to brominated flame retardants (PBDEs) and PFOS levels in females from the same population (Baixada Santista, SP)

(Alonso, 2008; Alonso et al., 2012a; Lailson-Brito et al., 2011; Leonel et al., 2008; Yogui et al., 2011). However, considering the highest pyrethroid levels in the same species that were in fetal muscle, PYR presented levels similar to PCBs, 1 order of magnitude higher than PBDEs and DDTs and 2 orders of magnitude higher than PFOS in females from the same population (Alonso, 2008; Leonel et al., 2008; Yogui et al., 2011). In addition, the lowest concentration among all matrices analyzed was found in milk (mean 3.40 ng/g lw, ±1.35, Fig. 1d). The mean level of PYR in breast milk of women from São Paulo was very similar to those found in the milk of dolphins from the same area (5.25 ng/g lw, ±3.00) (Corcellas et al., 2012), suggesting a similarity in PYR levels in marine and terrestrial mammals from the same region.

Pyrethroid concentrations in Guiana dolphins in the present study from Sepetiba Bay (RJ) were similar to DDTs and 1 order of magnitude lower than PCBs in females from the same population (Lailson-Brito et al., 2010), and were similar to PBDEs and PFOS values in Guiana females from RJ (Dorneles et al., 2010). Guiana dolphins from C3 region (CE) presented PYR levels between 5 and 6 orders of magnitude higher than DDTs and PCBs levels analyzed in mature females from the same population (Santos-Neto et al., 2014). The present data showed a pronounced concern in PYR accumulation in Franciscanas fetus from São Paulo coast and in Guiana dolphin females from Sepetiba Bay due to a number of other contaminants also present in both environments and in Ceará where pyrethroids had much higher contribution than organochlorines previously monitored. Therefore, our findings warrant a need for a regular monitoring of PYR in Brazilian coastal dolphin populations (especially Franciscana and Guiana dolphins), as well as pyrethroids are organohalogens (with exception of tetramethrin), and should be monitored along with other halogenated organic contaminants previously reported, in order to evaluate its long-term status in marine mammals as well in the marine environment.

3.2.2. Accumulation between tissues: blubber vs. muscle

A relevant point is that comparing the PYR levels in blubber and muscle of the two dolphin species analyzed in the present study, muscle was the organ that presented the highest concentrations ($p < 0.001$, Fig. 1a, b and c). To our knowledge, this is the first time that such observation is described. Total PYR concentrations were inversely proportional to the lipid content of the organs. Samples rich in lipids, such as blubber and milk, presented the lowest PYR concentrations, whereas organs with low fat content e.g. muscle, cord and placenta; showed the highest levels of the insecticides (Franciscana $p = 0.024$, $r = -0.66$; Guiana $p = 0.009$, $r = -0.91$). These data may indicate a pattern of preferential accumulation of PYR in muscle tissue, unlike other organohalogen contaminants that are highly lipophilic and tend to accumulate preferentially in tissues with higher percentage of lipids, such as blubber (Raach et al., 2011; Vetter, 2001). PFOS presented the same behavior in harbor porpoises from Red Sea, where muscle presented higher level than blubber and the highest concentration was measured in fetus (Van De Vijver et al., 2007). As it is known, PFOS has a different accumulation pattern from the other organohalogens, as it preferentially binds to blood proteins and accumulates in different tissues (e.g. liver, kidney and muscle) (Van De Vijver et al., 2007). As the results showed, pyrethroids seem to behave similarly with PFOS, since they are also organohalogen compounds, containing fluorine, bromine and/or chlorine (with exception of tetramethrin that is not an halogenated) (Feo et al., 2011).

3.2.3. Pattern of distribution

Permethrin was the PYR predominant in 68% of the dolphin samples in this study, at concentrations ranging from below mLOD (a blubber sample from a pregnant and lactating female) to 5280 ng/g lw (a muscle sample from a fetus) as showed in Table 1 and in Figure (Fig. S2). Permethrin is known as being more acutely toxic to children than to adults and female rats exposed to this insecticide during pregnancy generated calves who exhibited neurological effects (Cox, 1998; Horton et al., 2011; Nasuti et al., 2014; Sinha et al., 2004). The effects of pyrethroids in marine mammals are unknown, but the hypothesis of a synergistic effect of those entire chemical cocktail in dolphin fetuses cannot be discarded. More studies are needed in order to evaluate the toxicological effects of pyrethroids insecticides in marine mammals.

The next most abundant PYR was cypermethrin, which appeared in 32% of the samples as the predominant PYR (<mLOQ to 1110 ng/g lw). The highest levels of cypermethrin were found in placenta and muscle (Table 1, Fig. S2). USEPA (2002) and Assayed et al. (2010) reported abnormalities in the offspring and decreased calf survival percentage in mammals fed cypermethrin in their diets during pregnancy, which demonstrate the potential of cypermethrin to induce health risks for females and their progeny. From 1996 to 2003, cypermethrin was the mainly insecticide used in Brazil as malaria control (Santos et al., 2007) and it has been heavily used for dengue control since 1999 (Da-Cunha et al., 2005). However, the most abundant pyrethroid in the samples from this study was permethrin. Both pyrethroids have similar log *Kow* (permethrin 6.5 and cypermethrin 6.6), though permethrin exhibits relatively longer half life in aerobic and anaerobic soils (39.5 days and 197 days, respectively) compared with cypermethrin (27.6 days and 55 days, respectively) (Feo et al., 2010b). Furthermore, permethrin is classified as Type I pyrethroid (lack of cyano group), and cypermethrin as Type II (containing a cyano group in its molecule), which raises the hypothesis that cypermethrin can be more readily degraded compared to permethrin. This hypothesis was supported in studies on sediments from Southern California estuaries, where permethrin was among the most abundant of pyrethroids analyzed; in contrast, the abundance of cypermethrin was half of its average usage, suggesting a relatively lower degree of persistence (Lao et al., 2012, 2010).

Milk was the compartment that presented the most diverse pattern among the matrices analyzed (Fig. S2). In this Figure is possible to observe a minor contribution of cypermethrin in milk samples compared with all other matrices, while delta/tralomethrin and es/fenvalerate had a higher contribution in milk samples compared to maternal blubber samples, as observed in Alonso et al. (2012b). Due to limited available milk samples ($n = 3$), it is difficult to assume, however one reason may be attributed to lipophilicity and persistence of delta/tralomethrin and es/fenvalerate (longer half-lives, up to 209 and 546 days, respectively) compared to cypermethrin, that could might influenced in the pyrethroid transport from blubber to milk.

Bifenthrin was detected in most Franciscana and Guiana tissue samples. In contrast, cyfluthrin was detected in blubber but not in muscle of Guiana dolphins. In urban creeks of California, bifenthrin found in sediments was the responsible for most amphipod mortality (Amweg et al., 2006; Weston et al., 2009). Weston et al. (2009) concluded that cyfluthrin, cypermethrin and permethrin individually were below acutely toxic concentrations; however together they have synergistically toxicity effects.

3.3. Maternal transfer of UV filters

3.3.1. Concentration levels: fetuses vs. mothers

UVFs were detected in all mother-fetus pair from Guiana and Franciscana dolphins in this study and the concentrations of 4-methylbenzylidene camphor (4MBC), 2-ethyl-hexyl-4-trimeth oxycinnamate (EHMC), 2-ethyl-hexyl-4-dimethyl-aminobenzoate

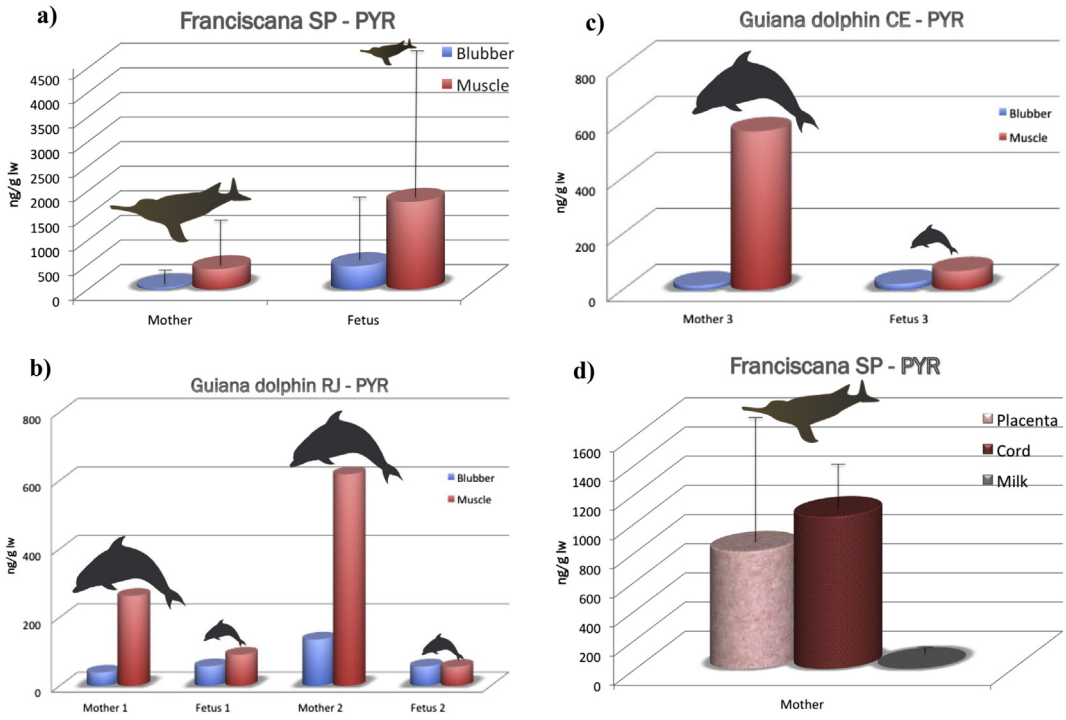

Fig. 1. **a.** ∑PYR concentrations (mean and standard deviation, expressed in ng/g lw) in maternal and fetal blubber and muscle of Franciscanas from Baixada Santista (SP), Brazil, South Atlantic. **b.** ∑PYR concentrations (two mother-fetus pairs, expressed in ng/g lw) in maternal and fetal blubber and muscle of Guiana dolphins from Sepetiba Bay (RJ), Brazil, South Atlantic. **c.** ∑PYR concentrations (one mother-fetus pair, in ng/g lw) in maternal and fetal blubber and muscle of Guiana dolphins from Canoa Quebrada (CE), Brazil, South Atlantic. **d.** ∑PYR concentrations in placenta, cord and milk (mean and standard deviation, ng/g lw) of Franciscanas from São Paulo coast, Brazil.

(OD-PABA) and octocrylene (OCT) are shown in Table 2. Mean ΣUVF concentrations in Franciscana maternal blubber was 55.8 ng/g lw (SD ± 39.0); in maternal muscle 381.7 ng/g lw (SD ± 389); in fetal blubber 126.3 ng/g lw (SD ± 95); and in fetal muscle 4108 ng/g lw (SD ± 6101). ΣUVF concentrations in Guiana dolphin from RJ and CE are reported in Table 2.

Fetus/mother ratios (F/M) of blubber and muscle ΣUVF concentrations were calculated, in order to assess the maternal transfer of these contaminants during gestation. F/M in Franciscanas were 0.07, 1.9, 3.52 and 8.19 in blubber tissue (n = 4 pairs) and 5.47 and 267 (n = 2 pairs) in muscle tissue, indicating a higher potential for bioaccumulation of sunscreen agents in the fetus than in their mothers. Similar findings in relation to HCB occurred in long-finned pilot whales (*G. melas*) from Tasmania (F/M from 1 to 1.5) (Weijs et al., 2013) and PFOS in Guiana dolphins from RJ (F/M = 2.75 and 2.62) (Dorneles et al., 2008a), but in a lower scale. A similar trend was observed for Franciscana in relation to PYR, suggesting that the fetus receives the major levels of these emerging organic pollutants in comparison to the adults.

In the other hand, a dramatic lower UVF input from female to fetus in Guiana dolphins occurred. F/M ratios of ΣUVF concentrations in *S. guianensis* were <mLOD and 0.06 for blubber and 0.25 and 0.03 for muscle in RJ dolphins, and 0.51 and 0.20 blubber and muscle, respectively in CE mother-fetus pair. ΣUVF concentrations in maternal muscle of Guiana dolphin were significantly higher ($p = 0.035$) than in Franciscana females. Guiana dolphins from both areas (RJ and CE) presented higher ΣUVF concentrations in maternal tissues (blubber and muscle) and lower in fetal tissues (F/M < 1) compared to Franciscanas. This fact may be due to Guiana dolphins could have a different (i) UVFs bioaccumulation and/or (ii) UVFs transplacental transfer and/or (iii) UVFs metabolic/detoxification rates and/or (iv) number of pregnancies, in relation to Franciscanas (Fig. 2a, b and c, Table S1). In Table S1 is possible to observe that Guiana females from Sepetiba Bay in this study had 17 and 13 pregnancies (Pair 1 and Pair 2, respectively) according to the number of corpus albicans and luteum in their ovaries, and Franciscanas probably had one or two pregnancies due to the number of corpus luteum, their age (2–4 years old), gestation period (mean of 10.22 months) and maturity age (1.2–1.8 years old) (Bertozzi, 2009). The lower transfer rates from mother to fetus in Guiana compared to Franciscana dolphins can be related to Guiana females have transferred their contaminant loads to previous offsprings, and Franciscanas were transferring for the first progeny that receives the higher amount of contamination compared with later birth order (Weybridge, 2012).

Although the higher UVF levels in Guiana dolphins compared with Franciscana can be related to (i) a higher bioaccumulation in Guiana tissues and/or (ii) higher biomagnification in Guiana trophic chain, and or (iii) a lower detoxification in Guiana, and/or (iv) females' age and/or (v) regional differences. Guiana dolphin females in this study were older (Mother 1 was 17 years old and Mother 2 was estimated to 18 years old) than Franciscanas (2–4 years old) and have accumulated UVF for a longer period of time in their tissues (Table S1 – Guiana dolphins estimative of age was based on 13 pregnancies, 11.4 months per gestation and maturity age of 6 years old (Ramos et al., 2000)). Another hypothesis might be a regional difference among dolphin populations, since Guiana dolphins were collected in RJ (22° S) and CE (5° S) that are tropical areas in Brazil where the use of sunscreen by the human population might be higher than in São Paulo (24° S) where Franciscanas were collected which is a subtropical area.

ΣUVF concentrations in muscle (11,530 and 10,475 ng/g lw, Franciscana fetus from Pair 1 and Guiana mother from Pair 2, respectively) were the highest-ever reported in biota. The two pregnant Guiana dolphins from Rio de Janeiro coast were found dead, and necropsy results revealed that both fetuses, although

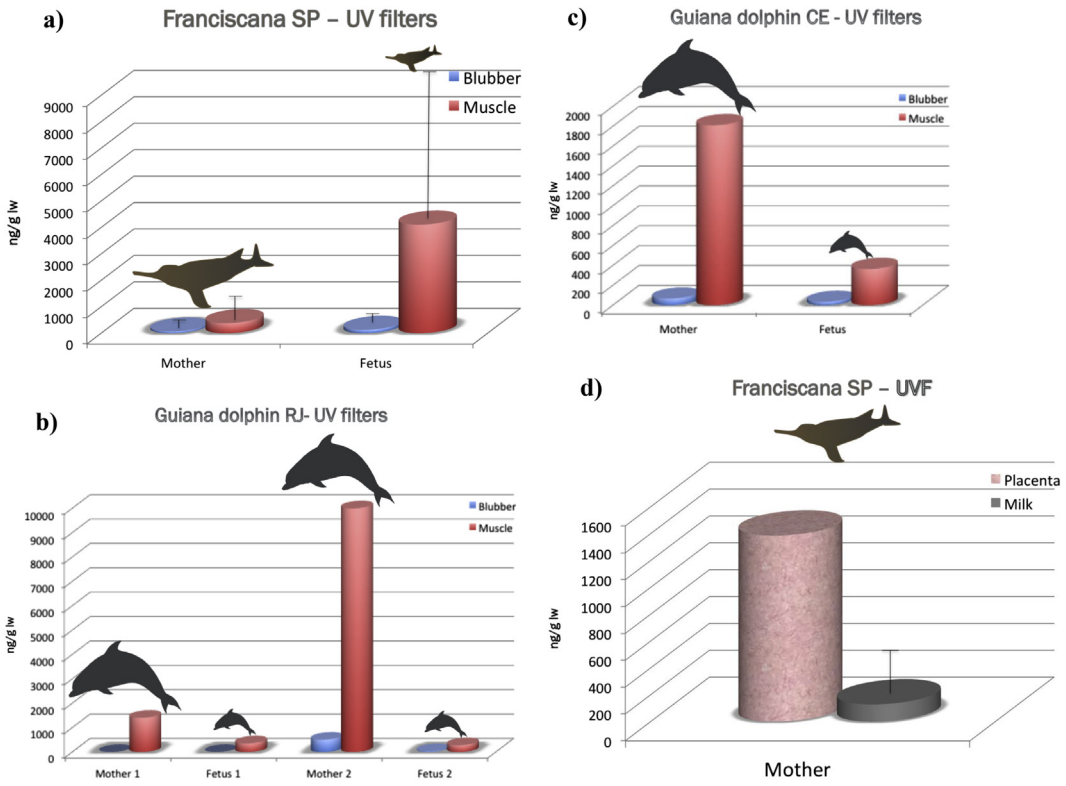

Fig. 2. **a.** ∑UVF concentrations (mean and standard deviation, expressed in ng/g lw) in maternal and fetal blubber and muscle of Franciscanas from Baixada Santista (SP), Brazil, South Atlantic. **b.** ∑UVF concentrations (two mother-fetus pairs, expressed in ng/g lw) in maternal and fetal blubber and muscle of Guiana dolphins from Sepetiba Bay (RJ), Brazil, South Atlantic. **c.** ∑UVF concentrations (one mother-fetus pair, in ng/g lw) in maternal and fetal blubber and muscle of Guiana dolphins from Canoa Quebrada (CE), Brazil, South Atlantic. **d.** ∑UVF concentrations in placenta and milk (mean and SD, ng/g lw) of Franciscanas from São Paulo coast, Brazil.

almost completely formed, had open abdominal cavity, incompatible with life (Marigo personal comm.). The agent responsible for the lesion in both fetuses is still unknown, but this developmental anomaly occurring in both animals from the same population, may suggest a teratogenic effect of the pollutants found in high concentrations in those pregnant females. Guiana dolphin populations that inhabit Rio de Janeiro state waters are highly exposed to all organic pollutants already targeted in samples from their tissues (e.g. PCBs, PBDEs, PFOS, PCDDs, PCDFs, OCPs and OTs) (Dorneles et al., 2013, 2010, 2008a, 2008b; Lailson-Brito et al., 2010), which can lead these individuals to be subject to adverse health effects associated with chronic exposure to a cocktail of POPs.

UVF concentrations in Franciscanas maternal blubber and muscle were similar to PBDEs, DDTs and PCBs (Alonso, 2008; Alonso et al., 2012a) and were higher than PFOS concentrations in blubber and liver of females from the same population previously analyzed (Alonso, 2008; Alonso et al., 2012a; Leonel et al., 2008; Yogui et al., 2011). Maternal blubber ∑UVF levels in Guiana dolphins from Sepetiba Bay (RJ) were 1 order of magnitude lower than PCBs, but in similar levels than DDT reported in mature female from the same population (Lailson-Brito et al., 2010). However, in maternal muscle from the same animals, ∑UVF concentrations were 1 order of magnitude higher than hepatic concentrations of PBDEs and PFOS in mature female from Rio de Janeiro (Dorneles et al., 2010, 2008a). Organochlorinated compounds were analyzed in Guiana female from C3 area in Ceará and the concentrations in blubber of mature females were 6–7 orders of magnitude lower than UVF concentrations found in this study (Santos-Neto et al., 2014). In other words, it is possible to observe that UVF levels verified in this study should be monitored in cetacean populations in future studies, in order to verify their behavior in a long range term.

In order to investigate the maternal transfer of UVFs in dolphins, placenta and milk samples of Franciscana dolphins were analyzed (Fig. 2d). All four UV filters analyzed in this study (OD-PABA, EHMC, 4MBC and OCT) were observed in Franciscanas' milk. ΣUVF concentrations in fetus from both cetacean species showed a transplacental discharge of these sunscreen agents, as well as they were present in milk.

3.3.2. Accumulation between tissues: blubber vs. muscle

The highest concentrations of sunscreen agents (UVFs) were observed in the muscle in both dolphin species analyzed from the Brazilian coast (Franciscana $p < 0.01$ and Guiana $p = 0.03$, Fig. 2). The lipid content was inversely proportional to UVF concentrations in both species (Franciscana $r = -0.66$ and Guiana $r = -0.83$). Log K_{ow} values of the four UVFs analyzed (OD-PABA, EHMC, 4MBC and OCT) ranged from 4.95 to 6.90 (Gago-Ferrero et al., 2012). In a previous study, 4MBC exhibited species-specific accumulation in fish and was detected at higher levels in muscle than in offal (Nagtegaal et al., 1997). And a review paper about organic UVFs in aquatic biota concluded that muscle has been the preferred sample for analysis in fish, despite that individuals showed preferential accumulation pattern according to species (Gago-Ferrero et al., 2012).

3.3.3. Pattern of distribution

The detection frequency of UVFs in the samples analyzed was as follows: EHMC 76%, 4MBC 68%, OD-PABA 47%, and OCT 34% (Fig. S3). Due to a lack of information on UVFs in marine mammals, concentration comparisons of the data in the present study to others cannot be made. The only other study was published by our group, and it comprised the determination of hepatic OCT in Franciscana dolphins, with levels in liver tissues up to 780 ng/g lw

(Gago-Ferrero et al., 2013).

The pregnant Franciscana females from this work correspond to individuals from the previously studied population (Gago-Ferrero et al., 2013). The currently presented OCT concentrations in fetal muscle are 15 times higher than those presented in liver from adult and juveniles animals. We previously suggested that UVFs have the potential to undergo maternal transfer based on the detection of OCT in one paired placenta and liver sample and in the calves analyzed (Gago-Ferrero et al., 2013). Further, the data presented here provides strong evidence for maternal transfer of UVFs based on detection in all paired samples of Franciscana (n = 5 pairs, 100%) and Guiana dolphins (n = 3 pairs, 100%), in the fetus and maternal bubbler and muscle, and in both the placenta and milk samples.

When OCT was detected in dolphin tissues, it corresponded to the highest contribution of ΣUVFs (Table 2, from 50 to 11,130 ng/g lw). Muscle was the predominant tissue to accumulate OCT.

The samples of Guiana dolphins collected from the Ceará coast belong to a region of marine algal banks and phanerogams (*Halodule wrightii*). This area is also known by the banks of calcareous algae (*Halimeda genus*), natural habitat of lobsters, one of the main fish stocks in the region (CEARA, 2014). This indicates that sunscreens are also present in this marine ecosystem in Brazil. Ecotoxicological studies have demonstrated estrogenic activity of the most commonly used sunscreen agents, additionally, with the potential for synergistic effects there is an urgent need for long-term occurrence studies in biota (Buser et al., 2006; Díaz-Cruz and Barceló, 2009; Gago-Ferrero et al., 2012; Schreurs et al., 2005).

4. Conclusions

This is the first time where tissue samples of mother-fetus pair from wild mammals were analyzed to evaluate the occurrence of PYR and UVF contaminants, proving the prenatal transfer of these compounds that are in heavy use worldwide. Muscle is the preferential organ for PYR and UVF bioaccumulation in comparison to the blubber. It differs from the former reports concerning well-known organic chlorinated and brominated contaminants, where the blubber was the target tissue for the analysis. However, they showed a similar behavior to PFOS, which preferentially bind to blood proteins. More studies are necessary in order to identify the ideal tissue for monitoring these novel contaminants in sentinel species such as dolphins.

Fetal tissue samples contained higher levels of PYR and UVF than their respective mothers in Franciscanas. Fetal blubber samples contained higher levels of PYR than their respective mothers and maternal muscle samples contained higher levels of UVF than their respective fetuses in Guiana dolphins. Fetal exposure to this cocktail of pesticides and chemical sunscreen agents may result in adverse teratogenic effects in calves, as the organ growth and development is at its maximum rate and vulnerability during the prenatal period.

The concentrations of PYR and UVFs detected in this study are of concern for those dolphin species from Brazilian coastal waters, as these compounds have been shown to be risk factors for cancer, immune deficiency, and reproductive abnormalities. Franciscana presented higher concentration of PYR than Guiana dolphins and the opposite is true for UVF. Additionally, both dolphin species inhabit anthropogenically disrupted environments they face a number of known and unknown threats, but also represent a good sentinel species for a regulated and targeted environmental monitoring program.

To our knowledge, this is the first study to evaluate the occurrence of UVFs in paired maternal and fetal tissues. Our results suggest prenatal transfer of these compounds, since ΣUVF concentrations were found in fetal tissues. Whereas ΣPYR concentrations were also observed in fetal samples, we can define PYRs and UVFs as transplacental contaminants.

Since dolphins are considered good sentinels for human exposure to marine pollutants, the same transfer might be occurring in humans. Future studies are warranted in order to demonstrate if the same pattern of the transplacental transfer occurs in humans as was indicated in the present study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.09.039.

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UV filters bioaccumulation in fish from Iberian river basins

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HIGHLIGHTS

- First evidence of UV filters in fish from Iberian rivers
- Biota-sediment accumulation factors (BSAFs) were always below 1.
- Predator species presented higher UV-F concentrations suggesting trophic magnification.

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ABSTRACT

The occurrence of eight organic UV filters (UV-Fs) was assessed in fish from four Iberian river basins. This group of compounds is extensively used in cosmetic products and other industrial goods to avoid the damaging effects of UV radiation, and has been found to be ubiquitous contaminants in the aquatic ecosystem. In particular, fish are considered by the scientific community to be the most feasible organism for contamination monitoring in aquatic ecosystems. Despite that, studies on the bioaccumulation of UV-F are scarce.

In this study fish samples from four Iberian river basins under high anthropogenic pressure were analysed by liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). Benzophenone-3 (BP3), ethylhexyl methoxycinnamate (EHMC), 4-methylbenzylidene camphor (4MBC) and octocrylene (OC) were the predominant pollutants in the fish samples, with concentrations in the range of ng/g dry weight (d.w.). The results indicated that most polluted area corresponded to Guadalquivir River basin, where maximum concentrations were found for EHMC (241.7 ng/g d.w.). Sediments from this river basin were also analysed. Lower values were observed in relation to fish for OC and EHMC, ranging from below the limits of detection to 23 ng/g d.w. Accumulation levels of UV-F in the fish were used to calculate biota-sediment accumulation factors (BSAFs). These values were always below 1, in the range of 0.04–0.3, indicating that the target UV-Fs are excreted by fish only to some extent. The fact that the highest concentrations were determined in predators suggests that biomagnification of UV-F may take place along the freshwater food web.

1. Introduction

UV filters (UV-Fs) are emerging environmental pollutants of recent concern for which there is currently a lack of knowledge about their occurrence, fate and effects on the environment (Richardson, 2010). These compounds are used extensively in a variety of personal care products as well as in many industrial goods to protect products against photodegradation. UV-Fs enter the aquatic environment by direct inputs from recreational activities but mainly through the effluents of wastewater treatment plants (WWTPs) (Cuderman and Heath, 2007). These chemicals have been widely detected in surface water and wastewater at high concentrations, up to 19000 ng/L and 4000 ng/L in influent and effluent wastewater, respectively (Balmer et al., 2005; Kasprzyk-Hordern et al., 2008; Gago-Ferrero et al., 2013a) and up to 3000 ng/L in surface water (Rodil et al., 2009; Negreira et al., 2010). They are present in high concentrations in sewage sludge and sediments (Plagellat et al., 2006; Gago-Ferrero et al., 2011a; Gago-Ferrero et al., 2011b; Amine et al, 2012), due to their high lipophilicity and poor degradability. Their widespread occurrence has raised serious concern because of the known effects of these chemicals on various organisms. Several UV-Fs have an endocrine disrupting capacity, including benzophenone-3 (BP3), ethylhexyl methoxycinnamate (EHMC), octocrylene (OC) and 4-methylbenzylidene camphor (4MBC) (Schlumpf et al., 2004; Kunz and Fent, 2006; Calafat et al., 2008; Blüthgen et al., 2012). Adverse effects on fecundity and reproduction

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have been observed for BP3 and other benzophenone derivatives in fish and rodents (Calafat et al., 2008; Kunz and Fent, 2009).

Ecological factors, including aquatic species, size (weight and length), body lipid content, and sampling location, may affect bioaccumulation of chemicals (Yu et al., 2012). Aquatic organisms store chemical substances either directly from the surrounding environment or from their diet. Humans are consumers of fish and sea food. Exposure assessment currently considers fish and sea food as a potential route of human exposure to chemicals in the environment (Binelli and Provini, 2004). So far very little data is available on the bioaccumulation of UV-F in aquatic organisms from marine and fresh water, which was reviewed by Gago-Ferrero et al. (2012). Reported concentrations in fish ranged from 9 to 2400 ng/g lipid weight (l.w.) (Nagtegaal et al., 1997; Balmer et al., 2005; Fent et al., 2010; Spiric et al., 2010) in some monitoring studies conducted in different rivers and lake waters from Germany and Switzerland. Higher concentrations were found in mussels (Bachelot et al., 2012; Picot Groz et al., 2014), and relevant values of OC (89–782 ng/g l.w.) were recently determined in marine mammals (*Franciscana* dolphins (*Pontoporia blainvillei*)) along the Brazilian coast (Gago-Ferrero et al., 2013b).

When a substance is not metabolized or excreted at the pace that it is ingested, it accumulates and biomagnification may occur through the food web as shown in the study by Fent et al. (2010) for some UV-F, including EHMC. For this compound, values up to 22.50 ng/g l.w. were detected in crustacean and mollusks, and values as high as 300 ng/g l.w. in fish. The highest concentrations, above 700 ng/g l.w., however, were determined in fish-eating birds (*Phalacrocorax sp.*) suggesting the trophic transfer of EHMC in the aquatic ecosystem.

In this scenario, the aim of this study was to investigate for the first time the presence and concentration of UV filters in freshwater fish from four Iberian river basins as well as the sediments of the most polluted river basin. The concentration determined allowed us to estimate the bioaccumulation factors (BAFs) for the bioconcentrated compounds.

2. Materials and methods

2.1. Chemicals

Table 1 lists the target compounds and some of their relevant physicochemical properties. BP3, OC, ethylhexyldimethyl PABA (OD-PABA), 2,4-dihydroxybenzophenone (BP1), 4-hydroxybenzophenone (4HB), 4,4′-dihydroxybenzophenone (4DHB) and the isotopically labelled compound benzophenone-C_{13} (BP-C_{13}) were of the highest purity (>99%) and were obtained from Sigma-Aldrich (Steinheim, Germany); 4MBC (99% purity) was supplied by Dr Ehrenstorfer (Augsburg, Germany); and EHMC (98%) by Merck (Darmstadt, Germany). The isotopically labelled compounds 2-hydroxy-4-methoxy-2′,3′,4′,5′,6′-d_5 (BP3-d_5) and 3-(4-methylbenzylidene-d_4)camphor, used as internal standards (>99%), were obtained from CDN isotopes (Quebec, Canada). Solvents including methanol (MeOH), acetone, dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (AcEt) and HPLC grade water, as well as formic acid (98% purity), aluminium oxide and Florisil were provided by Merck. N_2 and Ar purchased from Air Liquide (Barcelona, Spain) were of 99.995% purity. Pressurized liquid extraction cellulose filters used were obtained from Dionex Corporation (Sunnyvale, CA, USA). Isolute C18 (500 mg, 3 mL) cartridges used for solid phase extraction (SPE) were obtained from Biotage (Uppsala, Sweden).

Individual stock standard solutions as well as the isotopically labelled internal stock standard solution were prepared on a weight basis in MeOH at 200 mg/L. The solutions were stored in the dark at −20 °C. A mixture standard solution at 20 mg/L in MeOH of each compound was prepared weekly and working solutions were prepared daily by appropriate dilution of the mixture stock standard solution in MeOH.

2.2. Sample collection and preparation

Fish samples analysed in this study were collected in four Iberian river basins: Llobregat, Ebro, Jucar and Guadalquivir in 2010. These rivers have a Mediterranean regime and are exposed to a high anthropogenic impact. Detailed information about each sampling point can be found in http://www.scarceconsolider.es/publica/P000Main.php. Five sampling stations were selected distributed along each river basin except for Guadalquivir River, where four were selected (see Fig. 1).

In order to obtain a representative sample of different trophic levels within the aquatic community, specific fish species were targeted (see Table 2). For each river basin two fish species were selected, i.e. carp and barbel. However, it was not always possible to find them, and then other species were considered. Altogether, 49 individuals were collected using electro-fishing, and were weighed and measured, wrapped in aluminium foil, and immediately frozen for transport to the laboratory. In the particular case of *Luciobarbus sclateri* individuals lower than 30 cm were considered as juveniles. Once in the laboratory, the fish were composited (thawed, ground, homogenized and lyophilized) according to species and sampling point. The lyophilized samples were stored in sealed containers at −20 °C until analysis.

Sediment samples were collected in the same sampling stations as the fish samples located in the Guadalquivir River basin. Around 250 g of sediment was taken using a Van Veen grab sampler (500 mL capacity); they were transferred and wrapped into an aluminium foil and were frozen at −20 °C overnight before freeze-drying for approximately one week. Then, they were ground, sieved (2 mm) and finally stored at −20 °C until analysis.

2.3. Analytical methods

2.3.1. Quality assurance and quality control

Background contamination is a common problem in the determination of UV filters at environmental levels. To avoid it, all glassware used was washed and heated overnight at 380 °C, and further sequentially rinsed with different organic solvents and HPLC grade water. Furthermore, gloves were worn during sample preparation; separate solvents and only previously unopened packages of solvents, chemicals and other supplies were used. Many of the compounds analysed undergo photodegradation. Therefore, stock standard solutions and samples were always covered with aluminium foil and stored in the dark.

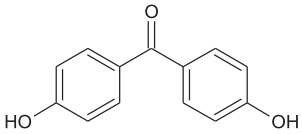
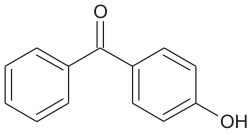
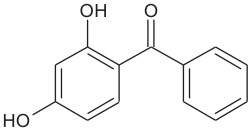
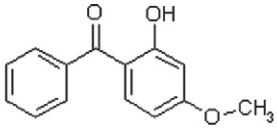
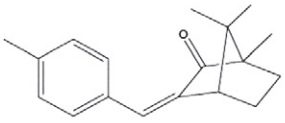
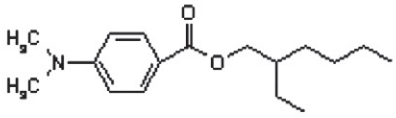
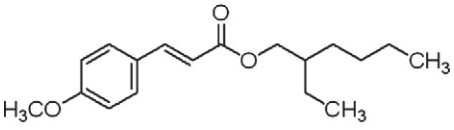
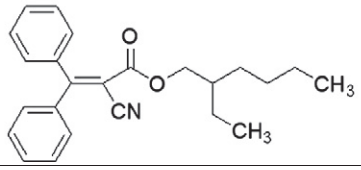
With every six samples, a methodological blank was analysed. Concentration of the target UV-F in the blanks was always <LOD. Linearity was satisfactory ($r^2 > 0.9$) for all the compounds. Recoveries in fish were >66% for all the compounds except for OD-PABA (36%). In sediment samples, recoveries were >58% for all the analytes. More details on QA/QC can be found in the Supporting Information in Appendix A.

The method performances of the methodologies for the analysis of UV-F in fish and in sediments are summarized in Table S1 and Table S2, respectively of the Supporting Information.

2.3.2. Analysis of fish

The analysis of UV-F and derivatives in the fish samples was carried out following a previously developed analytical methodology based on pressurized liquid extraction (PLE) and LC–MS/MS described elsewhere (Gago-Ferrero et al., 2013c). Briefly, the extraction of the analytes was performed using an ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, USA). One cellulose filter followed by 1 g of Florisil was placed at the bottom of the cells. Aliquots of 1 g of freeze-dried fish (spiked with the surrogate standard mix solution) was mixed in the extraction cells with Florisil. Extraction was implemented in 4 cycles of 5 min of static time each at 100 °C and 1500 psi using AcEt/DCM (1:1, v/v) as extracting solvent. The PLE extract obtained (~25 mL) was diluted to 200 mL with HPLC grade water (MeOH < 5%), and further purified by solid phase extraction (SPE)

Table 1
Target compounds: Names, abbreviations, CAS numbers, structures and Log Kow.

Name (INCI nomenclature)[a]	Abbreviation	CAS no.	Structure	Log K_{ow}
4,4´–Dihydroxy benzophenone	4DHB	611–99–4		2.19[b]
4–Hydroxybenzophenone	4HB	1137–42–4		2.92[c]
Benzophenone–1	BP1	131–56–6		3.15[c]
Benzophenone–3	BP3	131–57–7		3.79[b]
4–Methylbenzylidene camphor	4MBC	36861–47–9		4.95[b]
Ethylhexyl dimethyl PABA	OD–PABA	21245–02–3		5.412[c]
Ethylexyl methoxycinnamate	EHMC	5466–77–3		5.8[b]
Octocrylene	OC	6197–30–4		6.88[b]

[a]INCI (International Nomenclature for Cosmetic Ingredient) elaborated by CTFA and Cosmetics Europe (former COLIPA).
[b]Experimental values, from database of physicochemical properties. Syracuse Research Corporation: http://www.syrres.com/esc/physdemo.htm.
[c]Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (©1999–2011 ACD/Labs).

using Isolute C18 (500 mg, 3 mL) cartridges from Biotage. The compounds were eluted sequentially with AcEt/DCM (1:1, v/v) and 2 mL of DCM at 1 mL/min flow rate. Finally, the SPE extracts were evaporated and reconstituted with 1 mL of ACN containing the isotopically labelled internal standards.

HPLC–MS/MS analyses were carried out in a system consisting of an Agilent HP 1100 pump (Agilent Technologies, Palo Alto, CA, USA) connected to a 4000 Q TRAP™ MS/MS system from Applied Biosystems-Sciex (Foster City, California, USA). The chromatographic separation was achieved on a Hibar Purospher® STAR® HR R-18 ec. (50 mm × 2.0 mm, 5 μm) from Merck, preceded by a guard column of the same packaging material. A gradient using a mixture of HPLC grade water and ACN, both 0.15% formic acid, at a flow rate of 0.3 mL/min was used as the mobile phase.

The MS/MS detection of UV-F was performed in positive (PI) electrospray ionization (ESI) mode under selected reaction monitoring (SRM) mode. Two major characteristic fragments of the protonated molecular ion $[M + H]^+$ were monitored per analyte for improved sensitivity and selectivity. The most abundant transition was used for quantification, whereas the second most abundant was used for

Fig. 1. Sampling locations of fish and surface sediments in the four river basins evaluated.

confirmation. Other experimental conditions can be found elsewhere (Gago-Ferrero et al., 2013c).

Determination of lipid contents of fish was based on the method described by Spiric et al. (2010).

2.3.3. Analysis of sediments

The analysis of UV-F in the sediments of the Guadalquivir River basin was carried out using the method previously developed by Gago-Ferrero et al. (2011a). The extraction and in-cell purification was performed by PLE. One gram of freeze-dried and sieved sediment was mixed in the extraction cells with aluminium oxide. PLE extraction was carried out using MeOH and a mixture MeOH/water (1:1 *v*/*v*). The PLE extract (≈20 mL) was brought to 25 mL with MeOH. Two milliliters of this solution was passed through 0.45 μm filters to LC-vials, evaporated and finally were reconstituted in 250 μL ACN.

Instrumental analysis was performed by ultra high resolution liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), using an Acquity UHPLC chromatograph coupled to a TQD mass spectrometer (Waters) according to a previously developed methodology (Gago-Ferrero et al., 2011a).

In order to investigate the distribution of the selected UV-F between the biota and the sediment, the biota-sediment accumulation factors (BSAFs) were calculated in the most polluted basin, Guadalquivir River, using the following equation (Jia et al., 2011):

$$BSAF = \left(C_b / f_{lip}\right) / \left(C_s / f_{oc}\right) \quad (1)$$

where C_b is the UV-F concentration (ng/g wet weight) in fish, f_{lip} is the lipid content in fish (g lipids/g wet weight), C_s is the UV-F concentration (ng/g d.w.) in surficial sediment, and f_{oc} is the organic carbon content in sediment (g organic carbon/g sediment d.w.).

3. Results and discussion

3.1. Levels and distribution profiles of UV-F in fish and sediments

3.1.1. Spatial distribution

Table 2 summarizes the UV-F concentrations in different fish species collected at each sampling site. Method limits of detection (LOD, lowest analyte concentration with a signal to noise (S/N) ratio of 3) and method limits of quantification (LOQ, concentration with S/N ratio of 10 and imprecision lower than 20%) ranged from 0.1 to 6.0 ng/g d.w. and from 0.3 to 20.0 ng/g d.w., respectively (see Table 3).

Of the eight compounds analysed, four, i.e. BP3, EHMC, 4MBC and OC (the most lipophilic ones except OD-PABA) were detected with frequencies ranging from 5.6% to 80%. Table 3 shows the detection frequencies, ranges and median concentrations for each compound. The total detection frequencies were under 21.3% except for EHMC and BP3 in Guadalquivir River that attained 60% and 80%, respectively, showing big variations depending on the river basin. The total UV-F concentrations ranged from not detected (<LOD) to 363 ng/g d.w.

Guadalquivir River, with a length of 657 km, was by far the most polluted of the four basins investigated. This river basin is of particular ecological value because of the Doñana National Park, an important and protected wetland area. The river is navigable up as far as Seville (about 90 km upstream), a major inland port, which leads to a serious environmental problem due to erosion and pollution. The lower Guadalquivir River basin is also impacted by reservoirs and dams and its regime is rather artificial. Highest levels were observed in fish of the species *L. sclateri*, endemic of the Iberian Peninsula, where UV-F concentrations above 290 ng/g d.w. were observed. For this river basin, sediments collected in the same sampling points as the fish were also analysed and positive results for the compounds EHMC and OC were found. EHMC was detected at concentrations of 7.5, 22.9 and 18.9 ng/g d.w. at the sampling points GUA3, GUA4 and GUA5, respectively. OC was detected at 22.5 ng/g d.w. at the sampling point GUA4 and under the limit of quantification at GUA5. UV-F residues were not

Table 2
Locations and number of fish samples, fish species and concentration of the detected UV filters (ng/g d.w.) of target UV F along the four studied river basins.

Sampling station	Fish lipid content (%)	Common name	Scientific name	BP3	EHMC	4MBC	OC
Llobregat							
LLO3 (n = 3)	13.6	Ebro barbel (juvenile)	*Luciobarbus graellsii*	n.d.	n.d.	n.d.	n.d.
LLO4 (n = 3)	14.1			n.d.	n.d.	n.d.	n.d.
LLO6 (n = 3)	15.6			n.d.	n.d.	n.d.	n.d.
LLO3 (n = 3)	19.9	Ebro barbel (adult)	*Luciobarbus graellsii*	n.d.	n.d.	n.d.	n.d.
LLO4 (n = 2)	26.9			n.d.	n.d.	n.d.	n.d.
LLO6 (n = 3)	20.8			n.d.	n.d.	n.d.	n.d.
LLO3 (n = 3)	26.6	Common carp	*Cyprinus carpio*	n.d.	n.d.	n.d.	n.d.
LLO4 (n = 1)	n.a.			n.d.	n.d.	n.d.	n.d.
LLO5 (n = 3)	20.7			n.d.	n.d.	n.d.	n.d.
LLO6 (n = 3)	25.5			n.d.	n.d.	n.d.	n.d.
LLO7 (n = 3)	22.9			n.d.	n.d.	n.d.	<LOQ
Ebro							
OCAn (n = 4)	12.1	Ebro barbel (juvenile)	*Barbus graellsii*	n.d.	n.d.	n.d.	n.d.
EBR2 (n = 3)	11.8			n.d.	n.d.	n.d.	n.d.
EBR3 (n = 3)	12.3			n.d.	n.d.	n.d.	n.d.
EBR4 (n = 3)	12.6			n.d.	n.d.	n.d.	n.d.
EBR5 (n = 3)	n.a.			n.d.	n.d.	n.d.	n.d.
OCA (n = 3)	17.1	Ebro barbel (adult)	*Barbus graellsii*	n.d.	n.d.	n.d.	n.d.
EBR2 (n = 3)	24.1			n.d.	n.d.	n.d.	n.d.
EBR3 (n = 3)	n.a.			2.2	n.d.	2.7	n.d.
EBR4 (n = 3)	n.a.			n.d.	n.d.	n.d.	n.d.
EBR5 (n = 2)	15.4			n.d.	n.d.	n.d.	n.d.
EBR2 (n = 1)	11.2	Common carp	*Cyprinus carpio*	n.d.	n.d.	n.d.	n.d.
EBR3 (n = 3)	9.4			n.d.	n.d.	n.d.	n.d.
EBR4 (n = 3)	8.3			n.d.	n.d.	n.d.	n.d.
EBR5 (n = 3)	12.6			n.d.	n.d.	n.d.	<LOQ
EBR4 (n = 2)	24.8	Wels catfish	*Silurus glanis*	n.d.	12.2	n.d.	<LOQ
EBR5 (n = 2)	26.6			<LOQ	30.4	<LOQ	25.7
Guadalquivir							
GUA1 (n = 1)	27.1	Andalusian barbel (adult)	*Luciobarbus sclateri*	n.d.	n.d.	n.d.	n.d.
GUA3 (n = 9)	29.3			n.d.	19.0	n.d.	<LOQ
GUA4 (n = 9)	40.6			24.3	241.7	n.d.	30.4
GUA5 (n = 9)	34.5			16.5	63.0	n.d.	n.d.
GUA3 (n = 9)	9.0	Common carp	*Cyprinus carpio*	11.2	<LOQ	n.d.	n.d.
Jucar							
JUC1 (n = 3)	47.7	Brown trout (adult)	*Salmo trutta*	4.6	n.d.	n.d.	n.d.
JUC2 (n = 2)	19.3	Iberian nase	*Pseudochondrostoma polylepis*	n.d.	n.d.	n.d.	n.d.
JUC2 (n = 13)	n.a.	Iberian gudgeon (juvenile)	*Gobio lozanoi*	n.d.	n.d.	n.d.	n.d.
JUC4 (n = 10)	n.a.			n.d.	n.d.	n.d.	n.d.
JUC4 (n = 4)	n.a.	Iberian gudgeon (adult)	*Gobio lozanoi*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 4)	n.a.			n.d.	n.d.	<LOQ	n.d.
JUC4 (n = 6)	13.0	Black bass	*Micropterus salmoides*	n.d.	n.d.	n.d.	n.d.
JUC5 (n = 5)	n.a.			n.d.	n.d.	n.d.	<LOQ
JUC6 (n = 2)	18.9			n.d.	n.d.	n.d.	n.d.
JUC5 (n = 6)	n.a.	Bleak	*Alburnus alburnus*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 16)	n.a.			n.d.	n.d.	n.d.	n.d.
JUC5 (n = 3)	11.3	European eel	*Anguila anguila*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 3)	44.6			n.d.	<LOQ	n.d.	30.0
JUC6 (n = 1)	n.a.	Pumpkinseed	*Leponis gibbosus*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 2)	12.3	Mediterranean barbel (juvenile)	*Barbus guiraonis*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 1)	14.6	Mediterranean barbel (adult)	*Barbus guiraonis*	n.d.	n.d.	n.d.	n.d.
JUC6 (n = 1)	8.4	Pike	Esox lucius	n.d.	n.d.	n.d.	n.d.

LOQ values (ng/L d.w): 4.0 (BP3), 16.7 (EHMC), 2.3 (4MBC), 20.0 (OC). n.a.: not available

detected at GUA1, located in the upper river. Fig. 2 shows the total concentration of UV-F in fish (species: *L. sclateri*) and in sediments detected in the Guadalquivir River basin. The location of fish samples with high UV-F levels corresponded to the sites where the highest UV-F values were determined in sediments. The highest concentrations were found for both fish and sediments in the sample point GUA4, which is located downstream the Cordoba city WWTP (serving 350,000 inhabitants), from which it receives large volumes of wastewater. Wastewater discharge is considered to be an important source of UV-F for aquatic environments and aquatic organisms. Important loads of these contaminants have been directly associated with dense populations and proximity to wastewater effluent discharges (Buser et al., 2006) and it seems that the concentrations detected in the sample point GUA4 constitutes an example in this regard. The second most polluted sampling location was GUA5, which is located in the main stream in Peñaflor, a municipality of around 4000 inhabitants. In GUA3 only EHMC was determined, whereas GUA1 appeared not to be contaminated by the target UV F. These two sampling sites were located in the main stream close to two small villages, Marmolejo and Mogón. The last one is included into the Sierra de Cazorla National Park.

The levels detected in fish samples are significantly higher than the ones in the corresponding sediments, showing an increased accumulation of these lipophilic compounds in fish over sediment. However, when normalizing the respective concentrations to lipid content and TOC (from 0.7 to 1.2%), the calculated BSAFs were always below 1, in the range of 0.04–0.3, which suggests that estimates based on bioavailability of the contaminant by the fish are lower than those based on the adsorption onto the sediments. This may be explained by the metabolization and elimination in the fish (Rüdel et al, 2006). These results suggest a positive correlation between UV-F concentration and

Table 3
Summary of UV filter results in fish from the selected Iberian rivers.

			Llobregat (11 samples)				Ebro (16 samples)				Guadalquivir (5 samples)				Jucar (17 samples)			
UV-F	LOD (ng/g d.w.)	LOQ (ng/g d.w.)	Freq. (%)	Positive Samples (LOQ)	Range (ng/g d.w.)	Median[a] (ng/g d.w.)	Freq. (%)	Positive Samples (LOQ)	Range (ng/g d.w.)	Median[a] (ng/g d.w.)	Freq. (%)	Positive Samples (LOQ)	Range (ng/g d.w.)	Median[a] (ng/g d.w.)	Freq. (%)	Positive Samples (LOQ)	Range (ng/g d.w.)	Median[a] (ng/g d.w.)
BP3	1.2	4.0	0.0	0	–	.	12.5	2 (1)	LOD–2.2	LOQ	60.0	3 (3)	11.2–24.3	16.5	5.6	1 (1)	LOD–4.6	4.6
EHMC	5.0	16.7	0.0	0	–	.	12.5	2 (2)	LOD–30.4	LOQ	80.0	4 (3)	LOD–241.7	41.0	5.6	1 (0)	LOD–LOQ	LOQ
4MBC	0.7	2.3	0.0	0	–	.	12.5	2 (1)	LOD–2.7	1.75	0.0	0	–	–	5.6	1 (0)	LOD–LOQ	LOQ
OC	6.0	20.0	9.1	1 (0)	LOD–LOQ	LOQ	18.8	3 (2)	LOD–25.7	LOQ	40.0	2 (1)	LOD–30.4	LOQ	11.1	1 (0)	LOD–30.0	LOQ
Total UV-F[a]			9.1	1		7	25	4	4.9–66.3[a]	13.1	80.0	4	17.1–296.4[a]	52.7	22.2	4	0.8–35.9[b]	5.8

LOD: Method limit of detection; LOQ: Method limit of quantification, Freq: Frequency of detection; LOD: Below limit of detection; LOQ: limit of quantification. LOQ: Below limit of quantification.
[a] Only positive values were used for this calculation. For positive results below LOQ, calculations were performed by assigning a value corresponding to [(LOQ–LOD)/2].

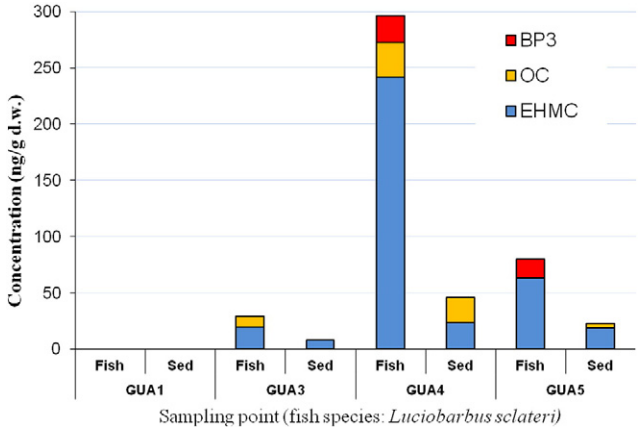


Fig. 2. Comparison of UV-F levels obtained in samples of sediment and fish in the Guadalquivir River basin. For results below the limit of quantification a value corresponding to [(LOQ–LOD)/2] was assigned. Sediment total organic carbon (TOC) values: GUA1 (0.66%), GUA3 (0.69%), GUA4 (1.20%) and GUA5 (0.98%).

lipid weight (see Table 2), and also between UV-F concentration and TOC.

The Llobregat River was found to be the lowest contaminated basin among the rivers studied. Only one out of the eleven samples taken along the basin showed OC, but at low concentration (between 6 and 20 ng/g d.w.) in the sampling station close to the large city of Barcelona. This low detection draws attention considering that Llobregat is a high industrialized river basin with a low average flow (19 m^3/s) (data from Agencia Catalana de l'Aigua) and high values of UV F have been previously detected in this river basin (Gago-Ferrero et al., 2013a), although the sampling points were not the same. However, as the Llobregat River shows a Mediterranean hydrological pattern, its flow can fluctuate considerably from dry to rainy periods. At high flow contaminants dilution occurs.

Similar findings were also observed in Jucar river basin (20% detection frequency) where only OC, the most lipophilic one, was found at a concentration above the LOQ in one *Anguila anguila* sample (30.0 ng/g d.w.). According to previous studies, this species tend to bioaccumulate more substances than the other species due to the high percentage of lipids in its body (Sancho et al., 1998).

Ebro River is regulated by dams and channels, which have altered its hydrological and sedimentary regime. Abstraction of ground and surface water, irrigation and industrial activities concentrated close to the main cities in the basin have also deteriorated soil and water quality. This river shows the highest average flow (600 m^3/s) (data from Confederación Hidrográfica del Ebro, CHE) among the rivers studied which contribute to the dilution of the contamination. UV-Fs were observed in 25% of the samples from the Ebro River. The samples of the species *Silurus glanis* showed the highest concentrations for EHMC and OC at the sampling points EBR4 and EBR5. Both sampling sites are located downstream the WWTP close to the cities of Logroño (154,000 inhabitants) and Tudela (36,000 inhabitants), respectively. BP3 and 4MBC were also determined in *Barbus graellsii* in EBR3 in La Rioja, a well-known vineyard region.

Summarizing, the highest frequency of detection was observed for EHMC in Guadalquivir River (found in 80% of the samples), whereas OC was the most frequently found compound in the whole study, being present in all four river basins. EHMC is extensively used in several personal care products and has shown an estrogenic activity (Kunz and Fent, 2006) and effects on the global gene expression in fish (Zucchi et al., 2011) at relatively low concentration (2.2 μg/L).

The highest mean concentration was determined for the compound EHMC (82.4 ng/g d.w.). BP3 showed a mean concentration of 17.3 ng/g d.w. and in the case of OC and 4MBC this parameter was <LOQ. The contamination level (accumulated mean concentrations

of total UV F) order between the four river basins was: Guadalquivir (104.7 ng/g d.w.) > Ebro (26.3 ng/g d.w.) > Jucar (12.1 ng/g d.w.) > Llobregat (7 ng/g d.w.). For positive results < LOQ, calculations were performed by assigning a value corresponding to [(LOQ–LOD)/2].

3.1.2. Fish species distribution

In the present study, the influence of fish size, i.e. between juvenile and adults on the UV-F fish concentration was not observed.

EHMC occurred in several fish species (*L. sclateri*, *S. glanis*, *Anguila anguila* and in *Cyprinus carpio*) having different diet and strata preferences. All these fish species, except for *S. glanis*, which is a predator, are bottom feeding omnivorous species. Taking into account these data, it is not clear whether biomagnification might play a role in the concentration of UV-F in fish. However, in the Ebro River, only *S. glanis* (trophic levels of 4.3–4.7) (Encina and Granado-Lorencio, 1991), a predator at the top of the food chain in that ecosystem, showed detectable UV-F concentrations. In Guadalquivir River, EHMC accumulation was also most pronounced in *L. sclateri* (trophic level of 2.64) (Syvaranta et al., 2010) than in *C. carpio* (trophic level of 2.79) (Yu et al., 2012). In a previous study, Fent et al. suggested that biomagnification occurs for this compound in the aquatic environment (Fent et al., 2010). In that study biomagnification was suggested in the predator/prey pair cormorant and fish (barb, chub and brown trout) and between the omnivorous barb feeding on *Gammarus*. For a reliable correlation data between UV-F concentrations and for instance morphometric data of analysed fish (length, weight, gender or maturity level), a more extensive sampling in each site following a different strategy should be carried out.

The herein reported results are in agreement with those of previous studies performed in other European river basins studying fish and other fresh water organisms (Balmer et al., 2005; Mottaleb et al., 2009; Fent et al., 2010; Vela-Soria, 2011; Gago-Ferrero et al., 2013d). The concentrations needed to induce known adverse effects on organisms are higher than those observed in this study and typically reported in surface waters.

The analysis of vitellogenin (VTG) in rainbow trout (*Oncorhynchus mykiss*) and Japanese medaka (*Oricias latipes*) after aqueous exposure to BP3 indicated that high effective concentrations in the range of 620–749 μg/L were needed for its induction (Coronado et al., 2008). In male Japanese medaka, the levels of VTG and choriogenin, another known estrogen-responsive gene product, were found to increase after exposure of the fish to 4-MBC and EHMC (Inui et al., 2003), with high estrogenic potency being displayed by 4-MBC.

4. Conclusions

The present findings revealed that several fish species from four Iberian rivers contained detectable concentrations of UV-F. However, the target compounds were detected with low frequencies of detection. These results constitute the first data on bioaccumulation of UV-F in fish from Iberian rivers. Among the eight target sunscreens, only the lipophilic compounds (Log Kow > 3.5) were accumulated. The detected levels are comparable with the values reported in previous studies conducted in few European rivers and lakes indicating a similar pattern of use of these compounds. The highest concentrations were detected in fish from the Guadalquivir River, which accumulated BP3, EHMC and OC. The sediments corresponding to the same sampling locations where the fish were collected were contaminated only with the two most lipophilic compounds EHMC (Log Kow 5.8) and OC (Log Kow 6.88). In general, the highest UV-F contamination level in fish was observed downstream WWTPs close to populated urban areas along the basins. The BSAF values estimated for OC and EHMC (always < 1) indicated that the target UV-F tend to bioaccumulate in fish but are also eliminated to some extent. Predator species occupying a higher position in the trophic chain showed higher levels of UV-F, which suggests that biomagnification may play a certain role in the accumulation of these chemicals in fish. Nevertheless, due to the short food chain available in the present study, further investigation at longer food chains is still needed to clearly identify the trophic magnification potential of UV filters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.03.026.

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The role of direct photolysis and indirect photochemistry in the environmental fate of ethylhexyl methoxy cinnamate (EHMC) in surface waters

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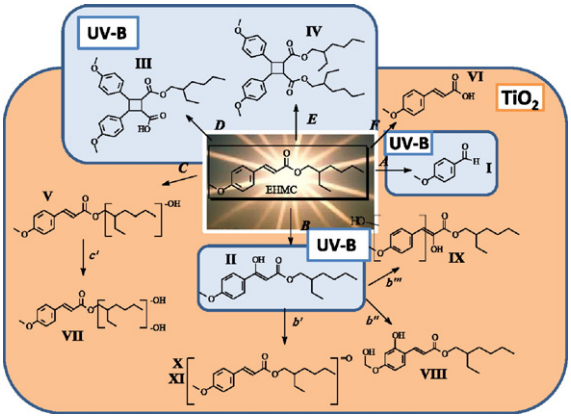
HIGHLIGHTS

- Study of the photolytic and photocatalytic transformation of EHMC in water.
- The role of direct and indirect photolysis was evaluated.
- Predicted EHMC lifetime is of the order of hours to a few days in fair-weather summertime.
- We identify 11 phototransformation products using HPLC–HRMS.

GRAPHICAL ABSTRACT



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ABSTRACT

The aquatic environmental fate of ethylhexyl methoxy cinnamate (EHMC), one of the most used UVB filters worldwide, was studied by assessing its environmental persistence and photoinduced transformations. The role of direct and indirect photolysis was evaluated. Direct photolysis was shown to play a key role, and this process is expected to be the main attenuation route of EHMC in sunlit surface waters. In contrast, the reaction with •OH radicals would be negligible and that with ^{3}CDOM* would at most be a secondary process.

The measurement of the quantum yield of direct photolysis and of the rate constants of reaction with photogenerated transient species (or, sometimes, the use of reasonable values for the latter) allowed the prediction of the EHMC half-life time in surface waters, by means of a validated photochemical model. The predicted EHMC lifetime is of the order of hours to a few days in fair-weather summertime, and the main factors controlling the EHMC phototransformation in sunlit surface waters would be the water depth and the dissolved organic carbon (DOC) content.

The formation of transformation products (TPs) was followed as well via HPLC/HRMS. Three TPs were detected in the samples exposed to UVA radiation, while one additional TP was detected in the samples exposed to UVB

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radiation. The detected TPs comprised 4-methoxybenzaldehyde, a hydroxylated derivative and dimeric species. Through the use of heterogeneous photocatalysis with TiO_2, seven additional TPs were identified, most of them resulting from the further degradation of primary TPs formed through direct photolysis and that might be detected in aquatic systems as well. The photodegradation of EHMC in the presence of TiO_2 yielded more toxic TPs than the parent compound (as determined with the *Vibrio fischeri* Microtox assay). The increased toxicity is partially accounted for by the formation of 4-methoxybenzaldehyde.

1. Introduction

Organic UV filters are considered as pseudo-persistent environmental contaminants, although at present little is known about their distribution and impact on aquatic systems. Despite the fact that most of them are characterized by a high lipophilicity ($\log K_{OW} > 3$), they can be washed away by water, thus ending up in the environment. Therefore, several sunscreens have been detected at ppb or ppt levels in surface water (Poiger et al., 2004; Rodil and Moeder, 2008) and wastewater (Kupper et al., 2006; Magi et al., 2013), with maximum concentrations in summertime. Their hydrophobicity could also lead to accumulation in biota or sediments. Several studies have actually shown the occurrence of UV filters in aquatic organisms: the 4-methyl-benzylidene-camphor has been detected in the muscle tissue of trout in Swiss and German waters (Balmer et al., 2005), while traces of ethylhexyl methoxy cinnamate and octocrylene have been found in shellfish in the Mediterranean and Atlantic coasts of France (Bachelot et al., 2012). Furthermore, eighteen organic sunscreens were found in sediments of Japanese rivers and lakes, at concentrations ranging from 2 to about 3000 ng/g (Kameda et al., 2011). The accumulation of organic UV filters in living organisms is of major concern because some of them (and their metabolites) can act as endocrine disruptors both in vitro and in vivo (Schlumpf et al., 2001).

The present study was focused on ethylhexyl methoxy cinnamate (EHMC), also known as Eusolex 2292 and Uvinul MC80. It is one of the most used UVB filters worldwide, and it is included in the so-called High Production Volume Chemicals (HPVC) list that includes chemicals produced or imported in the EU at a rate of more than 1000 tons per year. Although EHMC is well tolerated by the skin, it has some side effects including its ability to produce reactive oxygen species (ROS) and to penetrate in the human skin after exposure to UV light (Hanson et al., 2006; Janjua et al., 2008). The occurrence of EHMC in the environment has already been reported in many aqueous, solid and biological samples (Bachelot et al., 2012; Balmer et al., 2005; Goksoyr et al., 2009; Kameda et al., 2011; Kupper et al., 2006; Magi et al., 2013; Poiger et al., 2004; Rodil and Moeder, 2008). EHMC has also been found in shellfish, fish and cormorants at ng/g levels, which suggests that it can be accumulated in the food chain (Fent et al., 2010). EHMC has also proved responsible for coral bleaching by promoting viral infections (Danovaro et al., 2008).

From the toxicological point of view, EHMC has estrogenic properties both in vitro and in vivo (Schlumpf et al., 2001). For instance, exposure to this compound caused the increase of the weight of the uterus in rats. Prenatal exposure to EHMC can affect both the reproductive and neurological development in the offspring of rats, which can be a cause for concern because humans are routinely exposed to this compound through the use of sunscreens and other cosmetics (Axelstad et al., 2011).

The widespread environmental occurrence of EHMC and its negative health effects account for the importance of assessing its environmental persistence and transformation. The present work focuses on photochemical processes, which are an important class of abiotic transformation reactions that involve xenobiotics in surface waters. Previous works have studied the photostability of EHMC under cosmetic conditions (trying to simulate the behavior of the compound in solar lotions), at relatively high concentrations and without any attempt to assess or extrapolate EHMC photochemistry to sunlit surface waters (Carlotti et al., 2005, 2007; Hauri et al., 2004). The latter issue is accounted for by the fact that the previously studied systems, conditions and additives were representative of cosmetic sunscreens instead of the natural environment. Moreover, the photogenerated TPs were either not identified, or they were not tested for health or environmental effects. Therefore, previous studies do not allow an assessment of the environmental significance of EHMC phototransformation in aqueous media. The present paper has the goal of filling in the above-mentioned knowledge gaps.

Photochemical reactions in surface waters can be divided into direct photolysis and indirect photochemistry. In the case of direct photolysis, sunlight absorption by the pollutant triggers its transformation. As far as indirect photochemistry is concerned, sunlight is absorbed by photoactive compounds called photosensitisers, such as chromophoric dissolved organic matter (CDOM), nitrate and nitrite. Upon sunlight absorption, these compounds produce reactive transients such as the hydroxyl radical (•OH), singlet oxygen ($^1O_2$) and CDOM triplet states (^{3}CDOM*), which can induce pollutant transformation (Vione et al., 2014). The role of direct photolysis and indirect photochemistry in the environmental fate of EHMC in surface waters was thus assessed, as well as the phototransformation half-life time under summertime irradiation conditions that are most significant for the environmental occurrence of the studied UV filter. The TPs of EHMC were studied as well. In particular, in addition to identifying the compounds formed upon direct photolysis of the substrate, the environmental photodegradation was also simulated by the use of heterogeneous photocatalysis with titanium dioxide (TiO_2). The latter approach allows the generation of TPs that are similar to those formed by photochemical processes in the environment, as documented in several studies (Calza et al., 2010, 2011; Konstantinou et al., 2001; Kouloumbos et al., 2003), and it is particularly suitable for TPs identification.

2. Experimental section

2.1. Materials

TiO_2 P25 was used as photocatalyst, after being subjected to irradiation and washings with ultrapure water in order to eliminate the potential interference caused by adsorbed ions such as chloride, sulfate and sodium. EHMC (CAS 5466-77-3, 98%), methanol (≥99.9%), acetonitrile (≥99.9%), formic acid (99%) and acetaminophen (also known as acetyl-para-aminophenol, APAP, ≥99.0%) were purchased from Sigma-Aldrich, Italy. Rose Bengal was purchased from Alfa Aesar, Italy.

2.2. Irradiation procedures

2.2.1. Direct photolysis

Due to the low water solubility of EHMC, its aqueous solutions were prepared by methanol spiking (Rodil et al., 2009) using two different procedures: 1) Samples used for intermediate analyses were prepared by adding 0.4 mL of a concentrated methanol solution of EHMC (1000 mg/L or 3.6 mM) to ultra-pure water, to obtain a final EHMC concentration of 4 mg/L (14 μM); and 2) Samples used to determine the direct photolysis quantum yield of EHMC, were prepared at an initial concentration slightly lower (10 μM). In alternative and in comparison with methanol spiking, tests were carried out with acetonitrile spiking: fully comparable results as for methanol were obtained.

Five milliliters of aqueous solutions prepared as described above were introduced into cells of Pyrex glass (cylindrical, 4.0 cm diameter, 2.3 cm height) and subjected to irradiation. Two different lamps were used, namely a 40 W Philips TLK 05 lamp with maximum emission at 365 nm and a 20 W Philips TL 01 RS lamp with emission maximum at 313 nm. The latter lamp was also used to measure the quantum yield of EHMC direct photolysis. Due to its limited water solubility EHMC has the tendency to adsorb on the cell walls and, for this reason, the irradiated solutions were recovered from the cells by adding 5 mL of methanol that achieves desorption from the glass walls (Li et al., 2007) and allows a nearly quantitative recovery.

Fig. 1 reports the absorption spectrum of EHMC, measured with a Varian Cary 100 Scan double-beam UV–vis spectrophotometer, equipped with quartz cuvettes (Hellma, 1.000 cm optical path length), as well as the emission spectrum of the TL 01 RS lamp (spectral photon flux density $p°(\lambda)$), taken with a CCD spectrophotometer (Ocean Optics USB 2000, calibrated with a DH-2000-CAL radiation source). The lamp spectrum was normalized to the results of chemical actinometry with 4-nitrobenzaldehyde. The followed procedure is described in detail by Marchisio et al. (2015).

2.2.2. Indirect photolysis

The above-described technique of methanol spiking in aqueous solution was used in the case of indirect photolysis as well (see procedure 2 described in Section 2.2.1). To determine the reaction rate constants of EHMC with •OH, $^1O_2$ and CDOM triplet states, acetaminophen (APAP) was used as model compound because its reaction rate constants with the above transients are known (De Laurentiis et al., 2014). In this case, solutions containing EHMC and APAP at equal initial concentration (10 μM for both) were irradiated under suitable conditions (vide infra) to produce the transient species X (•OH, $^1O_2$ or ^{3}CDOM*). The time evolution of the two substrates was monitored, and the concentration vs. time data were fitted with the equation $C_t = C_o e^{-k\,t}$, where C_t is the substrate concentration at time t, C_o the initial concentration and k the pseudo-first order degradation rate constant. The initial transformation rate is $R = k\,C_o$. The reported error on the rates ($\pm\sigma$) mainly depended on the uncertainty on k, which represents the average of replicate runs.

If the degradation of the two substrates is mainly or exclusively accounted for by reaction with X, the ratio of the initial transformation rates of the two compounds can be expressed as follows:

$$\frac{R_{EHMC}}{R_{APAP}} = \frac{k_{EHMC,X}[X][EHMC]}{k_{APAP,X}[X][APAP]} = \frac{k_{EHMC,X}}{k_{APAP,X}} \quad (1)$$

where $k_{EHMC,X}$ and $k_{APAP,X}$ are the second-order reaction rate constants with X of EHMC and APAP, respectively, [X] is the steady-state concentration of the transient (note that EHMC and APAP are in the same solution) and [EHMC] = [APAP] = 10 μM are the initial concentration values of the two substrates. The equation can be simplified and one gets that the ratio of the initial rates is equal to the ratio of the second-order rate constants. Therefore, by knowing the rate constant $k_{APAP,X}$ (De Laurentiis et al., 2014) and by measuring the initial degradation rates, one gets $k_{EHMC,X} = k_{APAP,X} R_{EHMC} (R_{APAP})^{-1}$.

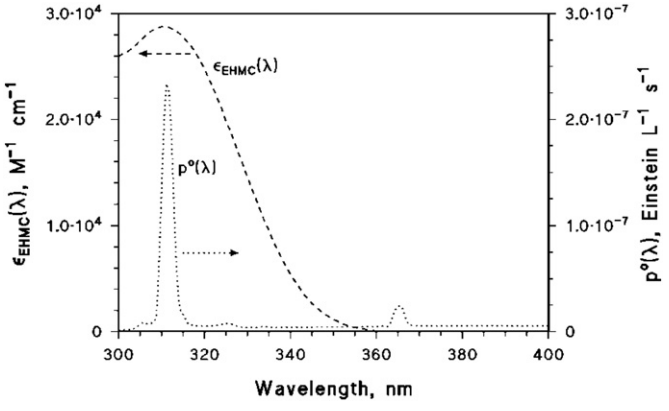


Fig. 1. Absorption spectrum (molar absorption coefficient $\varepsilon_{EHMC}(\lambda)$) of EHMC. Emission spectrum (spectral photon flux density $p°(\lambda)$) of the TL 01 RS lamp.

The radical •OH was produced by irradiating 1 mM H_2O_2 under the TLK 05 or the TL 01 RS lamp, while anthraquinone-2-sulfonate (AQ2S) was used as CDOM proxy to study the reactivity of ^{3}CDOM* (De Laurentiis et al., 2014). In this case, 1 mM AQ2S was irradiated under the TLK 05 lamp. Measures of reactivity with $^1O_2$ were performed using a lamp Philips TL D 18 W/16 with emission maximum at 545 nm. The dye Rose Bengal (10 μM initial concentration) was chosen as the source of singlet oxygen. Also in the indirect photolysis experiments, at the end of the irradiation, the content of the cells was recovered with 5 mL of methanol. In all the cases, the pH of the irradiated solutions was 6–6.5.

2.2.3. Photocatalysis

The irradiation with TiO_2 of a compound that is poorly water-soluble can be carried out by dispersing in water the photocatalyst loaded with the substrate. To do so, a EHMC stock solution was prepared in methanol at the concentration of 15 mg/L. The photocatalyst powder was added to obtain a TiO_2 loading of 200 mg/L, then the solvent was evaporated with a Büchi Rotavapor system so as to obtain a TiO_2 powder loaded with EHMC. The latter then was suspended in water to obtain an aqueous dispersion of EHMC.

Five milliliters of the suspension thus obtained was introduced into the Pyrex cells and irradiated under the Philips TLK 05 lamp. After irradiation the content of the cells was recovered with 5 mL methanol to prevent EHMC loss upon adsorption on the glass, and the slurry was filtered on a 0.45 μM filter (PTFE, Merck Millipore, Milan, Italy).

2.3. Analytical techniques

2.3.1. HPLC-UV

A VWR-Hitachi LaChrom Elite chromatograph, equipped with a L-2300 autosampler (injection volume 60 μL), a quaternary pump module L-2130, a L-2300 column oven (temperature 40 °C), a DAD detector L-2445, and a reverse-phase column (VWR RP-C18 LiChroCART, 4 mm × 125 mm × 5 μm) was used to measure the direct photolysis quantum yield of EHMC and its reaction rate constants with the transient species. To determine both EHMC and APAP, a gradient of methanol and phosphoric acid (3 mM) with 1 mL/min flow rate, by increasing the methanol percentage from 15 to 90% in 15 min, was used.

In other experiments, EHMC was monitored by using a Merck-Hitachi chromatograph equipped with a Rheodyne injector, L-6200 and L-6200A pumps for high-pressure gradients, a L-4200 UV–vis detector, and the same column as above. Isocratic elution (1 mL/min flow rate) was carried out with 20% of 10 mM phosphate buffer (H_3PO_4 + NaH_2PO_4) at pH 2.8 and 80% acetonitrile. The retention time of EHMC was 7.4 min and the detection wavelength was set at 310 nm.

2.3.2. HPLC–HRMS

The chromatographic separations, monitored using an MS analyzer, were run on a Phenomenex Luna C18 (2) 150 × 2.1 mm × 3 μm particle size, using an Ultimate 3000 HPLC instrument (Dionex, Thermo Scientific, Milan, Italy). The injection volume was 20 μL and the flow rate 200 μL/min. Gradient mobile phase composition was adopted: A/B was varied from 5/95 to 100/0 in 35 min (followed by 10 min of 100% A isocratic elution), where A = acetonitrile and B = formic acid 0.05% v/v in water, when run on ESI positive ion mode.

A LTQ Orbitrap mass spectrometer (Thermo Scientific, Milan, Italy) equipped with an ESI ion source was used. The LC column effluent was delivered into the ion source using nitrogen as both sheath and

auxiliary gas. The tuning parameters adopted for the ESI source were: capillary voltage 37.00 V, tube lens 65 V. The source voltage was set to 3.5 kV. The heated capillary temperature was maintained at 275 °C. The acquisition method used was optimized before and in the tuning sections for the parent compound (capillary, magnetic lenses and collimating octapole voltages) to achieve maximum sensitivity. Mass accuracy of recorded ions (vs. calculated) was ± 10 millimass units (mmu) without internal calibration. External calibration was performed with a mixture of caffeine, MRFA peptide and Ultramark 1621 (LTQ calibration solution, Thermo Scientific, Milan, Italy).

Analyses were run using full MS (50–1000 *m*/*z* range), MS^2 and MS^3 acquisition in the positive ion mode, with a resolution of 30,000 (500 *m*/z FWHM) in FTMS mode. The ions submitted to MS^n acquisition were chosen on the base of full MS spectra abundance without using automatic dependent scan. Collision energy was set to 30 (arbitrary units) for all of the MS^n acquisition methods. MS^n acquisition range was between the values of ion trap cut-off and *m*/*z* of the fragmented ion. Xcalibur 2.0.7 (Thermo Scientific, Milan, Italy) software was used both for acquisition and for elaboration.

2.4. Photochemical modeling

The assessment of the phototransformation kinetics was carried out with the APEX software (Aqueous Photochemistry of Environmentally-occurring Xenobiotics). It predicts photochemical half-life times as a function of water chemistry and depth, for compounds with known direct photolysis quantum yields and second-order reaction rate constants with transient species. APEX is based on a photochemical model, validated by comparison with field data of phototransformation kinetics in surface freshwaters (Bodrato and Vione, 2014; De Laurentiis et al., 2012; Maddigapu et al., 2011; Marchetti et al., 2013; Vione et al., 2011). The previous validation of the model against available field data of several pollutants justifies the use of APEX also in the present case, where data of EHMC phototransformation in surface waters are unfortunately unavailable.

APEX results apply to well-mixed water bodies, including the epilimnion of stratified lakes. The absorption of radiation by photosensitisers (CDOM, nitrate and nitrite) and xenobiotics is computed by taking into account competition for sunlight irradiance in a Lambert–Beer approach. Data obtained with APEX are averages over the water column of given depth, and they include the contributions of the well-illuminated surface layer and of darker water at the bottom.

Sunlight irradiance is not constant in the natural environment, because of meteorological issues (not included in APEX) and of diurnal and seasonal cycles. To allow easier comparison between model results and environmental conditions, APEX uses as time unit a summer sunny day (SSD), equivalent to fair-weather 15 July at 45° N latitude. Another issue is that sunlight is not vertically incident over the water surface, but refraction at the interface deviates the light path in water towards the vertical. The light path length *l* depends on the depth *d*: on 15 July at 45°N it is $l = 1.05\ d$ at noon and $l = 1.17\ d$ at ± 3 h from noon, which is a reasonable daily average (Bodrato and Vione, 2014).

2.5. Prediction of toxicity towards freshwater organisms

Insight into the possible acute and chronic toxicity of the TPS of EHMC was obtained by ECOSAR software (Ecological Structure Activity Relationship, http://www.epa.gov/oppt/newchems/tools/21ecosar.htm, last accessed April 2015), which gives a standard toxicity profile based on calculated acute and chronic effects on fish, daphnia and algae. Calculations are carried out with a quantitative structure–activity relationship approach, based on class-specific linear regressions (Tunkel et al., 2005).

2.6. Toxicity measurements (Microtox)

The toxicity of samples collected at different irradiation times was evaluated with a Microtox Model 500 Toxicity Analyzer (Milan, Italy). Acute toxicity was evaluated with a bioluminescence inhibition assay using the marine bacterium *Vibrio fischeri*, by monitoring changes in the natural emission of the luminescent bacteria when challenged with toxic compounds. Freeze-dried bacteria, reconstitution solution, diluent (2% NaCl) and an adjustment solution (non-toxic 22% sodium chloride) were obtained from Azur (Milan, Italy). Samples were tested in a medium containing 2% sodium chloride, in five dilutions, and luminescence was recorded after 5, 15 and 30 min of incubation at 15 °C. Because no substantial differences were found between the three contact times, hereafter the results related to 5 min of contact are reported. Inhibition of luminescence, compared with a toxic-free control to give the percentage inhibition, was calculated following the established protocol and using the Microtox calculation program.

3. Results and discussion

3.1. Assessment of EHMC phototransformation in surface waters

When irradiated alone under the TL 01 RS lamp under ~ neutral pH conditions, 10 μM EHMC showed an initial degradation rate $R_{EHMC} = (1.72 \pm 0.15)\cdot 10^{-9}$ M s^{-1}. The photon flux absorbed by EHMC is $P_a^{EHMC} = \int_\lambda p^\circ(\lambda)\,[1-10^{-\varepsilon_{EHMC}(\lambda) b [EHMC]}]\,d\lambda = 4.5 \cdot 10^{-8}$ Einstein L^{-1} s^{-1}, where $p^\circ(\lambda)$ and $\varepsilon_{EHMC}(\lambda)$ are reported in Fig. 1, $b = 0.4$ cm and $[EHMC] = 1.0\cdot 10^{-5}$ M. The photolysis quantum yield can be calculated as $\Phi_{EHMC} = R_{EHMC}(P_a^{EHMC})^{-1} = (3.8 \pm 0.3) \cdot 10^{-2}$. Because the used lamp shows an emission maximum that is close to the absorption maximum of EHMC and because the relevant band is also responsible for sunlight absorption by EHMC, the calculated photolysis quantum yield is representative of EHMC photodegradation under sunlight (Turro et al., 1978).

The reaction rate constant between EHMC and $^1O_2$ was measured by irradiating 10 μM of EHMC and 10 μM of APAP under the TL D 18 W/16 lamp, in the presence of 10 μM Rose Bengal as $^1O_2$ source. Under the reported conditions it was $R_{EHMC} = (5.26 \pm 0.85)\cdot 10^{-11}$ M s^{-1} and $R_{APAP} = (1.26 \pm 0.27)\cdot 10^{-10}$ M s^{-1}. Considering that the second-order reaction rate constant $k_{APAP,^1O_2} = (3.68 \pm 0.73) \cdot 10^7$ M^{-1} s^{-1} (De Laurentiis et al., 2014), one gets $k_{EHMC,^1O_2} = k_{APAP,^1O_2} R_{EHMC}(R_{APAP})^{-1} = (1.54 \pm 0.88) \cdot 10^7$ M^{-1} s^{-1}.

Unfortunately it was not possible to measure the reaction rate constants of EHMC with •OH and ^{3}AQ2S* (taken as representative of ^{3}CDOM*), because irradiation under UVB and UVA caused an important direct photolysis of EHMC. Under such circumstances, the prerequisites behind Eq. (1) are not valid and the reaction rate constants cannot be measured. In the case of $^1O_2$, the measurement was allowed by the fact that the used lamp emits yellow light that is not absorbed by EHMC. Anyway, possible values of the rate constants with •OH and ^{3}CDOM* were considered in photochemical modeling. Moreover, the important interference of direct photolysis in the above irradiation experiments suggests that the direct photoprocess could also play a key role in the environmental transformation of EHMC.

Fig. 2a reports the modeled half-life time of EHMC (in SSD units, namely summer sunny days equivalent to 15 July at 45°N latitude) as a function of water depth and of the dissolved organic carbon (DOC). The relevant calculations considered only the direct photolysis and the reaction with $^1O_2$, thus the results are actually upper limits for the EHMC lifetimes. Despite this, it can be noticed that the phototransformation would be very fast because $t_{½} < 1.5$ SSD even in relatively deep water with high DOC concentration. Also note that reaction with $^1O_2$ would play a negligible role compared to the direct photolysis, thus the reported results almost exclusively depend on the direct phototransformation.

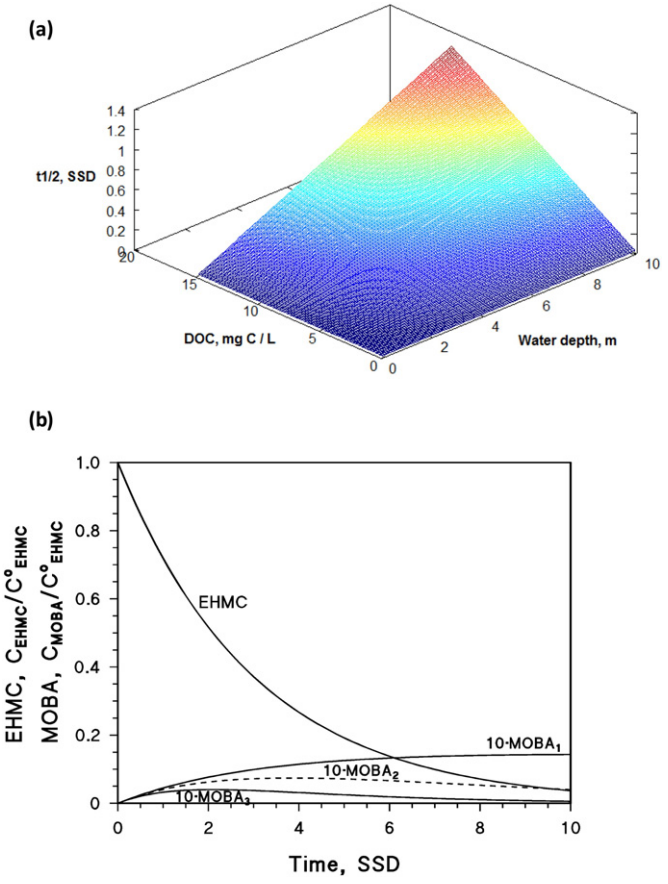


Fig. 2. (a) Modeled half-life time of EHMC (units of SSD = summer sunny days equivalent to 15 July at 45°N latitude) as a function of water depth and DOC. Other water conditions: 0.1 mM nitrate, 1 μM nitrite, 1 mM bicarbonate, 10 μM carbonate. (b) Modeled time trends of EHMC and MOBA (SSD units) for the following water conditions: 5 m depth, 5 mg C L^{-1} DOC, 0.1 mM nitrate, 1 μM nitrite, 1 mM bicarbonate, 10 μM carbonate. For plot readability issues, the MOBA concentration was multiplied by 10. $MOBA_1$: $k_{MOBA} = 0.01$ SSD^{-1} ($k°_{MOBA}$); $MOBA_2$: $k_{MOBA} = 2$ SSD^{-1}; $MOBA_3$: $k_{MOBA} = 6.6$ SSD^{-1} (k'_{MOBA}).

The figure shows that the half-life time increases with increasing depth and DOC. The trend with depth is accounted for by the fact that the bottom layers of deep water bodies are poorly illuminated by sunlight and, therefore, they are poorly photoactive. In deep waters, the high photoactivity of the surface layer is offset by the poor reactivity in the lower depths. As far as the trend with DOC is concerned, high-DOC waters usually contain abundant CDOM that competes with the pollutants for sunlight irradiance, thereby inhibiting the direct photolysis processes (Vione et al., 2014).

As mentioned above, the reaction rate constants of EHMC with •OH and ^{3}CDOM* are unfortunately not available. However, bimolecular reaction rate constants in aqueous solution have the diffusion control as an upper limit (Buxton et al., 1988). For this reason, the expected importance of the different phototransformation pathways was also assessed by assuming that $k_{EHMC,\cdot OH}$ could vary up to $2\cdot10^{10}$ M^{-1} s^{-1} and $k_{EHMC,^3CDOM*}$ up to $1\cdot10^{10}$ M^{-1} s^{-1}. The difference between the two values is due to the fact that •OH is generally more reactive than ^{3}CDOM* (Vione et al., 2014). With these assumptions it can be concluded that, under the surface-water conditions reported in Fig. 2a, •OH would never account for more than 1% of EHMC phototransformation and the relative role of ^{3}CDOM* would always be below 10%. As a consequence, the EHMC photodegradation would mainly take place upon direct photolysis, while the reaction with •OH would be negligible and that with ^{3}CDOM* would at most be a secondary process. The concentrations of nitrate, nitrite, carbonate and bicarbonate in water mainly affect the •OH reaction (Vione et al., 2014), thus the relevant concentration variations would not modify significantly the modeled half-life time of EHMC. In summary, the main factors controlling the phototransformation of this compound in surface waters would be the water depth and the DOC. APEX modeling predicts a very short lifetime for EHMC in surface waters. Unfortunately there are no field data against which to test this prediction, but significant photolability is not a rare occurrence for compounds that undergo efficient direct photolysis. For instance, Tixier et al. (2003) have reported a half-life time of around 8 days (which would correspond to just 4 SSD due to weather conditions at the time of their field study) for diclofenac in the surface water of Lake Greifensee, Switzerland. These data are not too far from our model predictions for EHMC which, therefore, do not look unreasonable.

Because the direct photolysis is expected to be the main EHMC phototransformation pathway in surface waters, it is important to identify the TPs that are produced during this process. Among the identified TPs (vide infra), 4-methoxybenzaldehyde (MOBA) was available as commercial standard. The transformation yield of EHMC into MOBA upon direct UVB photolysis was thus found to be $\eta_{MOBA} = 1.6\%$ (the disappearance of 13 μM EHMC yielded 0.21 μM MOBA). The yield datum allows the modeling of MOBA formation (Bodrato and Vione, 2014). Moreover, if the first-order degradation rate constant of MOBA (k_{MOBA}) were available, it would be possible to predict the time trends of both EHMC and MOBA due to photochemical reactions. Under the pseudo-first order approximation, the time trend of EHMC would be described by $[EHMC]_t = [EHMC]_o\, e^{-k_{EHMC}t}$, where $[EHMC]_t$ is the concentration of EHMC at the time t, $[EHMC]_o$ its initial concentration and k_{EHMC} the (APEX-modeled) pseudo-first-order degradation rate constant of EHMC. The time trend of MOBA (resulting from the budget of formation from EHMC and transformation) would be described by $[MOBA]_t = \eta_{MOBA}\, k_{EHMC}\, [EHMC]_o\, (k_{EHMC} - k_{MOBA})^{-1}\, [\exp(-k_{MOBA}\, t) - \exp(-k_{EHMC}\, t)]$, where $[MOBA]_t$ is the concentration of MOBA at the time t and the other variables have been already defined (Ruggeri et al., 2014). It was also assumed that $[MOBA]_o = 0$ M.

It is possible to make some assumptions on the possible value of k_{MOBA} by hypothesizing that MOBA only reacts with •OH ($k°_{MOBA}$), that $k'_{MOBA} = 2\, k_{EHMC}$ (that is, MOBA is twice more photolabile than EHMC), as well as intermediate cases. With surface-water conditions that middle-way in the plot of Fig. 2a (5 mg C L^{-1} DOC, 5 m depth, 0.1 mM nitrate, 1 μM nitrite, 1 mM bicarbonate and 10 μM carbonate) one gets $k_{EHMC} = 3.3$ SSD^{-1} and $[\cdot OH] = 1.6\cdot10^{-17}$ M. With these data, by assuming that MOBA reacts with •OH with a near-diffusion second-order reaction rate constant ($2\cdot10^{10}$ M^{-1} s^{-1}), one obtains $k°_{MOBA} = 0.01$ SSD^{-1} and $k'_{MOBA} = 6.6$ SSD^{-1}. Fig. 2b reports the time trends (SSD units) of the concentrations of EHMC and MOBA, referred to the above water conditions and for different values of k_{MOBA} (ranging from $k°_{MOBA}$ to k'_{MOBA}). The plot shows that $[MOBA]_t \le 0.02\, [EHMC]_o$, which could limit the possible environmental impact of MOBA. However, this possible conclusion is strongly questioned by toxicity measurements (vide infra).

3.2. Identification of the TPs of EHMC upon direct photolysis

Direct photolysis experiments were performed by subjecting an aqueous solution of EHMC (4 mg L^{-1}) to UVA and UVB radiation. The UVB radiation was more effective than UVA to degrade the sunscreen, as shown by the EHMC time trend reported in Fig. 3: half-life time passed from 250 min under UVA to 30 min under UVB. After 4 h of irradiation, EHMC was almost completely degraded under UVB irradiation, while only 50% was eliminated under UVA.

3.2.1. MS^n spectra of EHMC

EHMC was detected as $[M + H]^+$ 291.1952 in ESI positive mode. The MS^n study provided useful information to identify unknown TPs formed through the degradation process. The product ions generated from the

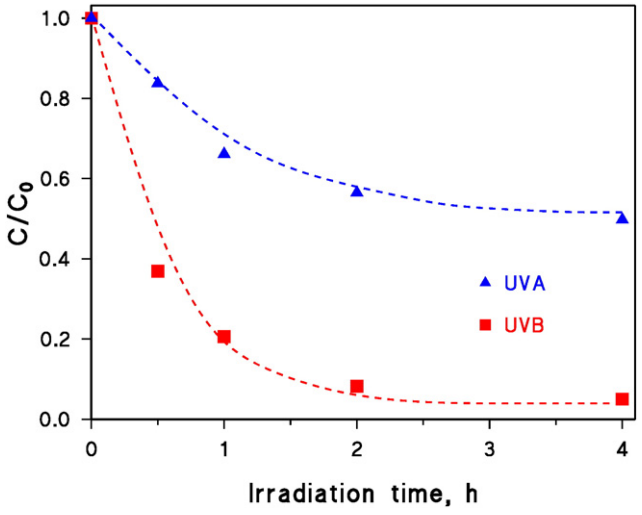


Fig. 3. EHMC degradation under UVA or UVB lamp.

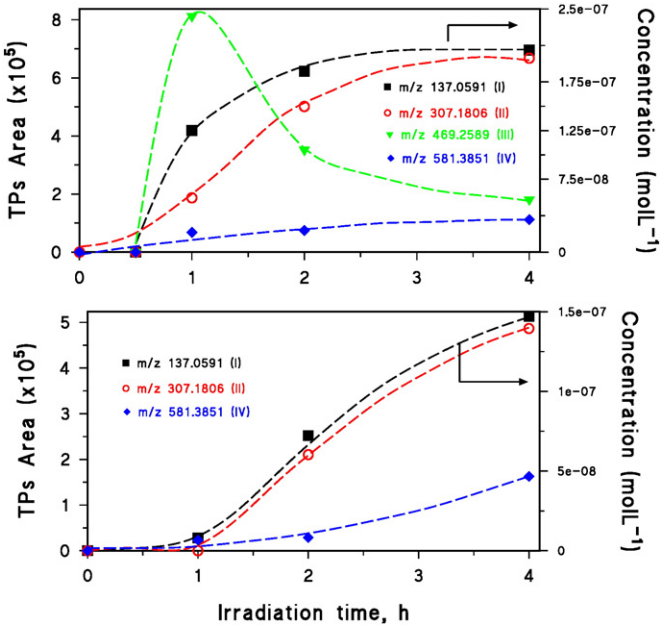


Fig. 4. Transformation products formed from EHMC over time under direct photolysis. The figure on the top shows the transformation products (TPs) formed under UVB, while in the bottom figure, the TPs formed under UVA are shown.

protonated molecular ions of EHMC are listed in Table S1 and a pattern of fragmentation for EHMC is shown in Scheme S1. The MS^2 spectrum shows the formation of two product ions at 179.0701 *m/z*, due to the loss of the alkyl chain, and at 161.0594 *m/z*, coming from the combined loss of the alkyl chain and a water molecule. These routes have been carefully considered in the structure attribution of the unknown compounds presented below.

3.2.2. Transformation products

Following EHMC degradation, several TPs were identified. In samples subjected to UVB radiation, the species at *m/z* 137.0591 (named **I**), 307.1806 (**II**), 469.2589 (**III**) and 581.3851 (**IV**) were identified. In the samples subjected to UVA radiation, only the species **I**, **II** and **IV** were detected. The detected intermediates are listed in Table 1, while their evolution over time is plotted in Fig. 4. In both cases, the most abundant TP was compound **I**.

HRMS analysis allowed the attribution of empirical formulas to these species, which were then characterized by MS^n spectra interpretation.

The species 137.0591 *m/z* (named **I**) was assumed to be the 4-methoxybenzaldehyde (MOBA), as already reported in the literature (Deblonde et al., 2011), which was confirmed by injection of a standard solution. The species **II** with 307.1806 *m/z* was attributed to monohydroxylated EHMC; the proposed pattern of fragmentation is shown in Scheme 1. The product ion at 195.0660 *m/z* results from the loss of an unmodified alkyl chain (C_8H_{16}), while the product ion 135.0442 *m/z* was formed through the combined loss of alkyl chain and acetic acid. Finally, 177.0551 *m/z* derives from the detachment of the alkyl chain and a molecule of water. Following the MS^n experiments, it was possible to recognize that hydroxylation involves the left side of the molecule and, in particular, we tentatively placed the hydroxyl group on the α carbon atom.

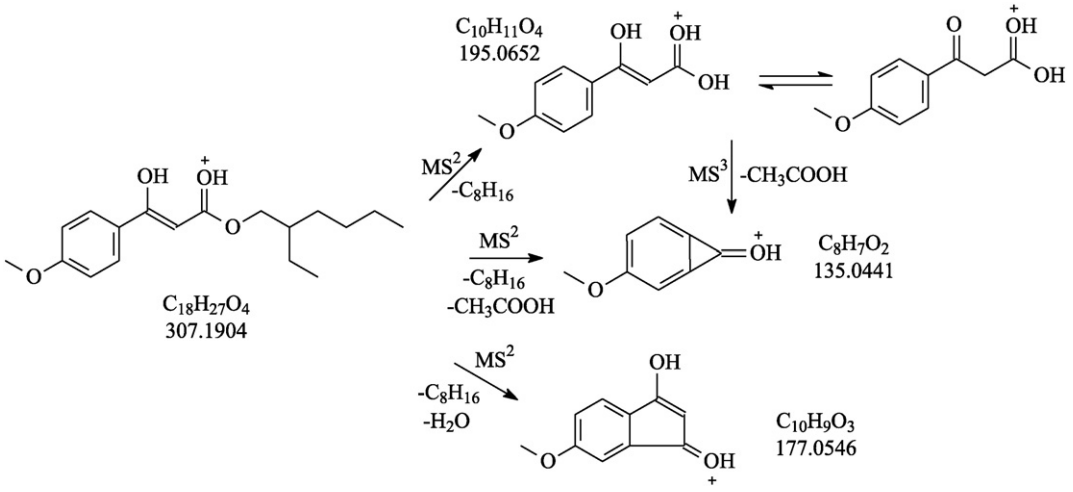
The other two TPs, named **III** and **IV**, involved an EHMC dimerization. TP with 581.3851 *m/z* (**IV**) with empirical formula $C_{36}H_{53}O_6$ and TP with 469.2589 *m/z* (**III**) with empirical formula $C_{28}H_{37}O_6$ are well-matched with the dimerization products already documented in the literature (Rodil et al., 2009). The hypothesized fragmentation pattern for TP **III** is illustrated in Scheme 2.

Interestingly, the dimerization of EHMC under UV irradiation has been reported in several studies (see for instance Hanson et al. (in press), and references therein). Curiously, we did not detect photoisomerization of EHMC, differently from previous reports, but it should be pointed out that our irradiation experiments were carried out in water, while the photoisomerization pathway is favored in organic solvents. In contrast, the aqueous solution favors alternative pathways that have only been marginally characterized in previous studies (Hanson et al., in press), and that the present work helps in elucidating. Also interestingly, the photostability of EHMC in organic solvents is attributed to the short lifetime of its excited states, and particularly of the triplet one (Kikuchi et al., 2014). However, triplet states in aqueous solution could react with the solvent to produce water adducts and, finally, hydroxy derivatives. For instance, in the case of anthraquinone-2-sulphonate, the reaction of its triplet state with water is so fast as to prevent the formation of $^1O_2$ from the triplet state itself and dissolved oxygen (Bedini et al., 2012). In the case of EHMC, the detection of several hydroxy derivatives upon direct photolysis in aqueous solution could be compatible with (although not making compelling evidence for) such a reaction with the solvent.

By applying the ECOSAR software to EHMC and to compounds **I–IV** it was possible to get some insight into the possible effects of the transformation intermediates on aquatic organisms, compared to the parent molecule (see Table S2(SM) for the detailed ECOSAR results). An interesting issue is that **I** (MOBA), the most abundant intermediate as far as peak area is concerned, is predicted to be much less toxic than EHMC considering all acute and chronic endpoints. Note, however, that ECOSAR predicts toxicity for fish, daphnid and algae. Quite different results were obtained in the case of *V. fischeri* bacteria (vide infra). The toxicity of **II** and **III** would be comparable to that of EHMC but a bit

Table 1
Transformation products formed during direct photolysis or heterogeneous photocatalysis.

$[M + H]^+$ and empirical formula	Number	Isomer	Δmmu	t_R (min)
137.0591 ($C_8H_9O_2$)	**I**	–	−0.656	17.3
307.180 ($C_{18}H_{27}O_4$)	**II**	**307 B**	−0.954	32.1
307.1806 ($C_{18}H_{27}O_4$)	**V**	**307 A**	−0.954	25.7
469.2589 ($C_{28}H_{37}O_6$)	**III**	–	0.935	31.9
581.3851 ($C_{36}H_{53}O_6$)	**IV**	–	1.404	40.9
179.0703 ($C_{10}H_{11}O_3$)	**VI**	–	−489	35.9
323.1850 ($C_{18}H_{27}O_5$)	**VII**	**323 A**	−0.270	20.4
323.1850 ($C_{18}H_{27}O_5$)	**VIII**	**323 B**	−0.270	24.4
323.1850 ($C_{18}H_{27}O_5$)	**IX**	**323 C**	−0.270	29.6
305.1732 ($C_{18}H_{25}O_4$)	**X**	**305 A**	−1.546	27.9
305.1732 ($C_{18}H_{25}O_4$)	**XI**	**305 B**	−1.546	29.6



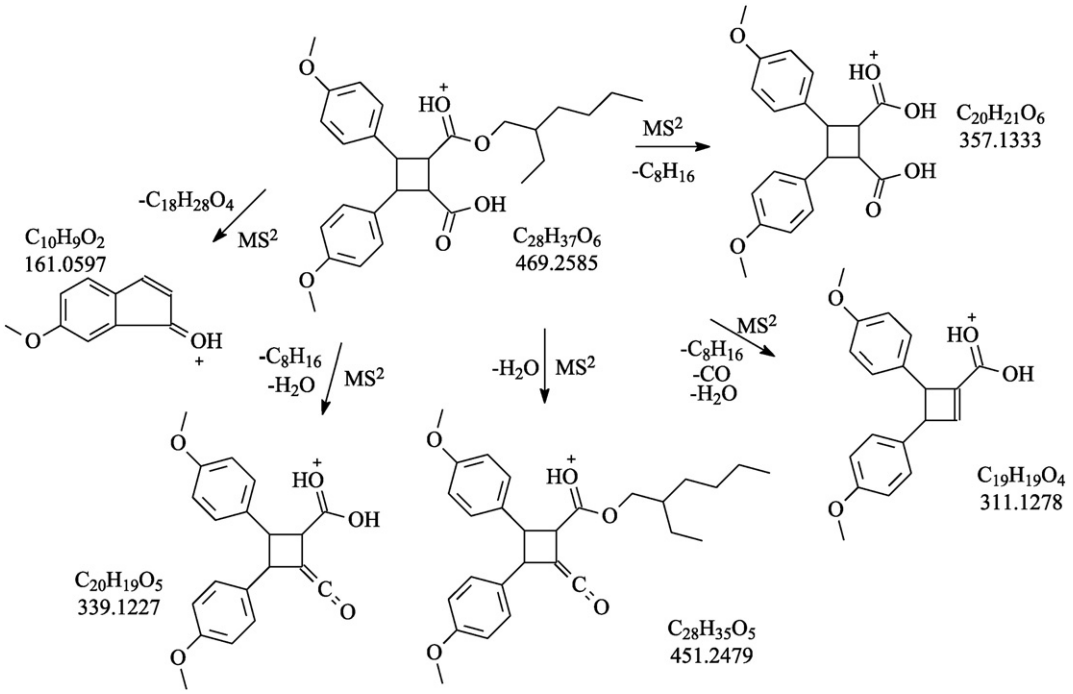
Scheme 1. Proposed fragmentation pathway for 307.1904 *m/z* (**II**).

lower (particularly in the case of **II**). The predicted LC50, EC50 and ChV values for these compounds would be in the ppb to ppm range. Finally, **IV** is predicted to be significantly more toxic than EHMC for aquatic organisms (all endpoints concerned). For both EHMC and compounds **II**–**IV**, the highest acute effects are expected for algae and the highest chronic effects for fish. In the case of **IV**, it should be considered that it is a dimeric species and that its formation from EHMC would strongly depend on the concentration of the parent compound. To allow easier identification of the intermediates, the used concentration of EHMC (4 mg/L) was considerably higher than its usual levels in surface waters (Magi et al., 2013; Poiger et al., 2004; Rodil and Moeder, 2008), where the probability to produce **IV** through direct photolysis would be much lower than in the investigated laboratory systems.

3.3. Heterogeneous photocatalysis

The addition of titanium dioxide increased the degradation rate of EHMC under UVA and its half-life time was shortened to 40 min, as shown in Fig. 5. Analyzing the formation of TPs, their time-trend profiles under heterogeneous photocatalysis are presented in Fig. 5. Species **I** and **II**, already detected through the direct photolysis process, were formed, while **III** and **IV** were absent. **I** was the main TP, as already reported under direct photolysis. Besides, the photocatalytic process triggered new pathways and seven additional TPs were identified. The species at 179.0703 *m/z* (named **VI**) showed a maximum after four hours of irradiation, then it decreased and it was eliminated within 16 h. MS^2 and MS^3 spectra analyses allowed its identification as 4-methoxycinnamic acid, confirmed by injection of a standard solution. Data are reported in Table S1, while the proposed fragmentation pathways are shown in Fig. S1.

An additional monohydroxylated derivative was formed, named **V**. Its MS^2 spectrum presents the product ions 179.0708 and 161.0600 *m/z*. Interestingly, the same ions were formed in the MS^2 spectrum of EHMC, and they are well-matched with the loss of the alkyl chain carrying a hydroxyl group. These ions allowed locating the hydroxyl group on the alkyl chain (see Fig. S2).



Scheme 2. Fragmentation pattern of the dimeric species 469.2589 (**III**).

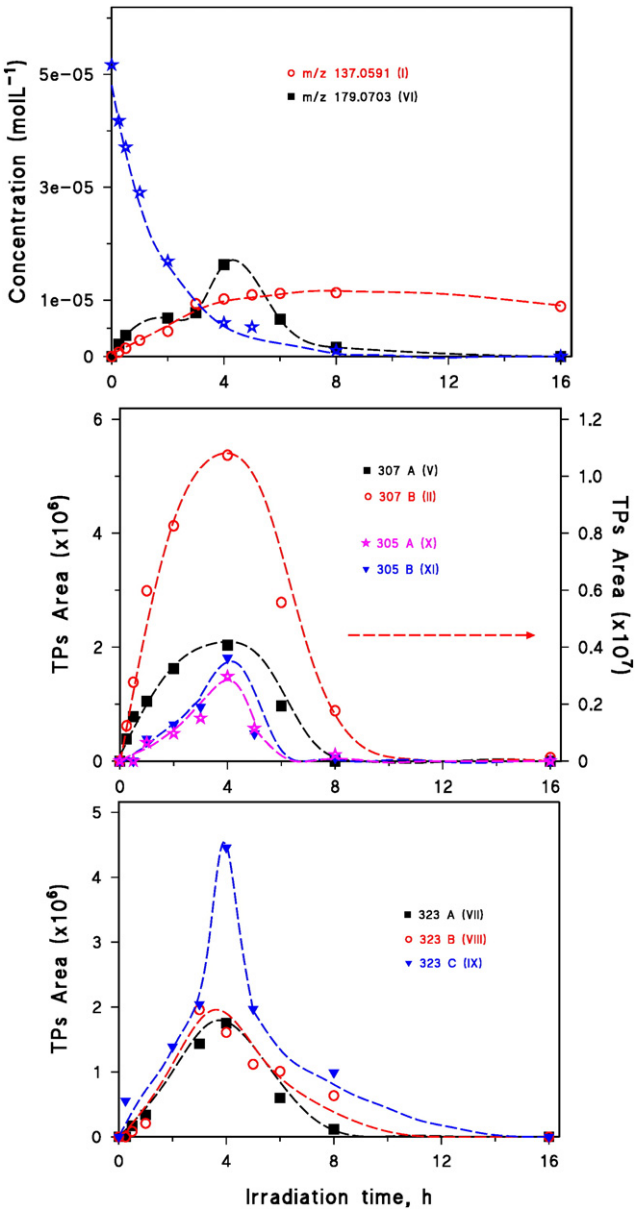


Fig. 5. EHMC degradation and transformation products formation profile over time in the presence of TiO_2. The top two figures show the most abundant TPs, while in the bottom one the less abundant TPs are shown.

Three isobaric species (named **VII–IX**), with 323.1850 *m/z* and molecular formula $C_{18}H_{27}O_5$ were found, well-suited to a double EHMC hydroxylation. The MS^2 and MS^3 spectra showed the formation of the product ions summarized in Table S1, while their patterns of fragmentation are shown below in Scheme 3. Considering isomer **A** (named **VII**), the presence of the structural-diagnostic ion at 161.0597 *m/z* in the spectrum of MS^2, resulting from the loss of the alkyl chain plus two oxygen atoms, suggests that the alkyl chain has undergone transformation.

Conversely for the isomers **B** and **C**, the formation of the product ion with 211.0610 *m/z* in the MS^2 spectrum, consistent with the loss of the unmodified alkyl chain, suggests that both hydroxylations are located on the left part of the molecule. For isomer **B** (named **VIII**), the MS^3 spectrum presents the other structural-diagnostic ion 121.0281 *m/z*, formed through a concerted loss of 2-hydroxypropenoic acid and water, which assesses the presence of a hydroxyl group on this moiety. Therefore, the occurrence of more than one hydroxylation on the aromatic ring can be excluded. For isomer **C** (named **IX**) the MS^3 spectrum presents the structural-diagnostic ion 135.0437 *m/z*, which allows the location of one of the two hydroxyl group on the carbon in positions β to the aromatic ring. The loss of formic acid, which gives rise to the ion 165.0550 *m/z*, further supports this hypothesis. The second OH group could be located in carbon α or on the aromatic ring.

Two isobaric species with 305.1732 *m/z* and molecular formula $C_{18}H_{25}O_4$, labeled **X** and **XI**, were identified and attributed to monohydroxylated/oxidized derivatives. The MS^2 spectra for both isomers allowed the proposal of oxidation on the alkyl chain, due to the presence of ions 161.0601 and 179.0709 *m/z*. Unfortunately, there is not enough information to properly locate the oxidized moiety.

A definite chemical formula could thus be suggested for the intermediates **VI** and **VIII**, and the ECOSAR software could be used to get insight into their possible acute and chronic toxicity. For all the considered endpoints, all these intermediates (and most notably **VI**) are expected to be much less toxic than the parent EHMC.

3.4. EHMC transformation pathways

All the characterized TPs resulting from EHMC photoinduced degradation could be linked together according to the pathways summarized in Fig. 6.

The identified TPs could be formed following six concomitant pathways: routes **A** and **F**, involving EHMC dealkylation; paths **B** and **C**, following mono or di-hydroxylation, as well as paths **D** and **E**, involving the molecule dimerization and occurring only through direct photolysis.

Route **A**, leading to the formation of MOBA, was the main transformation pathway under both direct photolysis and heterogeneous photocatalysis. The path **B**, involving mono hydroxylation, seems to play an important role in both photolysis and photocatalysis processes, with the predominant formation of the species **II**. The formation of 4-methoxycinnamic acid via dealkylation (path **F**) is a favored process, too. Taking into account the temporal evolution profiles, it can be assumed that dihydroxylated species (**VII**, **VIII** and **IX**) and hydroxylated/oxidized species (**X** and **XI**) resulted from the degradation of monohydroxylated TPs.

3.5. Toxicity assessment

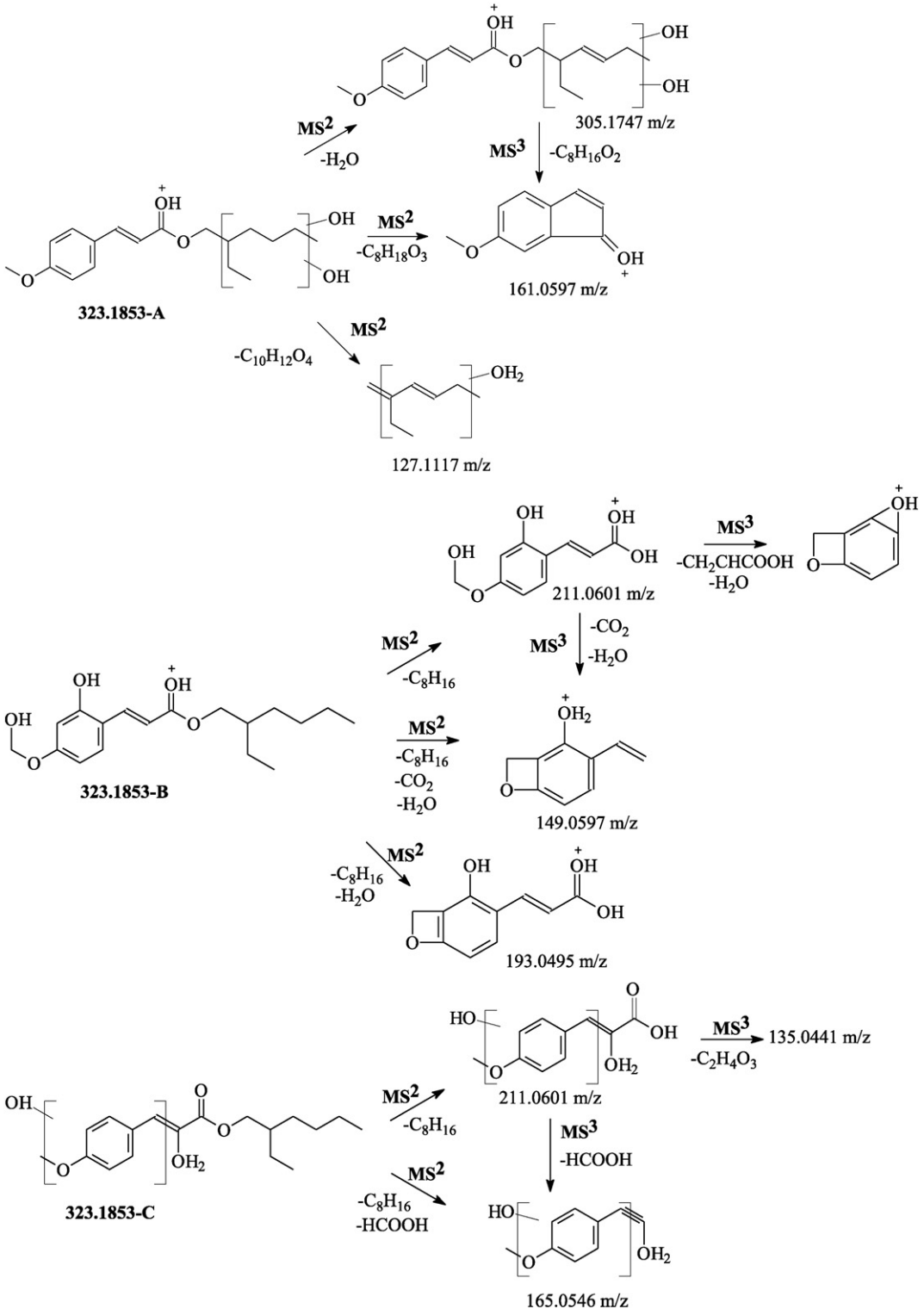
Acute toxicity was evaluated by monitoring changes in the natural emission of the luminescent bacteria *V. fischeri* when challenged with toxic compounds, and it was expressed as percentage of inhibition of the bacteria luminescence. Results obtained on samples subjected to heterogeneous photocatalysis are plotted in Fig. 7.

While EHMC was scarcely toxic (30% luminescence inhibition), its transformation proceeded through the formation of harmful compounds, as shown by the percentage of inhibition effect that increased as a function of the irradiation time. Indeed, acute toxicity increased up to 70–80% from 1 h onward and it remained almost stable for up to 24 h irradiation. This shape exhibits a good overlap (at least up to 8 h irradiation) with the time evolution of compound **I** (MOBA), which is more toxic than EHMC ($EC_{50} = 2.5$ mg/L) and seems to be the TP mainly responsible for the acute toxicity observed at $t_{irradiation} \leq 8$ h. This is very interesting when considering the relatively low transformation yield of EHMC into MOBA (see Fig. 5).

Another issue is that, while MOBA is quite toxic to *V. fischeri* bacteria, its modeled toxicity for higher organisms was pretty low. Therefore, its environmental impact might be very species-specific.

4. Conclusions

The results obtained suggest that the direct photolysis is the main removal route for EHMC in the aquatic environment, while the indirect photochemistry plays a negligible or minor role (degradation by ^{3}CDOM* could be somewhat important in deep and DOM-rich



Scheme 3. Fragmentation pathways of the species with 323.1850 *m/z* (**VII**, **VIII** and **IX**).

environments). The quantified intermediate **I** (MOBA) is formed in relatively low yield (1.6%) upon EHMC direct photolysis, but this issue does not necessarily imply a low environmental impact. The characterization of transformation products performed via HPLC–HRMS showed that EHMC is transformed into four TPs under UVB irradiation. Through heterogeneous photocatalysis, additional TPs were formed and identified: they may also be formed in aquatic systems.

The toxicity of the irradiated mixtures was directly measured with the Microtox assay. Moreover, by applying the ECOSAR software to EHMC and the identified TPs it was possible to get some insight into their possible effects on aquatic organisms (fish, daphnid, algae). The intermediate MOBA is more toxic than EHMC towards bacteria and it accounts for an important fraction of the measured toxicity at relatively short irradiation times, which is remarkable given its low formation yield from EHMC. However, MOBA might not be very toxic for higher

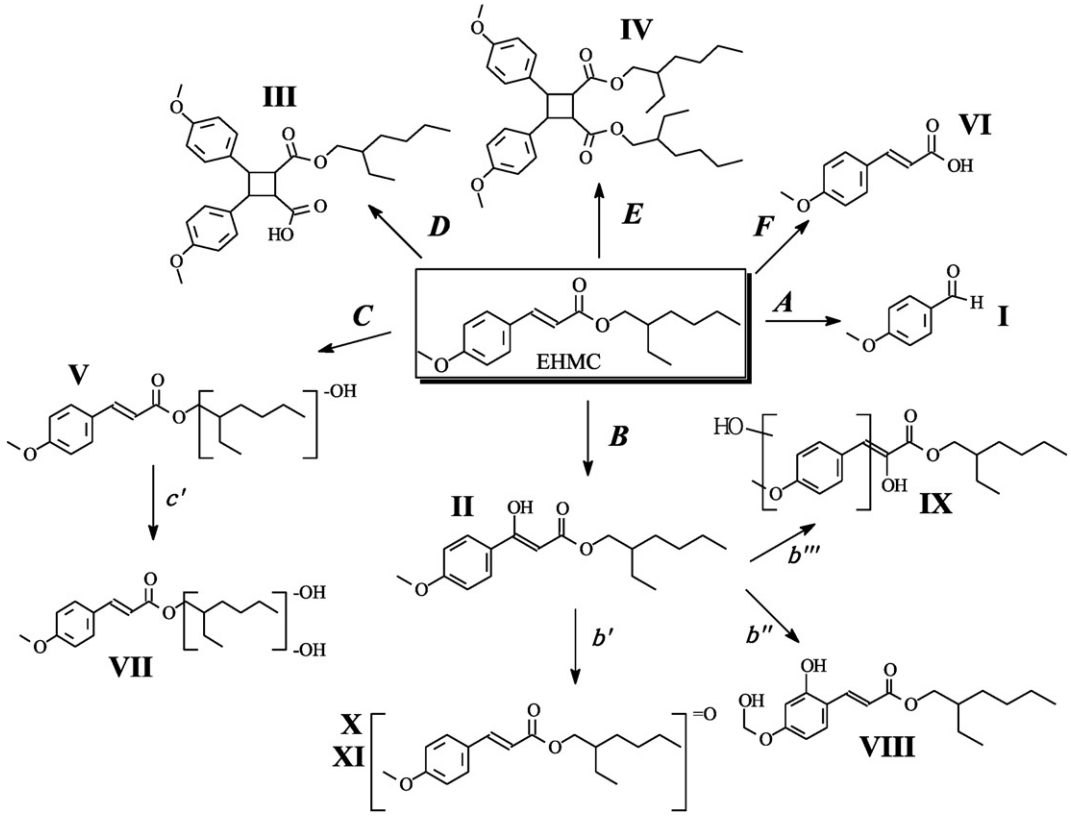


Fig. 6. Proposed photoinduced transformation pathways followed by EHMC.

organisms. The compound **IV** could be very toxic to aquatic organisms, but as a dimeric species its formation would presumably require much higher substrate concentrations than those actually found in natural surface waters.

Acknowledgments

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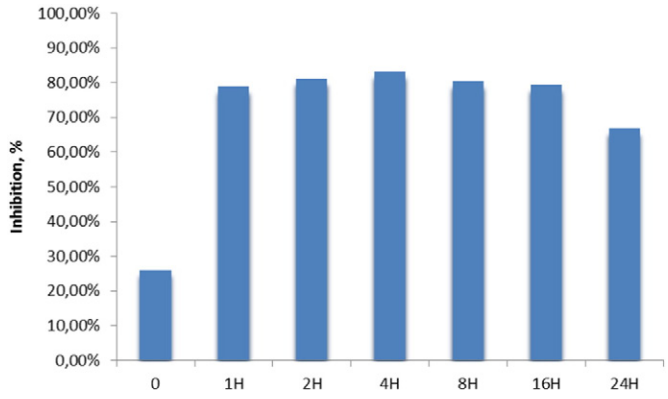

Fig. 7. Acute toxicity of EHMC as a function of irradiation time in the presence of TiO_2.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.08.002.

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OCTOBER 12, 2012
HOW DOES OCTINOXATE DEGRADE AVOBENZONE?
PERSONAL/INSPIRATIONAL
BY NICKI ZEVOLA BENVENUTI.
https://www.futurederm.com/how-does-octinoxate-degrade-avobenzone/

Our math is slightly more complicated than elementary addition!
In the past, I've briefly touched on the subject that octinoxate degrades avobenzone. Specifically, in Are Inorganic Sunscreens Better Than Organic Ones? Part III: Toxicity and Spotlight On: Vitamin B3 (Niacinamide and Nicotinic Acid), readers have seen the phrase "2 + 2 addition of cinnamates and alkenes" repeatedly used to explain this phenomenon. And in subsequent weeks, I've received many comments and messages inquiring about what exactly does this math mean? Therefore, this post will document what happens when octinoxate and avobenzone come together.

"But didn't I learn this in elementary school?"

Top row: 2 + 2 addition of cinnamates
Bottom row: 2 + 2 addition of alkenes
Well, the "2 + 2 addition of cinnamates and alkenes" is a bit more complicated than adding two groups of fruits or vegetables.

But getting back on topic, this phrase contains two elements: "2 + 2 addition of cinnamates" and "2 + 2 addition of alkenes." The first refers to when an octinoxate molecule forms a dimer with another octinoxate molecule. The resulting structure or dimer does not allow the octinoxate molecules to be photostable and therefore, meaningfully absorb UVB light. The top row shows the two main dimers that are formed from this "2 + 2 addition of cinnamates."

The second element, the "2 + 2 addition of alkenes" is when an octinoxate molecule binds to the double bond of the dominant form of avobenzone, resulting in the formation of a cyclobutane, which then undergo ring opening to form structures that don't allow either the octinoxate or avobenzone molecule to properly function. One of these structures can be seen in the bottom row of the picture shown.

As you can see, this degradation is caused by a structural transformation that's precipitated by the presence of the UV filter octinoxate. Furthermore, note that these two reactions only occur in the presence of UV light. Therefore, if your sunscreen contains both octinoxate and avobenzone, you don't have worry about them interacting in their container, assuming that it's completely opaque. But, you do still have to worry about this interaction after you actually apply the sunscreen!

"Okay that makes sense. But wouldn't photostabilizers like octocrylene reduce or even eliminate this octinoxate-induced degradation of avobenzone?

To answer this question, we need to understand how photostabilizers like octocrylene bring stability to avobenzone in the presence of UV light. When avobenzone absorbs a photon of UVA light, its electron goes into a triplet energy state. But avobenzone itself has no way of dissipating or quenching this excited electron. That's where octocrylene comes in. It accepts this "excited" energy, which allows avobenzone to return to its previously ground or "un-excited" state, where it's ready to receive another photon of UVA light. If there is no photostabilizer present, the excited electron will either destroy the avobenzone molecule, or avobenzone will pass it to whatever is nearby including the lipid bilayers of the skin, resulting in the generation of reactive oxygen species (free radicals), which leads to oxidative damage in the form of lipid peroxidation.

Now, as I stated in the last section, octinoxate degrades avobenzone by changing its very structure, which only occurs in the presence of UV light. Therefore, octocrylene would have some positive effect on the octinoxate-induced degradation of avobenzone because at times, it'd be able to accept the "excited" energy; other times, octinoxate accepts the energy first, leading to the inevitable "2 + 2 addition of alkenes."

A metaphor for all of this would be if there are hundreds of three-man squads– comprised of Privates Avobenzone, Octocrylene, and Octinoxate; trying to disarm a field of landmines (photons of UVA light). Only Pvt. Avobenzone knows how to disarm the mines. But by himself, Pvt. Avobenzone would blow up after a few successful disarmaments because he would tire and no longer move fast enough; land mines have timers, right? But with Pvt. Octocrylene at his side, the two would work as a team and disarm the mines without loss of life.

So how would Pvt. Octinoxate fit into the metaphor? He'd be the renegade soldier that binds Pvt. Avobenzone's and his hands together. So what good would Pvt. Octocrylene do then? He has no one to assist anymore. The landmines would then explode. Across the hundreds of squads, sometimes Pvt. Octinoxate would succeed in sabotaging the disarming process, and sometimes he would fail.

While this metaphor is far from perfect, (I mean how can 1 out of every 3 soldiers be a renegade?) I hope it gives you a better idea of what I'm talking about.

"Okay it does. But then why would manufacturers even include octinoxate and avobenzone in the same formulation?"

While the non-sunscreen ingredients are impressive and the packaging beautiful, I wouldn't recommend sunscreens like this because it contains both avobenzone and octinoxate, not to mention that it's expensive. Sorry Estee Lauder!

Here's where it gets a bit less certain. I can only speculate on why formulators include both ingredients; there are no hard facts.

They are lazy, uninformed, and/or simply don't care. This is the most unlikely reason, but it can't be ruled out.
Because avobenzone is the only organic UV filter approved in the US to adequately absorb UVA rays of all wavelengths, and because octinoxate is the most potent and effective UVB-absorbing organic filter, they include both because it's the most cost-effective combination.
They figure that because the octinoxate-induced degradation of avobenzone doesn't occur often enough to result in an overall or net loss in the level of protection that avobenzone and octinoxate inherently provide, it's preferable to use that combination rather than something with avobenzone and another less-potent UVB filter like homosalate.
They've found a way to prevent the octinoxate and avobenzone molecules from ever coming into contact with each other. This may be possible, though I doubt it since both molecules are so similar in terms of solubility; they're both very oil-soluble. Furthermore, why would manufacturers put in the extra effort to have these two similar compounds separated? They'd have to have the formulation be an oil-water-oil emulsion, which isn't very easy or cost-effective to make. Furthermore, how can they guarantee that these compounds stay separated during the most crucial time: once they've dried and set on the skin, which of course is exposed to UV light?
They assume that all their consumers follow the 2-hour reapplication rule decreed by the various dermatologic and medical associations. "So who cares if products aren't that photostable, since they're supposed to be reapplied every 2 hours anyways?" Obviously, very few consumers actually follow that rule. And like I've stated in the comments section of Are Inorganic Sunscreens Better than Organic Ones? Part IV: Level of Protection, and Practicality, the 2-hour rule was created because these medical associations want to cover all the bases. They know that there is such a heterogeneity or variety of behavioral and formulation-related aspects that factor into how much protection the average person achieves. Therefore, it'd be irrational for manufacturers to use this reason to justify the inclusion of octinoxate and avobenzone in a single formulation. But again, it can't be ruled out.
Finally, perhaps octinoxate is more cosmetically acceptable, cheaper, and/or easier to obtain than other organic UVB filters. I highly doubt that this is true since you can find UV filters in products from all price ranges. But once again, I can't completely rule this out.
"Okay that was a lot of information. What should I take away from this post?"

Octinoxate degrades itself and avobenzone via 2 + 2 addition of cinnamates and alkenes.
While photostabilizers like octocrylene do mildly reduce this octinoxate-induced structural degradation of avobenzone, but why would you want to deal with this anyways?

Because there are so many unknowns when considering the reason behind why octinoxate is used in conjunction with avobenzone, look for sunscreens that don't contain both octinoxate and avobenzone. It's just one less thing to worry about. Thank you for reading through this rather dense post!

31) Int J Dermatol. 2008 Nov;47 Suppl 1:35-7. doi: 10.1111/j.1365-4632.2008.03957.x.
Photoallergic contact dermatitis caused by ultraviolet filters in different sunscreens.
Collaris EJ1, Frank J.
Abstract
Over the last decade, a change in the public awareness regarding the possible danger of excessive sunlight exposure has resulted in an increased consumption of sunscreens. These products contain a broad spectrum of putative sensitizers that can cause contact dermatitis and, upon exposure to ultraviolet (UV) irradiation, photocontact dermatitis. Among these sensitizing compounds, UV filters are the most frequent cause of photoallergic reactions. Although rarely observed, we here describe the occurrence of a photoallergic contact dermatitis in a 55-year-old man after the use of two different sunscreens. Photopatch testing showed hypersensitivity reactions of the delayed type against three different chemical UV filters, 4-tert-butyl-4- methoxy-dibenzoylmethane (Parsol 1789), 2-ethylhexyl-p-methoxycinnamate (Parsol MCX), and isoamyl-p-methoxycinnamate (Neoheliopan).

32) Dermatology. 1998;196(3):354-7.
Photoallergic contact dermatitis due to combined UVB (4-methylbenzylidene camphor/octyl methoxycinnamate) and UVA (benzophenone-3/butyl methoxydibenzoylmethane) absorber sensitization.
Schmidt T1, Ring J, Abeck D.
Abstract
In a 71-year-old male Caucasian patient with persistent eczema on light-exposed skin, photocontact allergy was demonstrated to the UV filter substances 4-methylbenzylidene camphor (UVB), octyl methoxycinnamate (UVB), benzophenone-3 (UVA) and butyl methoxydibenzoylmethane (UVA) present in sunscreen products used by the patient over several years. A significantly reduced UVB sensitivity of 25 mJ/cm2 in this patient (normal minimal erythema dose in our laboratory = 70-130 mJ/cm2) was considered an early indication of a persistent light reaction. Topical anti-inflammatory treatment over 2 weeks together with consequent application of a sunscreen containing Mexoryl SX/titanium dioxide led to complete remission. Taking into account the widespread use of the above UV filter substances not only in sun protection products, but also in cosmetics such as antiaging lotions and day care products, the possible risk of allergy to these chemicals has to be taken seriously. The substitution of known photocontact sensitizers in UV filters by photostable compounds and detailed product information are the basis of preventive strategies.

33) Photodermatol Photoimmunol Photomed. 2006 Aug;22(4):189-92.
Causal agents of photoallergic contact dermatitis diagnosed in the national institute of dermatology of Colombia.
Rodríguez E1, Valbuena MC, Rey M, Porras de Quintana L.
Abstract
OBJECTIVE:
To describe and identify the photoallergens causing photoallergic contact dermatitis in the population attending the outpatient clinic of the Centro Dermatologico Federico Lleras Acosta (CDFLLA), the National Institute of Dermatology of Colombia.
MATERIALS AND METHODS:
Eighty-two patients with clinical diagnosis of photoallergic contact dermatitis enter the study. These patients attended the CDFLLA between August 2001 and May 2003. Photopatch tests were performed using the standard series of sunscreens (Chemotechnique Diagnostics) and 6-methylcoumarin. Cetyl alcohol, phenoxyethanol, methylparabene, propylene glycol, triethanolamine, propylparabene, trichlorocarbanilide and dichromate were also included. The allergens were applied in duplicate on the healthy skin of the back and covered with opaque tape withdrawn 24 h later, the panel on the right was irradiated with an ultraviolet A dose of 5 J/cm(2). The tests were read 24 h after the application of the allergens, 24 and 72 h post-irradiation. The readings were assessed according to the visual scoring system recommended by the International Contact Dermatitis Research Group.
RESULTS:
Twenty-six patients (31.7%) showed positive photopatch test responses to one or several allergens. Four of them showed positive results to three components of the series and four patients to two components. Thirty-eight photoallergic and 18 allergic reactions were observed. Ultraviolet filters were the substances which more frequently produced positive photopatch test responses (30.5%). The most common ultraviolet filter photoallergen was benzophenone-3 with 22/82 positive results (26.8%), followed by octyl methoxycinnamate (8/82), benzophenone-4 and mexenone (2/82), phenylbenzimidazole sulphonic acid, methylbenziliden camphor and octyl dimethyl PABA (1/82). One patient showed a photoallergic response to 6-methylcoumarin. There was a concordance between the allergen which elicited the positive response and the use of different substances which contained that molecule among its compounds in 17 patients (65.3%). 19.5% of the patients (16/82) showed positive results to one or several allergens in the irradiated panel as well as in the unirradiated control site. These cases were diagnosed as contact allergy, probably caused by aeroallergens, presenting a natural history and a clinical picture similar to photocontact allergy. The most common allergen was dichromate with 10 positive results.
CONCLUSIONS:

The results of this study confirm that sunscreens are the more frequently involved substances in photoallergic contact dermatitis in our population. Identification of the photoallergen is the key element for adequate disease control and patient education.

34) Am J Contact Dermat. 1998 Mar;9(1):42-4.
Sunscreen allergy in Singapore.
Ang P1, Ng SK, Goh CL.
Abstract
We report the epidemiology of sunscreen allergy over a period of 5 years at the National Skin Centre. A total of 61 patients with suspected allergy to sunscreen underwent patch or photopatch testing to our sunscreen series from 1992 to 1996. The results were retrospectively analysed and evaluated. Out of these 61 patients, 5 were found to have positive patch test reactions to sunscreens. 2 were photoallergic, and 3 were allergic to active ingredients in sunscreens. The main causative allergens were 2-ethylhexyl-4-methoxycinnamate (Parsol MCX) and 2-hydroxy-4-methoxybenzophenone (oxybenzone). We conclude that sunscreen contact allergy is uncommon in our practice

35) Br J Dermatol. 2001 Oct;145(4):597-601.
Photoallergic contact dermatitis is uncommon.
Darvay A1, White IR, Rycroft RJ, Jones AB, Hawk JL, McFadden JP.
Abstract
BACKGROUND:
Despite the enormous increase in sunscreen use, allergic contact (AC) and photoallergic (PA) reactions to ultraviolet (UV) filters are considered rare.
OBJECTIVES:
To analyse the data from 2715 patients who underwent photopatch testing at St John's Institute of Dermatology during the period 1983-98.
METHODS:
A retrospective analysis of all positive photopatch test episodes was undertaken with the results retrieved from the environmental dermatology database and further verified with the original archived patch test documentation for each individual patient.
RESULTS:
In 111 patients with positive reactions (4.1%), there were 155 AC or PA reactions to allergens in the photopatch test series. Eighty PA reactions were observed in 62 (2.3%) patients (32 men and 30 women, age range 28-75 years), with UV filters accounting for 52 positive reactions (65%), drugs 16 (20%), musk ambrette 11 (14%) and the antiseptic trichlorocarbanilide one (1%). The most common UV filter photoallergen was benzophenone-3 with 14 positive results, followed by benzophenone-10 (n = 9), isopropyl dibenzoylmethane (n = 6), p-aminobenzoic acid (PABA) (n = 5), octyl dimethyl PABA (n = 5), butyl methoxydibenzoylmethane (n = 4), isoamyl methoxycinnamate (n = 2), ethyl methoxycinnamate (n = 2), octyl methoxycinnamate (n = 2), amyl dimethyl PABA (n = 2) and phenylbenzimidazole sulphonic acid (n = 1). A similar number of AC reactions to UV filters was detected in this study. Thus 49 patients (1.8%) had a total of 75 reactions: 51 due to UV filters and 24 as a result of exposure to fragrances and therapeutic agents. Benzophenone-10 accounted for 13 AC reactions and benzophenone-3 for eight reactions. Twenty-two patients had a PA reaction alone, whereas 19 patients had chronic actinic dermatitis and 15 patients polymorphic light eruption (PLE) in addition. Thus, 34 of the 62 patients (55%) had a preceding underlying photodermatosis.
CONCLUSIONS:
These results show a low yield of positive photopatch tests. Thus, despite the large increase in the use of UV filters over the last decade, the development of PA reactions remains rare. Furthermore, most of the common UV filter photoallergens identified in this study, including PABA, amyl dimethyl PABA and benzophenone-10, are now rarely used in sunscreen manufacture, while isopropyl dibenzoylmethane was voluntarily removed from the market in 1993. Currently, benzophenone-3 is the commonest contact photoallergen still in widespread use. In contrast, the UVB filter octyl methoxycinnamate, used in a number of sunscreens, produced only two positive PA reactions in 12 years of testing. Nevertheless, although these reactions are extremely rare, patients with photodermatoses such as PLE and chronic actinic dermatitis do represent a group of patients at increased risk of developing photoallergy. Further photopatch test series should be regularly reviewed and updated, as the relevance of individual photoallergens changes over time. Currently, there is no evidence that PA reactions represent a common clinical problem.

36) Contact Dermatitis. 1997 Nov;37(5):221-32.
Contact and photocontact sensitivity to sunscreens. Review of a 15-year experience and of the literature.
Schauder S1, Ippen H.
Abstract
This review summarizes published and unpublished data of our 15-year experience with sunscreen allergy and photoallergy. From 1981-1996, 402 patients with suspected clinical photosensitivity were patch and photopatch tested with the commercial sunscreens and facial cosmetics that they had used and with chemical UV absorbers, fragrance materials, preservatives, and emollients. 80 patients (20%) (28 men, 52 women) demonstrated allergic and/or

photoallergic contact dermatitis to 1 or more UV absorber(s). In 47 patients with photodermatoses or photo-aggravated dermatoses and in 33 subjects with normal photosensitivity, 91 allergic and 84 photoallergic reactions to UV filters were observed. Over the years sunscreens were added to the test series, which since 1989 comprised the following 10 UV absorbers and which induced allergic (a) and photoallergic (pa) reactions (number, type of reaction): 4 UVA absorbers--isopropyldibenzoylmethane (30a/32pa); butyl methoxydibenzoyl-methane (15a/13pa); benzophenone-3 (3a/9pa); benzophenone-4 (0a/0pa); and 6 UVB absorbers--PABA (2a/2pa); octyl dimethyl PABA (1a/2pa); methylbenzylidene camphor (32a/5pa); octyl methoxycinnamate (3a/4pa); isoamyl p-methoxycinnamate (4a/10pa); and phenylbenzimidazole sulfonic acid (1a/7pa). The frequent (photo)sensitization to isopropyldibenzoylmethane was the reason that its production was discontinued in 1993. 47 patients reacted to fragrance materials, 11 to preservatives and 2 to lanolin alcohol. These constituents were contained in the commercial sunscreens and cosmetics that they had used. Continuous revision of the UV absorber photopatch test series was necessary to be closer to the real frequency of exposure and of reported (photo)allergy to newer sunscreens. Clinicians should consider contact and photocontact allergy, especially in patients with photodermatoses and photo-aggravated dermatoses, and they should perform photopatch testing. Once the culprit has been identified, its INCI (International Nomenclature Cosmetic Ingredients) designation should be given to the patient, who must be warned to avoid products containing the (photo)allergen.

37) Australas J Dermatol. 2001 Nov;42(4):257-9.
Report of 19 cases of photoallergic contact dermatitis to sunscreens seen at the Skin and Cancer Foundation.
Cook N1, Freeman S.
Abstract
We report on our experience with sunscreen allergy between 1992 and 1999 and also review the international literature on sunscreen allergy. There were a total of 21 allergic reactions to sunscreen chemicals observed in 19 patients over the 8 years. There were nine positive photopatch reactions to oxybenzone, eight to butyl methoxy dibenzoylmethane, three to methoxycinnamate and one to benzophenone. No positive reactions were observed to para aminobenzoic acid. Six patients also had positive patch tests to components of the sunscreen base. In our experience, sunscreen chemicals are the most common cause of photoallergic contact dermatitis.

38) Dermatitis. 2013 Jul-Aug;24(4):176-82. doi: 10.1097/DER.0b013e3182983845.
Patch test reactions associated with sunscreen products and the importance of testing to an expanded series: retrospective analysis of North American Contact Dermatitis Group data, 2001 to 2010.
Warshaw EM1, Wang MZ, Maibach HI, Belsito DV, Zug KA, Taylor JS, Mathias CG, Sasseville D, Zirwas MJ, Fowler JF Jr, DeKoven JG, Fransway AF, DeLeo VA, Marks JG Jr, Pratt MD, Storrs FJ.
Abstract
BACKGROUND:
Both active and inactive ingredients in sunscreen may cause contact dermatitis.
OBJECTIVES:
This study aimed to describe allergens associated with a sunscreen source.
METHODS:
A cross-sectional analysis of patients patch tested by the North American Contact Dermatitis Group between 2001 and 2010 was performed.
RESULTS:
Of 23,908 patients patch tested, 219 (0.9%) had sunscreen coded as an allergen source. Patients who were male, with occupational dermatitis, or older (older than 40 years) had significantly lower rates of allergic reactions to sunscreens; the most commonly affected areas were the face and exposed sites ($P < 0.0001$). The top 3 most frequent allergens in sunscreens were benzophenone-3 (70.2% for 10% concentration, 64.4% for 3% concentration), DL-alpha-tocopherol (4.8%), and fragrance mix I (4.0%). Less than 40% of positive patch test reactions were detected by the North American Contact Dermatitis Group screening series of 65 to 70 allergens.
CONCLUSIONS:
A supplemental antigen series is important in detecting allergy to sunscreens.

County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:11 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 4 of 9
Attachments: 39 Stein Environ Sci Process Impacts 2017 .docx; 40 Wang endocrine disruption of uv filters.pdf; 41 Gomez J Toxicol Environ Health A.docx; 42 Schlumpf 2001 article ED.pdf; 43 Balazs Hormal Activity of UV Filters.pdf; 44 Kunz Aquatic Toxicology Volume 79.docx; 45 Ozaez Ultraviolet filters differentially impact.pdf; 46 Kaiser Snails Environmental Pollution.docx; 47 Zucchi Global gene expression.pdf; 48 Schreurs Zebra Fish Asssay.docx; 49 Inui Effect of UV screens and preservatives.pdf; 50 Christen Effects of the UV-filter 2.pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

39) Environ Sci Process Impacts. 2017 Jun 21;19(6):851-860. doi: 10.1039/c7em00059f.

Photolysis and cellular toxicities of the organic ultraviolet filter chemical octyl methoxycinnamate and its photoproducts.

Stein HV1, Berg CJ, Maung JN, O'Connor LE, Pagano AE, MacManus-Spencer LA, Paulick MG.
Author information
1 Department of Chemistry, Union College, 807 Union St. Schenectady, NY 12308, USA. paulickm@union.edu macmanul@union.edu.

Abstract
Organic ultraviolet filter chemicals (UVFCs) are the active ingredients used in many sunscreens to protect the skin from UV light; these chemicals have been detected in numerous aquatic environments leading to concerns about how they might affect aquatic organisms and humans. One commonly used organic UVFC is octyl methoxycinnamate (OMC), better known by its commercial name, octinoxate. Upon exposure to UV light, OMC degrades rapidly, forming numerous photoproducts, some of which have been previously identified. In this study, we isolated and completely characterized the major products of OMC photolysis, including the two major stable OMC cyclodimers. One of these cyclodimers is a δ-truxinate, resulting from a head-to-head dimerization of two OMC molecules, and the other cyclodimer is an α-truxillate, resulting from a head-to-tail dimerization of two OMC molecules. Additionally, the cellular toxicities of the individual photoproducts were determined; it was found that the parent UVFC, OMC, 4-methoxybenzaldehyde, and two cyclodimers are significantly toxic to cells. The photoproduct 2-ethylhexanol is not cytotoxic, demonstrating that different components of OMC photolysate contribute differently to its cellular toxicity. This study thus provides an enhanced understanding of OMC photolysis and gives toxicity data that can be used to better evaluate OMC as a sunscreen agent.

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Review

Recent Advances on Endocrine Disrupting Effects of UV Filters

Jiaying Wang [1,2], Liumeng Pan [1], Shenggan Wu [3], Liping Lu [1], Yiwen Xu [1], Yanye Zhu [1], Ming Guo [4] and Shulin Zhuang [1,2,5,*]

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Academic Editor: Huixiao Hong
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Abstract: Ultraviolet (UV) filters are used widely in cosmetics, plastics, adhesives and other industrial products to protect human skin or products against direct exposure to deleterious UV radiation. With growing usage and mis-disposition of UV filters, they currently represent a new class of contaminants of emerging concern with increasingly reported adverse effects to humans and other organisms. Exposure to UV filters induce various endocrine disrupting effects, as revealed by increasing number of toxicological studies performed in recent years. It is necessary to compile a systematic review on the current research status on endocrine disrupting effects of UV filters toward different organisms. We therefore summarized the recent advances on the evaluation of the potential endocrine disruptors and the mechanism of toxicity for many kinds of UV filters such as benzophenones, camphor derivatives and cinnamate derivatives.

Keywords: ultraviolet filters; cosmetics; endocrine disrupting effects; nuclear receptor

1. Introduction

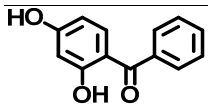
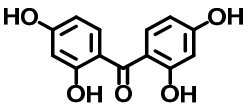
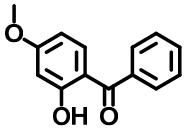
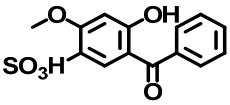
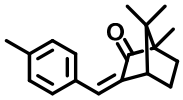
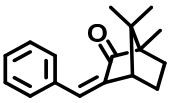
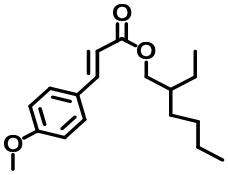
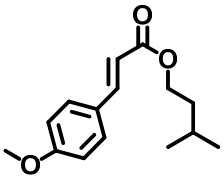
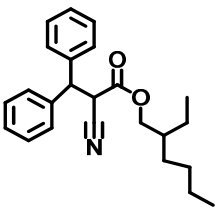
Ultraviolet (UV) filters are a class of chemicals that can absorb or reflect UV light in the ultraviolet A (UVA) range and ultraviolet B (UVB) range with specific wavelengths between 320 and 400 nm, 290 and 320 nm, respectively [1]. They can protect human skin against direct exposure to deleterious UV radiation [2,3]. Many kinds of organic UV filters were incorporated into cosmetics, plastics, adhesives and other industrial products to avoid potential UV-induced damage. There are 16 UV filters permitted to be used in cosmetics by the US Food and Drug Administration and 27 components permitted to be used in cosmetics by the EU Scientific Committee on Consumer Products [4].

Many UV filters were produced in large quantities and used widely [5]. Residues of UV filters have been detected in multiple environmental matrices including wastewater treatment plants, surface water, sewage sludge, river sediments, fish, human milk and placenta [6–9]. UV filters can be bioaccumulated in organisms due to their persistence, stability and lipophilicity [10,11]. They are now becoming contaminants of emerging concern [12–14]. UV filters were reported to induce acute toxicities, developmental toxicities and reproductive toxicities to different organisms [15–21].

Many kinds of UV filters (Table 1) have been identified as potential environmental endocrine disruptors [22]. There are an increasing number of studies on endocrine disrupting effects of UV filters.

Exposure to UV filters was increasingly reported to cause the disruption of the endocrine systems in many organisms such as rat, frog *Xenopus laevis*, Japanese quail (*Coturnix japonica*), *Chironomus riparius* (Meigen) and fish [22–31]. Considering increasing toxicological studies performed in recent years, a systematic compilation of current research status on their endocrine disrupting effects is necessary. We therefore compiled a state-of-the-art review on the endocrine disrupting effects of many type of UV filters (Table 2).

Table 1. The commonly used ultraviolet (UV) filters.

Compound	CAS No.	Chemical Structure	Kp (cm/h) *
BP-1	131-56-6		0.00917
BP-2	131-55-5		0.00458
BP-3	131-57-7		0.0271
BP-4	4065-45-6		0.0000511
4-MBC	36861-47-9		0.504
3-BC	15087-24-8		0.261
OMC	5466-77-3		0.264
IMC	71617-10-2		0.0477
OC	6197-30-4		0.549

BP: benzophenone; 4-MBC: 4-methyl benzylidene camphor; 3-BC: 3-benzylidene camphor; OMC: octyl methoxycinnamate; IMC: isopentyl-4-methoxycinnamate; OC: octocrylene. * Kp, the dermal permeability coefficient, calculated by Program (DERMWIN) v2.0, was calculated following the equation: Log Kp = −2.80 + 0.66 Log Kow − 0.0056 MW.

Table 2. Endocrine disrupting effects of the commonly used UV filters.

UV Filters	Endocrine Disrupting Effects		References
Benzophenones	**Estrogenic disrupting effects**	Activation of ERα, ERβ; Inhibition of the activity of 17β-Estradiol; Induction of proliferation of MCF-7 cell; Induction of VTG in fathead minnows; Reduce of the uterine weight in immature Long-Evans rats	[22,26,32–37]
	Androgenic disrupting effects	Antagonists of human AR transactivation; Repression of 4,5-dihydrotestosterone-induced transactivational activity; Inhibition of testosterone formation in mice and rats	[34,36–39]
	Disrupting effects toward other nuclear receptors	Inhibition of human recombinant TPO; Interference with THR; Inhibition of TPO activity in rats; Antagonists of PR	[38,40–42]
Camphor derivatives	**Disrupting effects toward estrogen receptor**	Activation of ERα, ERβ; Inhibition of the activity of 17β-Estradiol; Induction of proliferation of MCF-7 cell; Induction of pS2 protein in MCF-7 cells; Reduce of the uterine weight in rats; Induction of VTG in fish	[22,26,43–49]
	Disrupting effects toward androgen receptor	Repression of 4,5-dihydrotestosterone-induced transactivational activity; Inhibition of testosterone formation in HEK-293 cells; Antagonists of Human AR	[36,38,39,50]
	Disrupting effects toward progesterone receptor	Antagonists of PR; Increase of PR mRNA levels in rats; Inhibition of the expression of PR protein in rats; Disturbance of the expression of membrane-associate PR in insects	[38,47,51,52]
Cinnamate derivatives	**Disrupting effects toward estrogen receptor**	Activation of ERα; Inhibition of the activity of 17β-Estradiol; Induction of proliferation of MCF-7 cell; Reduce of the uterine weight in rats; Induction of VTG in fish	[22,36,43,45,48,49]
	Disrupting effects toward thyroid hormone receptor	Decrease of T4 level; Inhibition of the conversion of T4 to triiodothyronine in rats	[16,53,54]
	Disrupting effects toward other nuclear receptors	Antagonists of PR and AR; Inhibition of 4,5-dihydrotestosterone activity; Reduce of the prostate and testicular weight in rats	[16,36,38]

AR: androgen receptor; ER: estrogen receptor alpha; PR: progesterone receptor; T4: thyroxine; THR: thyroid hormone receptor; TPO: thyroid peroxidase; VTG: vitellogenin.

2. Endocrine Disrupting Effects of Typical UV Filters

2.1. Benzophenones

Benzophenone (BP)-type UV filters were used widely in many cosmetics for the protection of skin from UVA and UVB light [12]. Their molecular structures have a diarylketone scaffold with different substitute groups. Residues of BPs were detected in wastewater, surface water, soil, sediment, human urine and breast milk [55–58]. Many BPs were identified as endocrine disruptors and were involved in the disruption of the hypothalamic–pituitary–gonadal system [13,59]. As revealed by various in vivo, in vitro bioassays and in silico methods, BPs showed multiple endocrine disrupting effects toward estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR) and other nuclear receptors [22,32,38,39,60].

2.1.1. Estrogenic Disrupting Effects

BP-type UV filters could cause multiple estrogenic effects, developmental and reproductive toxicity as revealed by cell-based bioassay [26,33,34,43,61]. BP-3 was reported to cause a dose-dependent

increase of uterine weight of immature Long-Evans rats by the activation of ERα and ERβ [22,43]. BP-2 caused estrogenic effects such as vitellogenin (VTG) induction in fish [35]. BPs such as BP-1, BP-2, BP-3 and BP-4 caused estrogenic activity, developmental and reproductive toxicity to fish and rat [26,35,36,43]. BPs showed moderate potency to activate the proliferation of MCF-7 breast cancer cell and estrogen-responsive CHO cells at concentrations of the order of micromolar and lower [37,62]. This potency ranks as 4-hydroxy-benzophenone > 4,4′-dihydroxy-benzophenone > BP-8 > 2,3,4,4′-tetrahydroxy-benzophenone > BP-2 > 2,4, 4′-trihydroxy-benzophenone. However, there was no significant induction of proliferation induced by BP, BP-1, BP-3 and 2,3,4-trihydroxy-benzophenone. Molecular modeling can provide atomic-level information and has been well used to probe interactions of chemicals with biomacromolecules [39,63–66]. BPs interacted with residues Glu353, Arg394 or Phe404 of ERα ligand binding domain and such binding mode enhanced binding stability, contributing partly to their estrogenic activities [62].

Biotransformation or chemical transformation of BPs may have influence on their endocrine disrupting effects. BP-3 can be metabolized within human body and can be metabolized to various metabolites including BP-1 and BP-3. BP-1 was detected in human urine [37] and BP-1 and BP-8 were detected in rats [67,68]. BP-1 possessed higher estrogenic activity than that of BP-3 [32–34]. BPs were revealed to be converted to 4-hydroxybenzophenone after exposure to sunlight, indicating the potential estrogenic risk of BP-containing sunscreen in direct contact with the skin [62,69].

2.1.2. Androgenic Disrupting Effects

BP-1, BP-2 and BP-3 showed no agonistic activity toward AR [50]; however, they exhibited anti-androgenic activity in various cells-based bioassays [33,34,36,38]. BP-1, BP-2 and BP-3 showed complete inhibition of dihydrotestosterone activity in concentration-dependent mode. BPs disturbed the normal hormonal level of testosterone during male development of mouse and rat by inhibiting the conversion of androstenedione to testosterone [39,70]. BP-2 displayed antagonistic activity with non-monotonic dose–response curves. There were very weak or no inhibitory effects for BP-4, BP-7, BP-8 and BP-12 and weak effects for BP-2, BP-3 and BP-6 [39]. The androgenic disrupting effects of BPs were also affected by the biotransformation of BPs. BP-3 showed decreased androgenic activity after the metabolism mediated by rat and human liver microsomes [37]. BP-1 was the most potent anti-androgenic UV filter and concentration dependently inhibited 17β-HSD3.

2.1.3. Disrupting Effects toward Other Nuclear Receptors

BPs also exhibited disrupting effects towards PR and thyroid hormone receptor (THR). BP-3 exhibited antagonistic effects to PR and BP-2 interferes with the thyroid hormone (TH) axis in rats [38,40]. Although BP-3 did not activate PR, it is the antagonist of PR as revealed by PR CALUX1 bioassay [38]. BPs can also affect the TH axis by inhibiting the activity of thyroid peroxidase (TPO) or inactivate it, disturbing the biosynthesis of TH [71]. BP-2 was demonstrated as a very potent inhibitor of TPO activity [41]. The study revealed that BP-2-treated rats exhibited decreased thyroxine (T4) and increased thyroid-stimulating hormone (TSH) serum levels. BP-2 disturbed TH homeostasis by inhibiting or inactivating TPO as revealed by the stably transfected human recombinant TPO [40]. As evaluated by an in vitro reporter system containing a duplicated thyroid hormone response element of the HLA-DR4 serotype, BP-2 and BP-3 can induce luciferase activity, showing agonistic activity toward THR [42].

2.2. Camphor Derivatives

Camphor derivatives are highly effective UVB-absorbers incorporated in many kinds of cosmetics. These chemicals have high bioconcentration factors and can be bioaccumulated in tissues of organisms after prolonged exposure [72,73]. The camphor derivatives such as 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC) are very lipophilic and can be easily absorbed after direct

contact with the skin. 4-MBC and 3-BC were revealed as potential endocrine disruptors, adversely affecting the reproduction and development of many organisms [74,75].

2.2.1. Disrupting Effects toward Estrogen Receptor

4-MBC and 3-BC showed anti-estrogenic activity in fish, mammals and cell-based bioassays [22,44,76]. Exposure to 3-BC during the early development and postnatal life of rat, could lead to significant changes of the expression of ERs and estrogen target genes [77]. 4-MBC and 3-BC also showed specific estrogenic activity in the HELN ERα cell and MCF-7 cell line proliferation assay [22,45]. 3-BC exhibited estrogenic potency in immature rats [76] and also showed high estrogenic potency of inducing VTG in juvenile fathead minnow [26]. 4-MBC and 3-BC negatively affected the sex ratio of frog *Xenopus laevis* at environmental concentrations [23]. They were selective ER ligands [46] and bound preferentially to ERβ [76]. 4-MBC activated ERα weakly and showed higher potency toward ERβ mediated transactivation in Ishikawa cells [47]. 4-MBC could activate human ERα in concentration-dependent mode and also significantly activate transcription through human ERβ [43]. 4-MBC caused an increase of mRNA expression level of ERα and VTG in male Japanese medaka [48].

2.2.2. Disrupting Effects toward Androgen Receptor

4-MBC and 3-BC showed no agonistic activation toward AR [50]. They exhibited anti-androgenic activity toward AR in AR CALUX® cell line as revealed by the transcriptional-activation assay [38,50]. They could inhibit the activity of AR in a concentration-dependent mode as revealed by the recombinant yeast assays [36]. 4-MBC was proved to be a potent human AR antagonist and significantly inhibited luciferase activity [78]. 4-MBC and 3-BC also concentration-dependently prevented testosterone formation by inhibiting androgen-metabolizing 17β-HSD3 in HEK-293 cells [39].

2.2.3. Disrupting Effects toward Progesterone Receptor

The developmental 4-MBC exposure could cause an increase of PR mRNA levels in male medial preoptic area, but this change was not detected in female rat [52]. 4-MBC at very low dose can down-regulate the expression level of PR protein. With the increasing doses, the expression of PR protein returned to normal or slightly supranormal levels [77]. 4-MBC disturbed the expression of membrane-associate PR, measured by changes in mRNA levels at different developmental stages [51]. 4-MBC and 3-BC showed no PR transactivation in U2-OS cells, and these two UV filters at low concentrations were antagonists of PR [38].

2.3. Cinnamate Derivatives

Cinnamate derivatives are the most frequently used cosmetic UV filters with the high efficiency to absorb UVA or UVB light. Their molecular structures have a special unsaturated bond between the aromatic ring and the carboxyl group, allowing the molecule to better absorb the 305 nm wavelength UV [79]. Their residues were detected in wastewater, surface water, sewage sludge, fish and marine mammals [11,80,81]. Octyl methoxycinnamate (OMC) is one of the most commonly-used UV filters. It was listed as one of 27 UV filters approved for use in cosmetics formulations in the EU and US [82]. OMC has disrupting activities toward ER, AR, PR and THR as reported by multiple in vitro and in vivo studies [16,38,78].

2.3.1. Disrupting Effects toward Estrogen Receptor

The potential estrogenic activities of cinnamate derivatives have been reported by various in vivo and in vitro experiments [16,22,43]. OMC, isopentyl-4-methoxycinnamate (IMC) and octocrylene (OC) could completely inhibit the activity of E2 by yeast human assays [36]. OMC was found to moderately activate ERα but no obvious effect on ERβ by using reporter cell lines including HELN, HELN ERα, and HELN ERβ [45] was observed, in line with the transactivation bioassay using HEK293 cells in which

OMC dose-dependently activated ERα, but did not activate transcription of ERβ [43]. Exposure to OMC induced weak estrogenic effect to the uterus and the vagina of female Sprague–Dawley rats [49]. OMC could cause an increase of plasma concentration of VTG and up-regulate the mRNA expression levels of ERα in medaka fish [48].

2.3.2. Disrupting Effects toward Thyroid Hormone Receptor

Cinnamate derivatives also interfered with the TH axis in rats. The perinatal OMC-exposure could induce adverse effects on the reproductive and neurological development of rat offspring [16,53]. The treatment with OMC for 12 weeks caused a decrease of T4 level in the blood of ovariectomised female rats and inhibited the activity of 5′-deiodinase that converts T4 to T3 in the liver [53]. Exposure to OMC caused a dose-dependent decrease of serum concentrations of TSH, T4 or T3 in rats [54]. TPO activity was unaltered but T3-responsive hepatic type I 5′ deiodinase activity was reduced by OMC. OMC was found exerting effects on the TH axis by using female ovariectomized rats [83]. OMC affected TH via inhibition of type I 5′-Deiodinase activity and gene expression.

2.3.3. Disrupting Effects toward Other Nuclear Receptors

Cinnamate derivatives also induced disrupting effects toward other NRs such as PR and AR. OMC, IMC and OC showed obvious antagonistic effects toward PR and AR as revealed by cell-based bioassays and in vivo experiments using rats [16,38]. OMC showed antagonistic activity toward PR as determined by sensitive and specific reporter gene cell lines [38]. OMC, IMC and OC showed anti-androgenic activities as revealed by the yeast assay [36]. They inhibited 4,5-dihydrotestosterone activity in concentration-dependent mode.

3. Perspectives

With the continuous demand of cosmetics, plastics and various industrial products containing UV filters, the production and application of UV filters, especially new type of UV filters will be increased. For better risk assessment of these chemicals and their metabolites, the investigation of endocrine disrupting effects caused by direct and indirect exposure to UV filters is currently becoming a research hotspot. Adverse outcome pathways of emerging UV filters should be well characterized for their potential disruption of the hypothalamus-pituitary-gonadal endocrine axis. Although multiple in vivo and in vitro studies have investigated the adverse effects of UV filters, the underlying mechanism of endocrine disruption should be further explored at the atomic level. Considering the versatile role of computational toxicology for the study of physiochemical properties of organic contaminants and their interactions with various biomacromolecules [39,63,84,85], more in silico studies should be performed, primarily for UV filters to probe the molecular initiating event toward target receptors.

4. Conclusions

We reviewed the potential endocrine disruptors of typical UV filters including benzophenones, camphor derivatives and cinnamate derivatives. These UV filters are generally involved in the disruption of the hypothalamic–pituitary–gonadal system. As revealed by in vivo and in vitro assays, exposure to these chemicals induced various endocrine disrupting effects such as estrogenic disrupting effects, androgenic disrupting effects as well as the disrupting effects towards TR, PR. The underlying mechanism of endocrine disruption was summarized (Table 2). The minor structural changes of these kinds of UV filters have influence on the potency of their endocrine disrupting effects.

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Estrogenic activity of cosmetic components in reporter cell lines: parabens, UV screens, and musks.

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Abstract

In this work, the estrogenic effects of three classes of substances included in cosmetic formulations-parabens, ultraviolet (UV) screens, and musk fragrances-were studied. Their estrogenic activity was measured with the use of three reporter cell lines: HELN, HELN ERalpha, and HELN ERbeta. These three cell lines allowed for the measurement of estrogenic activity toward estrogen receptors alpha and beta (ERalpha and ERbeta, while taking nonspecific interactions into account. Eight of the 15 substances tested showed specific estrogenic activity with the following degree of potency on ERalpha butylparaben > propylparaben > homosalate = octyl-dimethyl-PABA = 4-methyl-benzylidenecamphor = octyl-methoxycinnamate > ethylparaben = galaxolide. Among these active substances, parabens activated ERalpha and ERbeta similarly, UV screens activated ERalpha moderately and had almost no effect on ERbeta, and fragrances did not activate ERbeta. Methylparaben, ethylparaben, musk moskene, celestolide, and cashmeran did not activate estrogenic responses up to 10(-5) M. Musk ketone and benzophenone-3 were not considered estrogenic at 10(-5) M.

In Vitro and *in Vivo* Estrogenicity of UV Screens

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Ultraviolet (UV) screens are increasingly used as a result of growing concern about UV radiation and skin cancer; they are also added to cosmetics and other products for light stability. Recent data on bioaccumulation in wildlife and humans point to a need for in-depth analyses of systemic toxicology, in particular with respect to reproduction and ontogeny. We examined six frequently used UVA and UVB screens for estrogenicity *in vitro* and *in vivo*. In MCF-7 breast cancer cells, five out of six chemicals, that is, benzophenone-3 (Bp-3), homosalate (HMS), 4-methyl-benzylidene camphor (4-MBC), octyl-methoxycinnamate (OMC), and octyl-dimethyl-PABA (OD-PABA), increased cell proliferation with median effective concentrations (EC_{50}) values between 1.56 and 3.73 μM, whereas butyl-methoxydibenzoylmethane (B-MDM) was inactive. Further evidence for estrogenic activity was the induction of pS2 protein in MCF-7 cells and the blockade of the proliferative effect of 4-MBC by the estrogen antagonist ICI 182,780. In the uterotrophic assay using immature Long-Evans rats that received the chemicals for 4 days in powdered feed, uterine weight was dose-dependently increased by 4-MBC (ED_{50} 309mg/kg/day), OMC (ED_{50} 935 mg/kg/day), and weakly by Bp-3 (active at 1,525 mg/kg/day). Three compounds were inactive by the oral route in the doses tested. Dermal application of 4-MBC to immature hairless (hr/hr) rats also increased uterine weight at concentrations of 5 and 7.5% in olive oil. Our findings indicate that UV screens should be tested for endocrine activity, in view of possible long-term effects in humans and wildlife. *Key words*: benzophenone-3, estrogenic activity, MCF-7 cell proliferation, 4-methylbenzylidene camphor, octylmethoxycinnamate, pS2 protein, rat, uterotrophic assay, UV screens. *Environ Health Perspect* 109:239–244 (2001). [Online 28 February 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p239-244schlumpf/abstract.html

Organic chemicals that absorb UVA (400–315 nm) or UVB (315–280 nm) radiation are added in concentrations up to 10% to sunscreen products for skin protection. Some of the compounds are also included in other cosmetics such as beauty creams, lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and bubble baths for product stability and durability.

Because of growing public concern about skin damage by UV light, the use of UV screens is increasing, even though the benefit with respect to prevention of melanoma remains controversial (*1,2*). Like other cosmetics such as musk fragrances (*3,4*), these chemicals are highly lipophilic and therefore can be expected to bioaccumulate in the environment. In 1991 and 1993, six different UV screens were identified in fish of the Meerfelder Maar lake (Eifel, Germany) at total concentrations of 2 mg/kg lipid in perch (summer 1991) and 0.5 mg/kg lipid in roach (1993) (*5*). Both fish species were contaminated with sunscreens, polychlorinated biphenyls and DDT at comparable levels. From these results it appeared that UV screens are relevant environmental contaminants (*5*).

Humans can be exposed to UV screens by dermal absorption (*6–9*) or through the food chain. The UV screen benzophenone-3 (Bp-3) and its metabolite 2,4-dihydroxybenzophenone have been detected in human urine from 4 hr after application of commercially available sunscreen products to the skin (*7,10*). Bp-3 has also been found to be readily absorbed from the gastrointestinal tract (*11*). Evidence for bioaccumulation in humans stems from analyses of human milk (*12*). In five out of six samples of human milk, Bp-3 and/or octyl methoxycinnamate were present in detectable amounts.

At present, the toxicologic classification of UV screens is rather heterogenous; they are classified as over-the-counter drugs in the United States, cosmetic ingredients in the European Union, and either cosmetics or quasi-drug products in Japan (*13*). Acute and subchronic systemic toxicity of these compounds is considered to be rather low (*7,14,15*), although some problems have arisen with photoallergic reactions (*16*). No values of acceptable daily intake or maximal tolerated intake of UV screens have been defined. However, the bioaccumulation potential of these lipophilic chemicals does not appear to have been considered in earlier published toxicologic long-term studies. The evidence of bioaccumulation in wildlife and humans raises the possibility of long-term exposure, including effects on reproduction and ontogeny. As a consequence, these compounds should be tested for endocrine activity.

We analyzed six frequently used UVA- or UVB-absorbing UV screens for estrogenic activity *in vitro* in MCF-7 breast cancer cells and *in vivo* in the immature rat uterotrophic assay. Estrogenic activity was demonstrated for five out of six compounds *in vitro* and for three out of six compounds *in vivo* by the oral route. The orally most active compound also increased uterine weight following dermal application.

Materials and Methods

Chemicals

The UV screens Bp-3 (2-hydroxy-4-methoxybenzophenone, oxybenzone, Eusolex 4360); butyl methoxydibenzoylmethane (B-MDM, Eusolex 9020); homosalate (HMS, 2-hydroxybenzoic acid-3,3,5-trimethylcyclohexyl ester, Eusolex HMS); 3-(4-methylbenzylidene) camphor (4-MBC, Eusolex 6300); octyldimethyl-*p*-aminobenzoic acid (OD-PABA, Eusolex 6007); and octyl-methoxycinnamate (OMC, Eusolex 2292) were purchased from Merck (Dietikon, Switzerland). 17β-Estradiol (E_2) and 17α-ethinylestradiol were obtained from Calbiochem (Lucerne, Switzerland), and ICI 182,780 (Astra-Zeneca) was purchased from ANAWA (Dübendorf, Switzerland).

In Vitro Studies on MCF-7 Cells

Cell line. MCF-7 human breast cancer cells (MCF7-Bos, originally from the Michigan Cancer Foundation, Detroit, MI, USA) were kindly provided by A. Soto (Tufts University, Boston, MA, USA). Cells were frozen every 10 passages. In the present experimental series, we used samples from frozen stock for a maximum of 6–13 passages. Mycoplasma status, which was regularly checked by the Institute of Virology of the Veterinary Faculty of the University of Zurich, was negative. Cells were cultured in Dulbecco's modified Eagle Medium (DME) supplemented with 5% fetal bovine serum (FBS; Gibco, Life Technologies, Basel, Switzerland) in 5% CO_2/95% air at 37°C under saturated

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humidity in 50 mL Falcon flasks. Sex steroids were removed from the serum by charcoal dextran treatment [steroid hormone-free FBS (CD-FBS)] (*17*).

E-SCREEN. The E-SCREEN was performed according to Soto and co-workers (*17,18*). Briefly, MCF-7 cells were trypsinized, mechanically dissociated, and plated into 24-well plates (Costar; INTEGRA Biosciences, Wallisellen, Switzerland) at an initial concentration of 40,000 cells/well. Cells were allowed to attach in the seeding medium (DME supplemented with 5% FBS) for 24 hr. The seeding medium was then aspirated and replaced by the experimental medium containing phenol red-free DME with 10% CD-FBS. Cells were incubated with test compound (final concentrations 10^{-4}–10^{-7} M), E_2 as positive control (10^{-8}–10^{-13} M), or chemical-free medium (control) (Table 1). For each concentration, 4 wells per plate were used. The number of independent *in vitro* experiments is given in Table 1 and in figures representing *in vitro* data. Each experiment was accompanied by a positive control (E_2). Concentrations of stock solutions in absolute ethanol were 2×10^{-3} M for E_2 and 10^{-2} M for UV screens; final concentrations of ethanol in culture medium were between 1.0% and 0.001% (v/v) with test compounds, and were ≤ 0.0005% (v/v) with E_2. No difference in the cell proliferation rate was observed in control experiments with chemical-free medium or medium with 1.0% ethanol. Therefore, we used chemical-free medium as a control. We tested antagonism by the pure antiestrogen ICI 182,780 (*19*) in MCF–7 cells exposed for 6 days to E_2 (10 pM) or to 4–MBC (10 μM) in the presence or absence of 1, 10, or 100 nM ICI 182,780.

Measurement of cell proliferation. Experiments were terminated after 6 days of incubation by removing the media from the wells. Cells were fixed with 10% trichloroacetic acid, washed with phosphate-buffered saline, and stained with 1% sulforhodamine blue (0.4% in 1% acetic acid, 1 mL/well) for 15 min at room temperature. Stained cells were dissolved in TRIS buffer (pH 10.6), and optical density (OD) was measured in a Anthos Labtec 2000 spectrophotometer (Anthos Labtec Instruments, Salzbury, Austria) at 492 nm. OD values were converted into cell numbers by a standard curve. Experimental readings were in the linear range of the standard curve (Figure 1).

pS2 protein assay. MCF-7 cells were incubated 72 hr with UV screens at 5, 10, and 50 μM; data are shown for 10 μM (except HMS 50 μM). E_2 (10 pM) served as a positive control. Culture media were harvested and centrifuged to avoid floating of detached cells. Samples were kept at –80°C until the radioimmunoassay for pS2 protein was performed according to the protocol of the manufacturer (ELSA - PS2; CIS Bio International, Gif-sur-Yvette, France). Media were analyzed in duplicate.

In Vivo Studies

Uterotrophic assay. Animal experiments were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Protection Ordinance under permit 190/98 of the Veterinary Office of the State of Zurich. We tested estrogenic *in vivo* activity of UV screens using the rat uterotrophic assay (*20,21*). Long-Evans rats were bred in our laboratory under controlled light and dark cycle (lights on from 0200 to 1600 hr) and temperature (22°C ± 1°C), with standard diet (chow 3430; Provimi Kliba AG, Kaiseraugst, Switzerland) and water *ad libitum*. All experiments were performed on offspring of time-pregnant rats. Receptive females were mated with a male between 1600 and 1900 hr. Sperm-positive females were housed in groups of two to three and separated 1 day before parturition.We defined the stage 24 hr after onset of the 3-hr mating period as gestational day (GD) 1 and the day of birth as postnatal day (PN) 1 (GD 23).

Peroral administration of test chemicals. From PN 16, the pups and their dam were habituated to powdered chow (chow 3430, Provimi Kliba), which continued after weaning at PN 20. Beginning on PN 21, female pups received chow 3430 containing one of several concentrations of test compound for 4 days, until 1200 hr on PN 25. For each experiment, chemicals were dissolved either in acetone or in 99% ethanol and added to powdered chow 3430. The mixture was prepared at least 48 hr before the experiment to allow for complete evaporation of the solvent. Evaporation was assisted by continuous stirring. We used ethinylestradiol (0.3–10 μg/kg) as a positive control. Vehicle controls received normal chow 3430. To limit the number of experimental animals, we adjusted the size of the various treatment groups according to statistical needs. We used the minumum group sample size of the three UV screens that we determined were inactive.

To avoid stress to the immature pups, we housed the animals in groups of four to six. We recorded body weight at the beginning and at the end of the treatment period. Animals from different litters were randomly assigned to the various treatment groups to give similar mean body weights to the various treatment groups at the onset of treatment, with no more than 15% deviation of individual animals from the mean. Mean body weights of experimental groups were in the same range as that of the controls (initial weight 38.0 ± 4.5 g, final weight 48.8 ± 3.8 g). We calculated the mean body weight of the 4-day treatment period for each animal. Food consumption of the group of four to six animals was measured for the 4-day period. The mean daily dose was calculated from the average amount of chemical consumed per animal (ingested powdered chow per animal × concentration of test compound in chow per mean body weight of a given animal). The advantage of using the average values of consumption is that the animals were not disturbed. The consistency

Table 1. Effect of UV screens and E_2 on MCF-7 cell proliferation *in vitro*.

Compound/ concentration (M)	Cells/well
E_2	
0	44,501 ± 5,079 (13)
1×10^{-13}	61,093 ± 12,200 (4)
5×10^{-13}	61,563 ± 2,090 (6)
1×10^{-12}	333,970 ± 51,026 (11)*
1×10^{-11}	743,296 ± 88,655 (11)#
1×10^{-10}	677,115 ± 91,301 (10)#
1×10^{-9}	655,969 ± 77,928 (10)#
1×10^{-8}	623,608 ± 72,292 (11)#
4-MBC	
0	49,028 ± 15,924 (6)
1×10^{-7}	132,292 ± 33,478 (6)
1×10^{-6}	147,396 ± 46,267 (6)
5×10^{-6}	583,299 ± 51,178 (6)#
1×10^{-5}	661,597 ± 66,740 (6)#
5×10^{-5}	330,157 ± 68,896 (6)**
OMC	
0	55,504 ± 13,373 (6)
1×10^{-7}	147,033 ± 25,657 (6)
1×10^{-6}	229,688 ± 65,150 (6)
5×10^{-6}	594,809 ± 74,438 (6)#
1×10^{-5}	566,945 ± 88,253 (6)#
5×10^{-5}	215,985 ± 58,542 (6)
Bp-3	
0	40,292 ± 2,422 (5)
1×10^{-7}	99,605 ± 28,489 (5)
1×10^{-6}	71,438 ± 29,796 (5)
5×10^{-6}	448,750 ± 78,557 (5)#
1×10^{-5}	704,750 ± 53,108 (5)#
5×10^{-5}	680,354 ± 63,914 (5)#
HMS	
0	96,292 ± 15,512 (5)
1×10^{-7}	155,771 ± 20,505 (5)
1×10^{-6}	279,833 ± 22,404 (5)**
5×10^{-6}	573,042 ± 50,308 (5)#
1×10^{-5}	652,917 ± 35,943 (5)#
5×10^{-5}	586,479 ± 14,416 (5) #
OD-PABA	
0	47,726 ± 14,068 (6)
1×10^{-7}	113,229 ± 24,761 (6)
1×10^{-6}	76,719 ± 6,740 (6)
5×10^{-6}	290,181 ± 59,554 (6)**
1×10^{-5}	435,827 ± 35,692 (6)#
5×10^{-5}	326,021 ± 63,239 (6)#
B-MDM	
0	28,125 ± 5,381 (5)
1×10^{-7}	99,104 ± 35,589 (5)
1×10^{-6}	29,833 ± 6,559 (5)
5×10^{-6}	73,250 ± 46,864 (5)
1×10^{-5}	120,846 ± 79,925 (5)
5×10^{-5}	54,297 ± 19,109 (5)

Values shown are mean ± SEM (number of independent experiments).
* $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ (ANOVA plus Bonferroni pairwise comparisons) as compared to control.

of the condition is indicated by an SD/mean ratio of uterine weights of 5.6% in controls and 9.8% in treated groups.

At the end of the treatment period, pups were decapitated under light ether anesthesia.The uterus was excised, trimmed free of fat and connective tissues, and blotted with sterile gauze to remove adherent fluid.The uterus was cut just above the junction between the cervix and vagina and at the top of the uterine horns. It was then weighed (wet weight) and either frozen in liquid nitrogen or fixed in buffered 4% formaldehyde for further analysis.

Dermal application of test chemicals. We studied possible effects of dermal application of UV screens in immature females of the Rat Nu (hairless) strain (Ico: OFA hr/hr). Parent animals were obtained from IFFA CREDO (Labresle, France) and kept under the same conditions as the Long-Evans rat colony (see above). One hr+/hr+ male was caged with three adult hr+/hr+ females. Pregnant dams were identified by weight gain, and the date of parturition (PN 1) was registered. Because of difficulties of the lactating hr+/hr+ dams to produce sufficient milk, the dam was replaced on PN 2 by a lactating Long-Evans dam. Pups were weaned at PN 20.

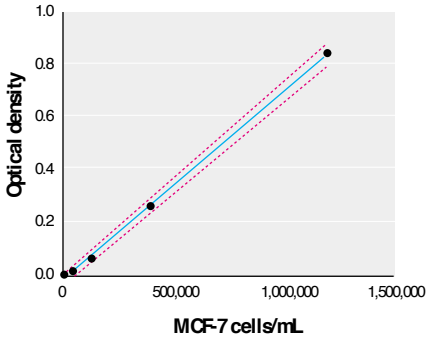

Figure 1. Representative standard curve of MCF-7 cell density (cells/mL) versus optical density (1% sulforhodamine blue staining). Linear regression with 95% confidence limits ($r^2 = 0.9065$). Circles represent mean values of 16 replicates.

Female rat pups were treated on 6 consecutive days, from PN 21 to PN 26. 4-MBC [2.5%, 5.0%, 7.5% (w/w) in olive oil] or olive oil (vehicle control) was applied twice daily at an interval of 3–4 hr. At 30°C ambient temperature, the animal was gently held by the neck and immersed up to its shoulders into a glass beaker containing olive oil with 4-MBC or pure olive oil for 15 sec. The pup was then transferred into a plastic box (one animal per box), where it remained on a paper towel for 30 min. During the 30-min period, an additional amount of the solution was applied twice onto the back of the animal with a soft brush. After 30 min, the skin appeared dry; the animal was transferred onto a clean paper towel to remove remaining solution and then returned to its home cage. We used separate plastic boxes for 4-MBC-treated pups and controls. The animals were continuously observed; they did not lick their skin, but remained in a quiet position. On PN 27, pups were weighed and decapitated under light ether anesthesia. The uterus was removed and weighed as described above. The treatment group was unknown to the person dissecting the uterus.

In the absence of toxicokinetic *in vivo* data, it is not possible to exactly determine the dose of 4-MBC taken up by dermal application. However, we determined the amount of olive oil applied during one treatment by weighing the animal before and after each manipulation. The average amount of oil retained after 15 sec immersion was 1.35 ± 0.13 g, and the additional amount applied by the brush was 1.4 ± 0.08 g. Thus, the total amount of oil was 2.75 g/treatment or 5.5 g/day, yielding total amounts of 4-MBC applied to the skin per day of 137.5, 275, and 412.5 mg for 2.5, 5.0, and 7.5% 4-MBC concentrations, respectively. According to *in vitro* data (*8*), the penetration of 4-MBC through hairless rat skin is 0.6% from oily gels or 0.4% from a water in oil (W/O) emulsion. Assuming 0.6% penetration, the dose absorbed from a 5% 4-MBC solution in oil can be tentatively calculated as 37 mg/kg/day based on mean body weight of the 5% group.

Data Analysis

In vitro studies. We calculated cell counts per well from optical density as described above. In every experiment, we analyzed each concentration in quadruplicate. From these values, we calculated the mean cell count of a given concentration of chemical or of chemical-free medium for each experiment. Cell counts from different independent experiments were compared using analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons (SYSTAT software; SYSTAT Intelligent Software, Evanston, IL, USA). The proliferative effect (PE) of a compound was defined as (maximal mean cell count obtained with the test chemical)/(mean cell count in the chemical-free medium), the relative proliferative effect (RPE) in relation to that of E_2 as (PE of test compound –1)/(PE of E_2 – 1) × 100 (*17*). For comparison with uterotrophic data, we expressed the increase in cell number as percentage of E_2 [(cell number with test compound – control)/(cell number with E_2 – control) × 100]. We calculated the median effective concentration (EC_{50}) values from the ascending part of the concentration–response curve using Graph Pad Prism2 software (Graph Pad Software, Inc., San Diego, CA, USA). For pS2 protein, treated groups and the chemical-free medium group were compared by ANOVA followed by Bonferroni pairwise comparisons (SYSTAT).

In Vivo Studies

Uterine weights of individual animals from different dose groups and vehicle controls were compared by ANOVA followed by Bonferroni pairwise comparisons. We calculated ED_{50} values using Prism2.

Table 2. Comparison of *in vitro* and *in vivo* activity of UV screens and steroidal estrogens.

	MCF-7 cell proliferation				Uterotrophic effect in immature rats		
Compound	PE[a]	RPE[b]	Maximal cell count increase (% of E_2)[c]	EC_{50}	Increase of uterine weight over control[d]	Maximal weight increase (% of EE_2)[e]	ED_{50}
E_2	16.70	100	100	1.22 pM			
EE_2					4.08	100	0.818 μg/kg/day
Bp-3	17.49	105.0	95.09	3.73 μM	1.23	7.60	1,000–1,500 mg/kg/day
4-MBC	13.49	79.54	87.66	3.02 μM	2.09	35.51	309 mg/kg/day
OMC	10.72	61.90	77.18	2.37 μM	1.68	22.21	934 mg/kg/day
OD-PABA	9.13	51.77	55.54	2.63 μM	1.04	1.15	Inactive
HMS	6.78	36.81	79.65	1.56 μM	1.12	3.79	Inactive
B-MDM	4.30	21.01	13.27	Inactive	1.06	2.01	Inactive

EE_2, ethinylestradiol.

[a]Proliferative effect over control; PE = (maximal cell count of experimental group)/(cell count of control). [b]Maximal proliferative effect (% of E_2); RPE = (PE of experimental group – 1)/(PE of estradiol – 1) × 100. [c](Cell count of experimental group – cell count of control)/(cell count of estradiol – cell count of control) × 100. [d](Uterine weight of experimental group)/(uterine weight of control). [e]Maximal weight increase (% of ethinylestradiol) = (uterine weight of experimental group – uterine weight of control)/(uterine weight of ethinylestradiol – uterine weight of control) × 100.

Results

Effect of UV Screens on MCF-7 Cells *in Vitro*

MCF-7 cell proliferation. Cell proliferation was dose-dependently increased by all UV screens tested except for B-MDM, with a bell-shaped dose–response curve (Tables 1 and 2, Figure 2). The effective concentration range (1–50 μM) and the maximum effect concentration (at around 10 μM) was similar for the various compounds. *In vitro* EC_{50} values of UV screens range between 1.56 μM (HMS) and 3.73 μM (Bp-3) (Table 2). According to their maximum effects on cell proliferation in relation to E_2, 4-MBC, OMC, OD-PABA, and HMS acted as partial agonists, whereas the maximum activity of Bp-3 reached the level of E_2. The proliferative effects of 4-MBC and the positive control E_2 were completely blocked by the pure estrogen receptor antagonist ICI 182,780 (Figure 3).

pS2 protein. Secretion of the estrogen-regulated protein pS2 into the culture medium was significantly increased by 4-MBC, HMS, and Bp-3 (Figure 4). Levels were also above control after incubation with OMC and OD-PABA, but the difference was not significant; B-MDM was clearly negative. At the concentration tested, 4-MBC induced the greatest response (43.9% of E_2). The correlation between the increase in proliferation and in pS2 secretion at the concentration used for pS2 protein induction was low ($r^2 = 0.6046$, not significant).

Effect of UV Screens on the Immature Rat Uterus *in Vivo*

Peroral administration. After administration in powdered feed for 4 days, three of the five chemicals active *in vitro* and the positive control ethinylestradiol elicited dose-dependent increases in uterine weight of immature Long-Evans rats (Table 3, Figure 2). The rank order of potency, 4-MBC > OMC > Bp-3, differed from the one observed *in vitro*; 4-MBC exhibited a significant increase in uterine weight at a dose of 119 mg/kg/day and an ED_{50} of 309 mg/kg/day (Tables 2,3). Two of the compounds with *in vitro* activity, HMS and OD-PABA, as well as B-MDM, were inactive *in vivo* at the doses tested. Mean body weights of the various treatment groups were similar (data not shown) and in the range of the vehicle control group (mean ± SD of 38.0 ± 4.5 g at PN 21 and 48.8 ± 3.8 g at PN25).

Dermal application of 4-MBC. Following dermal application of 4-MBC in olive oil twice daily for 6 days, immature rats of the hr/hr (hairless) strain exhibited a dose-dependent increase in uterine weight, with a significant increase above control induced by 5% and 7.5% 4-MBC (Figure 5). The mean uterine weight of the 5% 4-MBC group was also significantly higher than that of the 2.5 or 7.5% groups, yielding a bell-shaped dose–response curve. The control uterine weight of this strain appeared to be slightly lower than that of Long-Evans rats, even though the animals were 2 days older. Mean body weights (± SD) of the various groups were similar at the beginning (control, 34.78 ± 3.15; 2.5% 4-MBC, 32.54 ± 1.40; 5% 4-MBC, 34.44 ± 4.10; 7.5% 4-MBC, 34.51 ± 1.92) and at the end of the treatment period (control, 52.52 ± 7.41; 2.5% 4-MBC, 43.63 ± 0.81; 5% 4-MBC, 55.99 ± 5.38; 7.5% 4-MBC, 51.11 ± 5.07).

Discussion

The present study demonstrates *in vitro* and *in vivo* estrogenic activity for a number of UV screens with different chemical structures. The compounds tested are frequently used in sunscreens and cosmetics and have the potential for bioaccumulation.

In the *in vitro* system, five out of the six UV screens tested displayed significant dose-dependent estrogenic activity on MCF-7 cells. Bp-3 was most active on cell proliferation, followed by 4-MBC, HMS, OMC, and OD-PABA, whereas B-MDM was inactive. With maximum effects on MCF-7 cell proliferation at 5–10 μM and EC_{50} values between 1.5 and 3.7 μM (Table 2), the estrogenic activity of the five UV screens is in the range of other industrial chemicals identified as environmental estrogens (*17,22*). The strain of MCF-7 cells used displayed good sensitivity to E_2, with a maximum proliferation rate at 10 pM, comparable to previously published data (*17*). This enabled good discrimination between test chemicals. It cannot be determined whether the effects of the UV screens were caused by the parent compounds and/or by possible metabolites because MCF-7 cells express constitutive and inducible cytochrome P450 enzymes (*23*).

Complete blockage of the proliferative effect of 4-MBC by the pure estrogen receptor antagonist ICI 182,780 (*19*) indicates an estrogen receptor-mediated effect. The estrogenic activity of these chemicals is further demonstrated by the induction of the estrogen-regulated pS2 protein (*24*). The correlation between the effects of the six chemicals on cell proliferation and pS2 protein (expressed as percentage of E_2) was low ($r^2 = 0.6046$, not significant); however, the same compounds (4-MBC, Bp-3, and HMS) were most active on proliferation as well as pS2 protein induction, and B-MDM was clearly inactive on both parameters. It should be noted that the effect on pS2 protein was only analyzed for one concentration of test chemicals in the range of the maximum proliferative effect. The dose–response relationship and, hence, the maximum effective dose may be different for the two parameters.

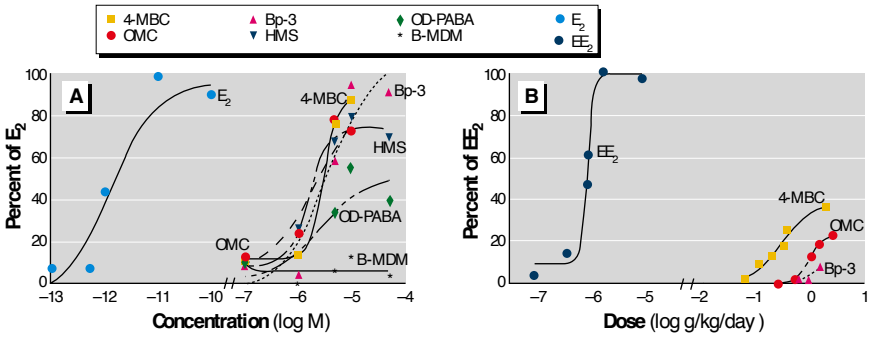

Figure 2. Dose–response relationship of estrogenic activity of different UV screens. (*A*) *In vitro* effect of UV screens on MCF-7 cell proliferation (cell number/well) as a percentage of the maximum effect of E_2. (*B*) Effect of oral UV screens on immature rat uterine weight as a percentage of the maximum effect of ethinylestradiol (EE_2).

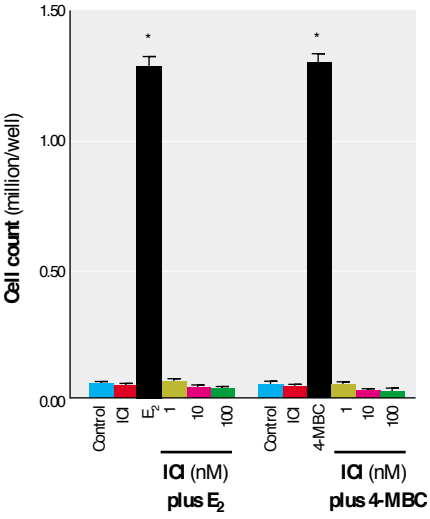

Figure 3. Antagonism of the proliferative effect of 4-MBC (10 μM) or E_2 (10 pM) on MCF-7 cells by increasing concentrations of the pure estrogen receptor antagonist ICI 182,780. Controls received chemical-free medium. ICI 182,780 alone was 100 nM. Values shown are mean ± SEM of four independent experiments.

*$p < 0.001$ (ANOVA followed by Bonferroni pairwise comparisons) as compared to all other experimental groups (control, ICI 182,780 alone, and E_2 or 4-MBC plus ICI 182,780 at all concentrations).

Three UV screens, 4-MBC, OMC, and Bp-3, were active by the oral route in an acute mammalian *in vivo* model for estrogenicity (*21*), eliciting dose-dependent increases of uterine weight in immature rats. Differences between individual UV screens were more pronounced than *in vitro*. 4-MBC was most active, with a significant increase in uterine weight at 119 mg/kg/day and an ED_{50} of 309 mg/kg/day; it also had the greatest maximal effect (Table 2). In contrast, Bp-3 displayed only weak activity *in vivo*. This compound has recently been reported to be inactive in the uterotrophic assay (*25*). The dose level used was also ineffective in our investigation; thus, the two data sets are in agreement. The *in vitro* (proliferation) and *in vivo* dose–response curves of 4-MBC and OMC suggest that they are partial agonists. Bp-3 is difficult to judge because it reached the level of the full agonist, E_2, *in vitro*, whereas the *in vivo* dose–response curve is incomplete.

Weak binding to estrogen receptors has been reported for unsubstituted benzophenone (*26*). One of the main metabolites of Bp-3, 2,4-dihydroxybenzophenone, binds to estrogen receptors with micromolar affinity, in contrast to its parent compound (*27*). This metabolite was detected in human urine after dermal application of a commercial sunscreen product (*10*). *O*-dealkylation also appears to be the major metabolic pathway of Bp-3 in rats (*28*). However, the relative roles of parent compounds and metabolites for *in vivo* estrogenic activities of the various UV screens remain to be clarified.

A comparison of *in vitro* and *in vivo* data indicates that the *in vitro* assay was useful for identifying estrogenic activity, but was of limited predictive value for the mammalian *in vivo* situation. Although all three chemicals that exhibited *in vivo* activity were strongly active *in vitro*, two compounds with high or moderate *in vitro* activity, HMS and OD-PABA, were completely inactive in the uterotrophic assay at the doses tested. The rank orders of activity also differed between *in vitro* and *in vivo* experiments. This may have resulted from pharmacodynamic and/or pharmacokinetic differences. A precise quantitative comparison of *in vitro* and *in vivo* effects is not possible because different tissues served as end points and different estrogens were used as positive controls. The differences between *in vitro* and *in vivo* data support the need for *in vivo* testing of chemicals after identification of endocrine activity *in vitro*.

The investigated chemicals are diverse in structure, but they share a common use as UVA or UVB screens and they have potential for bioaccumulation. Four of the UV screens with estrogenic activity, 4-MBC, Bp-3, HMS, and OMC, have been detected in fish (*5*), and so far two compounds, Bp-3 and OMC, have been detected in human milk (*12*). The total concentration of estrogenic UV screens in fish, where a larger data set is available, ranged between 1.6 μM and 7.8 μM in fat, or between 0.02 and 0.2 μM in whole fish (roach and perch, respectively). UV screens thus may contribute to the total body burden of endocrine active compounds in wildlife and humans.

The effective dose range of oral 4-MBC, OMC, and Bp-3 in the rat uterotrophic assay (119 mg/kg/day for 4-MBC to 1,500 mg/kg/day for Bp-3) compares with daily oral doses of bisphenol A (400 mg/kg) (*29*), methoxychlor (100–500 mg/kg), nonylphenol (190–1,000 mg/kg), and *o,p´*-DDT (1,000 mg/kg) (*21,30*) that increase rodent uterine weight. Uterine epidermal growth factor receptor was induced by 500 mg/kg/day methoxychlor (*31*). In one study on bisphenol A, Gould et al. (*32*) were unable to detect changes in uterine weight, but they reported an increase in peroxidase activity at 100 mg/kg/day and increased progesterone receptor levels at lower doses. These doses cannot be compared with actual exposure levels, as it is generally agreed that the acute high-dose rodent model cannot serve as a basis for risk assessment, but rather for identification of *in vivo* activity. Thus, bisphenol A has been found to disturb developmental processes at doses that are several orders of magnitude lower (2–50 μg/kg) (*33,34*).

As UV screens, the chemicals tested in this study present two different toxicologic aspects: On one hand, they may play an ecotoxicologic role in wildlife and humans, probably resulting mainly from intake via the food chain. On the other hand, they may also be transdermally active in humans when they are used as sunscreens. We observed an increase in uterine weight after dermal application of

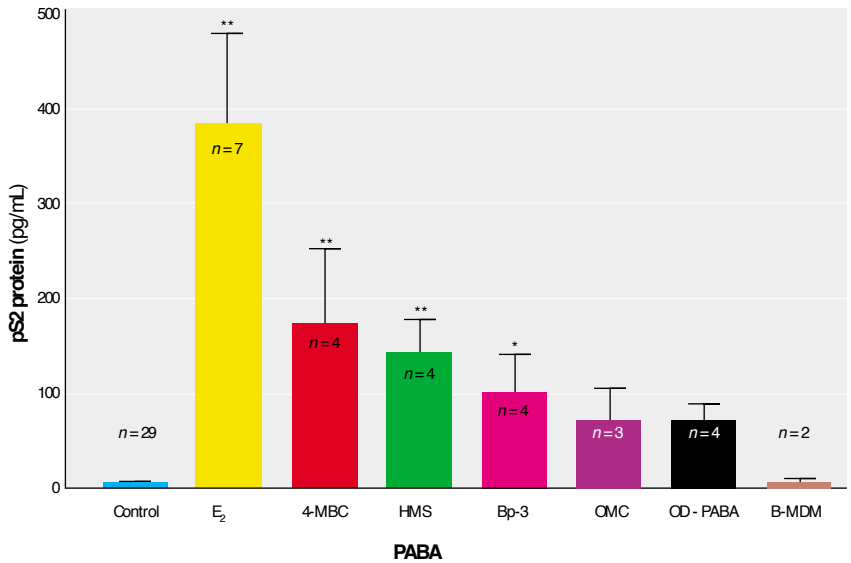

Figure 4. Effect of UV screens 4-MBC (10 μM), HMS (50 μM), Bp3 (10 μM), OMC (10 μM), OD-PABA (10 μM), B-MDM (10 μM), and E_2 (10 pM) on pS2 protein secretion by MCF-7 cells. Bars indicate mean pS2 protein concentration in culture medium (pg/ml) ± SEM; numbers inside the bars indicate the number of independent experiments).

*$p < 0.05$, **$p < 0.001$ (ANOVA followed by Bonferroni pairwise comparisons) as compared to controls that received chemical-free medium.

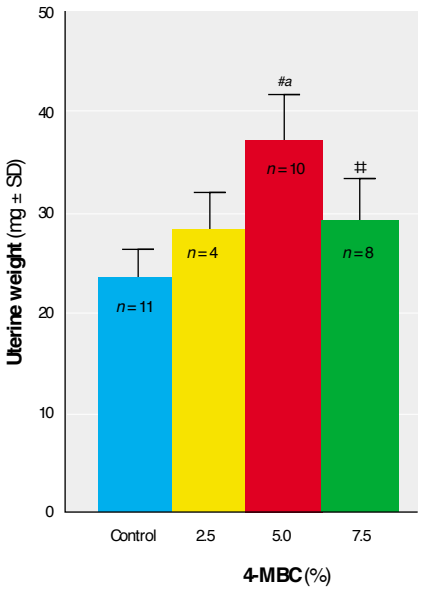

Figure 5. Effect of dermal application of 4-MBC (2.5, 5.0, or 7.5 % in olive oil, twice daily for 6 days) on uterine weight of immature hairless (hr+/hr+) rats. Controls received vehicle (olive oil). Bars indicate mean ± SD; numbers inside the bars indicate the number of animals.

[a]5% 4-MBC different from 2.5%, $p < 0.005$; different from 7.5%, $p < 0.001$ [#]$p < 0.001$, [‡]$p < 0.025$ as compared to controls (ANOVA followed by Bonferroni pairwise comparisons).

4-MBC in olive oil to immature hairless rats at concentrations allowed in sunscreen products. The dose–response curve of uterine weight was bell-shaped, suggesting more complex interactions. With 5% 4-MBC, the increase (159% of control) corresponded to the increase (154% of control) produced by an oral dose of 337 mg/kg/day, which is close to the oral ED_{50}. 4-MBC exhibits significant penetration through skin of hairless rats from a 6% solution in either a W/O emulsion or oily gels (*8*). BP-3 is also dermally absorbed in rats (*35*). Evidence for absorption by human skin has been presented for 4-MBC, Bp-3, and OD-PABA (*6,7,9,10*); 4-MBC also penetrates a folioxane membrane, a model for human skin (*8*). Skin penetration may vary between compounds, as indicated by lower penetration of OMC as compared to Bp-3 (*9,36*), and appears to be influenced by the formulation (*8,36*). Such kinetic differences may be of importance from a toxicologic point of view, but present knowledge is too incomplete to provide a picture of the general human exposure to UV screens.

Table 3. Effect of oral UV screens and ethinylestradiol on uterine weight of immature rats.

Dose	Uterine weight (mg)
Ethinylestradiol (µg/kg/day)	
0.085	29.15 ± 3.59 (6)
0.342	37.02 ± 1.05 (6)##
0.780	61.82 ± 5.00 (5)##
0.856	72.80 ± 5.77 (5)##
1.648	102.86 ± 13.09 (5)##
8.631	100.95 ± 3.28 (6)##
4-MBC (mg/kg/day)	
66	27.25 ± 1.72 (10)
119	32.43 ± 3.61 (13)‡
211	35.24 ± 5.84 (19)##
337	38.78 ± 6.36 (18)##
402	45.22 ± 8.23 (9)##
1,980	52.80 ± 11.8 (4)##
OMC (mg/kg/day)	
268	24.95 ± 2.50 (10)
522	26.81 ± 1.64 (13)
1,035	35.46 ± 8.74 (10)##
1,518	39.59 ± 7.58 (7)##
2,667	42.48 ± 1.25 (5)##
Bp-3 (mg/kg/day)	
611	26.84 ± 1.87 (5)
937	26.94 ± 2.26 (9)
1,525	31.14 ± 3.13 (5)##
HMS (mg/kg/day)	
491	28.18 ± 1.64 (6)
892	23.36 ± 0.96 (5)
OD-PABA (mg/kg/day)	
596	26.05 ± 0.95 (4)
761	24.75 ± 1.29 (6)
1,419	26.13 ± 3.10 (6)
B-MDM (mg/kg/day)	
421	26.80 ± 1.08 (6)
636	26.05 ± 0.97 (6)
Vehicle control	
0	25.24 ± 1.41 (28)

Values shown are mean ± SD (number of rats).
##$p < 0.0001$, ‡$p < 0.002$ (ANOVA plus Bonferroni pairwise comparisons) as compared to controls.

Conclusions

Our investigation revealed that several frequently used UV screens possess estrogenic activity *in vitro* and *in vivo*, in the range of other known xenoestrogens. With the exception of some benzophenones, these chemicals do not appear to have been considered as potential environmental endocrine disruptors (*37*). Considering the widespread use of UV screens, we suggest that toxicokinetics, in particular skin penetration, and systemic toxicology of these chemicals should be investigated more extensively. In view of possible long-term effects, screening for endocrine activity seems important. From our data and from observations in other fields (see above), it appears that there is a need to reconsider the potential benefits of extensive UV screen use both from a medical and an ecologic perspective.

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Hormonal activity, cytotoxicity and developmental toxicity of UV filters

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Zebrafish embryo toxicity test

ABSTRACT

Ultraviolet (UV) filters are commonly used compounds in personal care products and polymer based materials, as they can absorb solar energy in the UVA and UVB spectrum. However, they are able to bind to hormone receptors and have several and different types of hormonal activities determined by *in vitro* assays. One of the aims of this work was to measure the hormonal and cytotoxic activities of four frequently used UV filters using bioluminescence based yeast test organisms. Using *Saccharomyces cerevisiae* BLYES and BLYAS strains allowed the rapid and reliable detection of agonist and antagonist hormonal activities, whereas BLYR strain served to measure cytotoxicity. Results confirmed that all tested UV filters show multiple hormonal activities. Cytotoxicity is detected only in the case of benzophenone-3. Research data on the toxic effects of benzophenone-3, especially on aquatic organisms are scarce, so further investigations were carried out regarding its cytotoxic and teratogenic effects on bacteria and zebrafish (*Danio rerio*) embryos, respectively. Results revealed the cytotoxicity of benzophenone-3 not only to yeasts but to bacteria, as well as its ability to influence zebrafish embryo hatching and development.

1. Introduction

In recent years, a growing attention has been granted to the deleterious effects of ultraviolet (UV) radiation, as it may contribute to the development of skin cancer (IARC, 1992; Ichihashi et al., 2003) and deteriorate polymer based materials. Organic UV filters can absorb the energy of photons in the UVA (320–400 nm) and UVB (290–320 nm) interval, so they are widely used in personal care products (e.g. suntan lotions, body lotions, shampoos, lipsticks) and in technical materials for protecting human health and enhancing the light stability of products.

UV filters can enter surface waters through wastewater effluents and can be washed off from the skin during recreational activities. It can also be observed that there is a seasonal variation to the environmental concentration of these compounds. The highest concentrations are measured in summer in the case of benzophenone-3 (BP-3), 3-(4-Methylbenzylidene)camphor (4MBC), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) in water samples from lakes in Switzerland (Poiger et al., 2004). UV filters have been found in different matrices, for example wastewater influents (Loraine and Pettigrove, 2006), effluents (Li et al., 2007), rivers (Cuderman and Heath, 2007), lakes (Cuderman and Heath, 2007; Rodil and Moeder, 2008), soil (Jeon et al., 2006), sludge (Gago-Ferrero et al., 2011; Rodríguez-Rodríguez et al., 2012), sediment (Kameda et al., 2011) and biota (Buser et al., 2006; Fent et al., 2010) in trace level concentration (ng/L; ng/g; μg/g). Moreover, UV filters were also detected in human milk (Hany and Nagel, 1995; Schlumpf et al., 2010) and urine (Calafat et al., 2008).

Although UV filters can provide protection against the harmful effects of UV radiation, they also have some disadvantages. UV filters can bind to hormone receptors and show agonistic and/or antagonistic activities towards human estrogen receptor α (hERα) and human androgen receptor (hAR) (Klann et al., 2005; Kunz and Fent, 2006; Ma et al., 2003; Schlumpf et al., 2001; Schmitt et al., 2008; Suzuki et al., 2005). The most comprehensive study was carried out by Kunz and Fent (2006) who examined *in vitro* multiple hormonal activities of eighteen UV filters and one metabolite with recombinant yeast systems. All compounds proved to be hormonally active; moreover, ten UV filters showed three distinct hormonal activities each, and most of them elicited antiandrogenic and antiestrogenic activities.

There are only few assays that are concerned with the effects of UV filters to aquatic organisms but there are some evidence that the presence of UV filters in surface waters and sediment can be harmful to aquatic organisms. Coronado et al. (2008) experienced significant vitellogenin synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*) and male Japanese medaka (*Oryzias latipes*) at

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749 μg/L and 620 μg/L concentrations of BP-3. In addition to this, a 21-day treatment of the parent Japanese medaka with 620 μg/L BP-3 significantly decreased the percentage of hatched eggs. Kaiser et al. (2012) studied the effects of EHMC on zebrafish (*Danio rerio*) in a 48 h sediment assay. Exposing the fish to 1000 mg/kg dw EHMC resulted in serious developmental disorders. The freshwater populations of invertebrates can also be adversely affected by hormonally active UV filters. Schmitt et al. (2008) exposed *Potamopyrgus antipodarum* snails to 4MBC. The number of unshelled embryos was significantly enhanced and the mortality of snails significantly increased. The same mortality was detected in the case of *Lumbriculus variegatus* worms. Kaiser et al. (2012) found that EHMC significantly decreased the number of embryos per snail of *Potamopyrgus antipodarum* and *Melanoides tuberculata* species. Liu et al., (2015a) carried out an acute toxicity test with *Daphnia magna* testing thirteen benzophenone-type UV filters. The EC_{50} value was 2.01 mg/L for BP-3.

In this work, four commonly used UV filters were selected for the purpose of analyzing their hormonal and cytotoxic activities using bioluminescent yeast bioreporters, namely BLYES (Sanseverino et al., 2005), BLYAS and BLYR (Eldridge et al., 2007). These low cost, rapid bioassays have been validated for many compounds (Sanseverino et al., 2009), but not tested for UV filters yet. As the majority of *in vitro* tests focus only on the detection of agonistic activities, our aim was to measure antagonistic activities, too. When finding cytotoxic effect, that has not been referred yet our purpose was to investigate toxicity by standard aquatic test organisms, namely the Microtox test which uses *Aliivibrio fischeri* and the fish embryo toxicity test which uses zebrafish. These test organisms are widely used and zebrafish embryos have the advantage of transparency, so main morphological changes can be followed up easily (Hill et al., 2005).

2. Materials and methods

2.1. Chemicals

17ß-estradiol (E2) (purity ≥98%), 5α-dihydrotestosterone (DHT) (≥99%), 4-hydroxytamoxifen (4HT) (50:50 E:Z isomers), OC (certified reference material) and EHMC (≥98%) were purchased from Sigma-Aldrich Co. LLC. Flutamide (FT), BP-3 (≥98%) and 4MBC (≥99%) were purchased from VWR International LLC. FT and 4HT were used as positive controls. Dimethyl sulfoxide (DMSO) was bought from Fischer Scientific (analytical reagent grade) and used for preparing stock solutions that were applied in the Microtox test and in the acute embryo toxicity test. Methanol (purity 99.9%) was bought from Sigma-Aldrich Co. LLC and used for preparing stock solutions that were applied in BLYAS/BLYES/BLYR experiments.

2.2. The measurement of hormonal activities

2.2.1. Bioluminescent yeast systems

The BLYES and BLYAS strains served to detect estrogenic and androgenic activity, respectively. The constructions of these strains are detailed in Eldridge et al. (2007) and Sanseverino et al. (2005). Briefly, the BLYES and BLYAS strains contain the hERα and hAR gene in their chromosome, respectively; while two plasmids with estrogen and androgen response elements (EREs and AREs) and genes (*frp*, *luxCDABE*) are responsible for the production of bioluminescence. When an estrogenic or androgenic compound enters the cell and bind to the hormone receptor, receptor-molecule complexes form dimers and bind to the EREs or AREs to induce the transcription of genes (Eldridge et al., 2007; Sanseverino et al., 2005).

2.2.2. The measurement of estrogenic and androgenic activities using yeast bioreporters

Modified yeast minimal medium (YMM leu-, ura-) is applied for growing *Saccharomyces cerevisiae* strains (Routledge and Sumpter, 1996) at 30 °C and 200 rpm to an OD_{600} of 1.00. The stock solution of UV filters were serially diluted in methanol and 20 μl aliquots were transferred to sterile, flat bottom, black, 96-well microplates (Greiner Bio-one Gmbh, Germany), in 3 parallels. After methanol evaporated, 200 μl of yeast cells were added to the appropriate wells. The serial dilution of E2 and DHT served as positive controls. Negative controls (YMM and yeast cells) and solvent controls (YMM, yeast cells and methanol) were also applied. The bioluminescence was measured after 5 h of incubation at 30 °C by VictorX Multilabel Plate Reader (Perkin Elmer Inc.).

2.2.3. The antiestrogen and antiandrogen assay procedure

To measure antiestrogen and antiandrogen effect, the BLYES and BLYAS assay procedures were modified, as suggested by Sohoni and Sumpter (1998). The serial dilutions of UV filters were supplemented with E2 or DHT in concentration corresponds with EC_{65}. The known antiestrogen and antiandrogen 4HT and FT served as positive controls. Additionally, solvent controls (YMM, yeast cells, methanol) and negative controls as E2/DHT (EC_{65}) were also included.

2.3. The measurement of toxic effects

2.3.1. Saccharomyces cerevisiae BLYR strain

Cytotoxicity was followed up by the constitutive control BLYR strain. This strain also contains the genes responsible for bioluminescence but in spite of the BLYES and BLYAS strains, their transcription is continuous, so cytotoxic effects can lead to the decrease of bioluminescence (Eldridge et al., 2007). The same assay procedure was applied for the BLYR test as described in Section 2.2.2.

2.3.2. Aliivibrio fischeri (MicroTox™)

In order to prove unexpected cytotoxicity measured by BLYR test, further investigations were carried out using *Aliivibrio fischeri* (DSM-7151, NRLLB-11177) bacteria according to ISO 11348-3:2007. *A. fischeri* was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Stock solution of the tested compound was made in DMSO and was diluted in distilled water containing 2% NaCl. The final concentration of DMSO was only 1.665% v/v which is not toxic to *A. fischeri* bacteria. The NaCl and DMSO containing distilled water also served as solvent control. The test was repeated three times in two parallels. Relative bioluminescence was detected by Microtox™ 500 luminometer after 30 min incubation and bioluminescence inhibition was determined.

2.3.3. Modified fish embryo acute toxicity test (OECD 236.)

Fish were bred and maintained at the Institute of Aquaculture and Environmental Safety, Szent Istvan University in a recirculation system (ZebTEC, Tecniplast Inc.) under standard laboratory conditions (recirculation system water: 25°C, 525 ± 50 μS conductivity, with 14 h light–10 h dark cycle). Fish were fed twice a day with complete fish food (zebrafish Small Gran food, Dietex International Limited, Special Diets Services G.B.) supplemented by freshly hatched live *Artemia nauplii* twice a week. The Animal Protocol was approved by the Hungarian Animal Welfare Law (XIV-I-001/2303-4/2012).

The test method was based on the OECD guideline 236 (OECD, 2013) but to be able to detect developmental disorders, the test procedure had to be modified. The applied number of zebrafish embryos was 40 in each concentration in four replicates.

Experiment was prolonged until 120 h post fertilization (hpf) because this period includes time points at which different developmental states can be observed. The stock solution of BP-3 was prepared in DMSO and the serial dilution was made in recirculation system water. Six concentrations of BP-3 were prepared; the values were 1.10E-01 mM, 7.89E-02 mM, 5.26E-02 mM, 3.07E-02 mM, 2.19E-02 mM and 4.38E-03 mM. Every solution was supplemented by a certain volume of DMSO in order to reach the same DMSO concentration (3.52E+00 mM; 250 μl/L). For the facilitation of the solving of BP-3, each solution was ultra-sonificated for 3 min by 20% amplitude (Branson Sonifier 102 C). The system water served as negative control, whereas 3,4-dichloroaniline (2.47E-02 mM) served as positive control. A solvent control was also applied (DMSO). The 24-well plates were kept at 27 ± 1 °C and a 14 h light/10 h dark photoperiod was ensured during the whole experiment. The embryos were checked for the following endpoints every day: mortality, malformations, hatching and the inflation of the swim bladder.

2.4. Chemical analysis

Analytical measurements were carried out in order to determine whether nominal concentrations corresponded to measured concentrations. The analysis was performed on a Trace 1300 Gas Chromatograph-Single Quadrupole Mass Spectrometer. Separations were performed on a SLB™ – 5 ms fused silica capillary column (30 m × 0.25 mm × 0.25 μm). 10 μl of sample was diluted with 990 μl of methanol, and the solution was mixed with vortex mixer for 10 min. 1 μl of solution was injected in split injection mode (split flow rate 30 ml/min) at 200 °C. He (4.6) was used as carrier gas, and the flow rate of the stripping gas was 1.2 ml/min. With SIM measurement, the measured masses were 151, 227 and 228. The temperature of the detector and the ion source were 260 °C and 240 °C, respectively.

2.5. Statistical analysis

Data analysis was performed using the Graphpad Prism version 5.03 program for Windows (GraphPad Software Inc., San Diego California USA). The concentration-response curves were fitted by nonlinear regression and EC_{50}/IC_{50}/LC_{50} values were determined. In the case of hormonal tests, toxic equivalency quotients (TEQ) were calculated in case of full concentration-response curves by dividing the EC_{50} value of the tested compound by the EC_{50} value of the corresponding positive control. Significance analysis was performed by the Wilcoxon signed-ranked test to find difference between reported EC_{50} values for the UV filters. Differences were considered significant at $p < 0.05$. In the case of the *A. fischeri* test, the toxic unit (T.U.) value was determined according to the formula of Sprague and Ramsay (1965): $T.U. = (1/EC_{50})^{*}100$ and was classified according to Vengris et al. (2004): "not toxic" (T.U. < 0.4), "slightly toxic" (0.4 < T.U. < 1), "toxic" (1.1 < T.U. < 10), "very toxic" (11 < T.U. < 100), "extremely toxic" (T.U. > 100). In the case of the fish embryo toxicity test, the malformed embryos' number was normalized to the number of survivals.

3. Results

3.1. The results of the hormonal assays

3.1.1. Estrogen assays

In the case of BP-3, nonmonotonic concentration-response curve (Fig. 1A) could be experienced. The EC_{50} value was 6.44E-03 mM, which was determined from the linearly increasing portion of the inverted U-shaped curve.

According to Fig. 1A the 4MBC displayed submaximal concentration-response curve as the maximal response of the positive control E2 was about 70,000 cps (Fig. 1C). The EC_{50} value for 4MBC

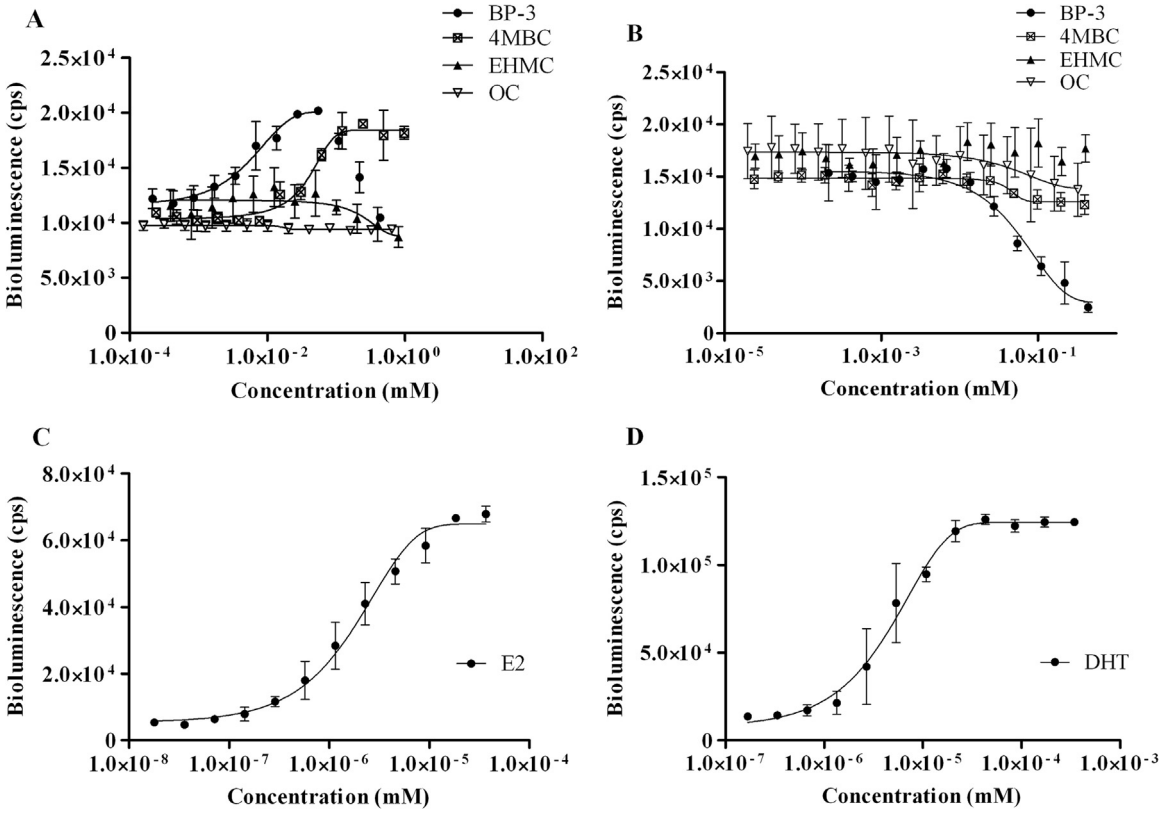

Fig. 1. The concentration-response curves of UV filters and positive controls in the estrogen (A, C) and androgen (B, D) tests.

was 4.04E-02 mM.

EHMC and OC did not prove to be estrogenic in the tested concentration interval (Fig. 1A).

3.1.2. Androgen assays

None of the tested UV filters could increase the bioluminescence of BLYAS strain in a wide concentration interval (Fig. 1B), thus androgenicity of BP-3, 4MBC, EHMC, OC is not proved by BLYAS.

3.1.3. Adapting BLYES/BLYAS tests to analyse antiestrogenic and antiandrogenic effects

The measurement of the antiestrogenic and antiandrogenic effects was carried out indirectly. The base of this procedure: an antagonist is able to decrease the background bioluminescence caused by an agonist. E2 and DHT served as agonists in the antiestrogen and antiandrogen assays. First of all the sensitivity of BLYES and BLYAS strains was tested to E2 and DHT. Both compounds showed full concentration-response curves in the estrogen and androgen experiments (Fig. 1C and D). The EC_{50} value for E2 was 2.02E-06 mM whereas the EC_{50} value for DHT was 4.89E-06 mM. Sanseverino et al. (2009) also reported EC_{50} values for E2 and DHT measured by the BLYES and BLYAS strains. The EC_{50} value for E2 was 6.31E-07 mM whereas the EC_{50} value for DHT was 1.08E-05 mM. There was no significant difference between the results of the two experiments either for E2 or DHT according to the Wilcoxon signed-ranked test.

In order to make the BLYES and BLYAS tests appropriate for measuring the antiestrogenic and antiandrogenic activities it was necessary to determine those concentrations (EC_{65}) of the agonists (E2/DHT) which generate a submaximal response of the BLYES/BLYAS strains. In the case of E2 this value was 3.66E-06 mM and it was 7.06E-06 mM for DHT in the test system. Thus, in antagonist tests, wells received 1.99E-07 mg E2 to analyse antiestrogenic effect, and 4.10E-07 mg DHT to analyse antiandrogenic effect.

3.1.4. Antiestrogen assays

According to Fig. 2A BP-3 showed sigmoidal concentration-response curve in the antiestrogen assay.

Full concentration-response curve could not be measured in the case of 4MBC, since solubility of 4MBC is not higher than 9.83E+01 mM in methanol, so 9.83E+00 mM was the highest tested concentration that was still in the linear part of the sigmoid shaped curve (Fig. 2A).

Although EHMC and OC displayed full concentration-response curves, their antiestrogenic effects were far lower than the effect of the positive control 4HT (Fig. 2A). The EC_{50} value of 4HT was 4.65E-04 mM, whereas the EC_{50} values of EHMC and OC were 7.46E+00 mM and 1.87E+01 mM, respectively. The calculated TEQ values were 16,043 and 40,215 for EHMC and OC, respectively. Interestingly, OC showed biphasic concentration-response curve (Fig. 2B).

3.1.5. Antiandrogen assays

According to Fig. 2/C BP-3 and 4MBC were as antiandrogenic as the positive control FT was. The EC_{50} value of FT was 9.38E-03 mM whereas the EC_{50} values of BP-3 and 4MBC were 1.02E-02 mM and 2.88E-02 mM, respectively. The TEQ values of BP-3 and 4MBC were 1.09 and 3.07, respectively.

EHMC could decrease the bioluminescence of BLYAS strain by 50% at 6.97E+00 mM concentration. The TEQ value was 742.91.

Similarly to the results of the antiestrogen assay OC showed biphasic concentration-response curve (Fig. 2D). The EC_{50} value was 7.29E+00 mM and the TEQ value was 777.78.

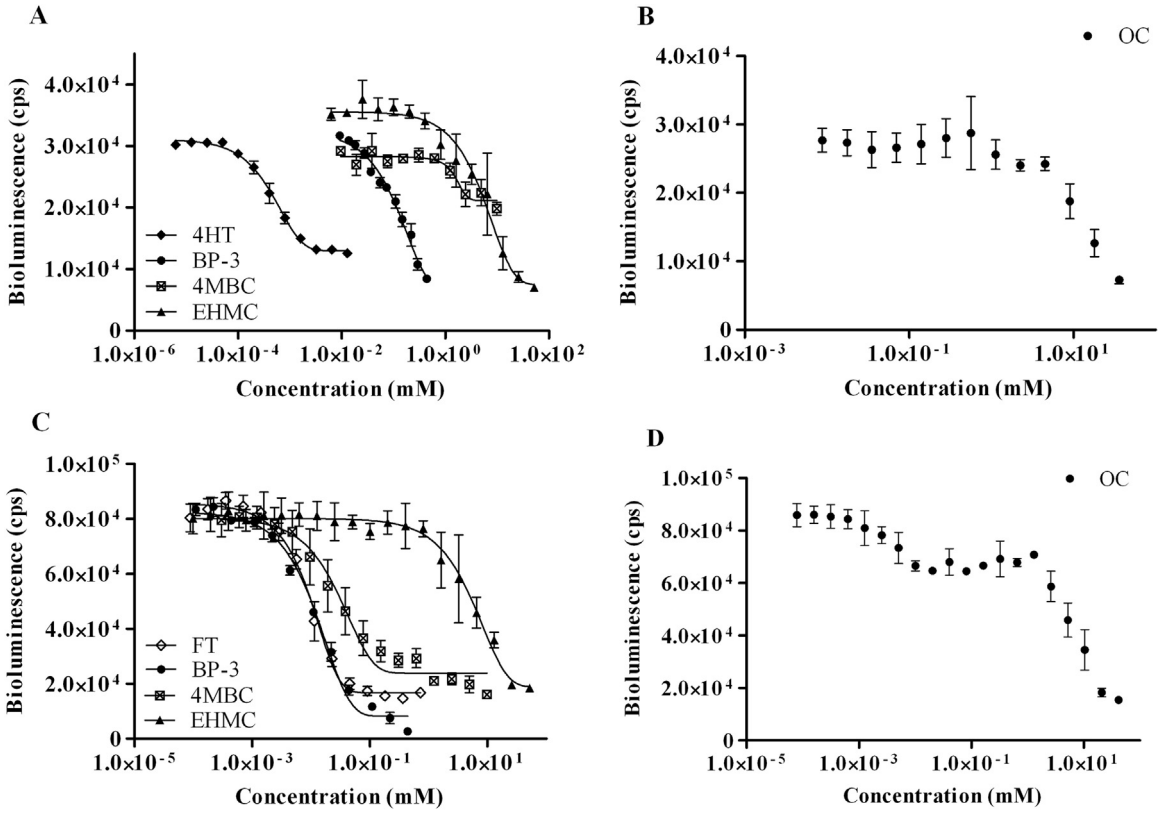

Fig. 2. The antiestrogenic (A) and antiandrogenic (C) activities of positive controls and UV filters. The biphasic concentration-response curves of octocrylene measured in antiestrogen (B) and antiandrogen (D) tests.

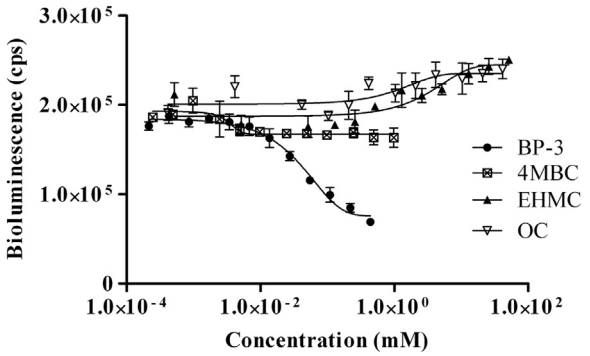

Fig. 3. Cytotoxic effects of the four UV filters measured by the BLYR strain.

3.2. Results of the toxicity tests

The stock solution of BP-3 (4.38E+02 mM nominal concentration) that was applied in the Microtox test and in the fish embryo toxicity test proved to be 5.16E+02 mM by GC–MS. This stock solution was diluted in the assays.

3.2.1. Saccharomyces cerevisiae BLYR strain

Among the four UV filters only BP-3 proved to be cytotoxic in the tested concentration interval according to the BLYR test (Fig. 3). The calculated IC_{50} value was 4.67E-02 mM. Interestingly, in the case of EHMC and OC a 50% increase of bioluminescence could be experienced at 4.34E+00 mM and 1.22E+00 mM, respectively (Fig. 3). Presumably these two UV filters could enhance the proliferation of the yeast cells at higher concentrations.

3.2.2. Aliivibrio fischeri (MicroTox™)

Out of the four UV filters only BP-3 showed cytotoxicity in the BLYR test (Fig. 3), so the toxic effect of BP-3 was tested on *A. fischeri*, too (Fig. 4). The IC_{50} value was 2.40E-02 mM and the T.U. was 3.30, so BP-3 can be ranked as a toxic compound.

3.2.3. Modified fish embryo acute toxicity test (OECD 236.)

Cumulative mortality was under 10% in the negative and solvent control groups at the end of the experiment, whereas it was 75% in the case of the positive control. The percentage of the hatched embryos was 95–95% in the negative and solvent controls, while there were no hatched embryos in the positive control. There were no malformations in the negative and solvent control groups except one individual in the solvent control at which no swim bladder could be observed.

The highest, middle and lowest nominal concentrations (1.10E-01 mM, 5.26E-02 mM and 4.38E-03 mM) were proved by GC–MS, which results are 1.16E-01 mM, 5.23E-02 mM and 5.35E-03 mM, respectively; thus deviations are 6.00E-03 mM, 3.00E-04 mM and 9.70E-04 mM.

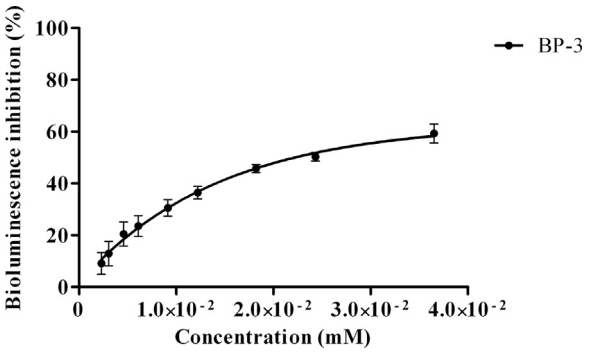

Fig. 4. Bioluminescence inhibition of benzophenone-3 in the *Aliivibrio fischeri* test.

Mortality was determined after the 72 hpf, 96 hpf and 120 hpf of exposure (Fig. 5A). The LC_{50} values were 7.66E-02 mM, 6.98E-02 mM and 5.73E-02 mM, respectively. The 95% confidence intervals (95% CI) were 6.97E-02–8.42E-02 mM (72 hpf), 6.14E-02–7.94E-02 mM (96 hpf) and 4.87E-02–6.75E-02 mM (120 hpf).

In the 4.38E-03 mM concentration group all embryos could inflate their swim bladder (Fig. 6B) but in higher concentration groups BP-3 caused the lack of swim bladder inflation in a concentration dependent way (Fig. 6C–F). The calculated EC_{50} value was 2.95E-02 mM (95% CI: 2.70E-02–3.22E-02 mM) after 120 hpf of exposure (Fig. 5B).

After 72 hpf of exposure the deformation of the tail (Fig. 6D and E) could be observed with the EC_{50} value of 4.19E-02 mM (95% CI: 3.35E-02–5.24E-02 mM) (Fig. 5C). In addition to the deformation of the tail the malformation of the somites could be experienced at the 5.26E-02 and 7.89E-02 mM concentrations.

BP-3 decreased the number of the hatched embryos (Fig. 5D) after 96 hpf. The EC_{50} value was 5.43E-02 mM (95% CI: 5.09E-02–5.79E-02 mM).

In addition to the above mentioned malformations some other deformations were detected but their incidence was not concentration dependent. BP-3 caused pericardial and yolk sac oedema to the embryos (Fig. 6E and F). Furthermore, some embryos had deformed jaw and ventricle or dilatated gut (Fig. 6C–E). Jaw deformity was mostly present in the 4.38E-03 mM concentration groups (Fig. 6B) but it also occurred at 3.07E-02, 5.26E-02 and 7.89E-02 mM concentrations (Fig. 6D and E).

4. Discussion

4.1. Hormonal assays

4.1.1. Estrogen assays

BP-3 showed nonmonotonic concentration-response curve in the BLYES test (Fig. 1A) as bioluminescence intensification was followed by the decrease of bioluminescence. BP-3 was cytotoxic to the BLYR strain in the same concentration interval (Fig. 3.) as where the descending part of the curve can be found in the BLYES test. The estrogenic activity of BP-3 had already been measured by the Yeast Estrogen Screen (YES) expressing hERα (Kunz and Fent, 2006), while Schlumpf et al. (2001) described the estrogenic activity of BP-3 by MCF-7 cell line. In addition, BP-3 could increase vitellogenin synthesis in fish but not at environmentally relevant concentrations (Coronado et al., 2008).

According to the result of the BLYES test 4MBC displayed partial estrogenic activity. In spite of this result Kunz and Fent (2006) did not find 4MBC estrogenic in the YES test. Schmitt et al. (2008) measured partial estrogenic activity of 4MBC but they used a modified YES test. The digestive enzyme lyticase was added to each well for enhancing the sensitivity of the YES test. Schmitt et al. (2008) calculated an EC_{50} value of 4.43E-02 mM for 4MBC that did not differ significantly from the EC_{50} value measured by the BLYES test. Estrogenic activity of 4MBC was measured on MCF-7 cell line, too. Klann et al. (2005) found 4MBC to be able to significantly increase the proliferation of MCF-7 cells at 9.99E-03 mM concentration.

Similarly to the results of the present experiment, Kunz and Fent (2006) detected no estrogenic activity in the case of EHMC. In spite of the results of *in vitro* experiments EHMC can have estrogenic activity *in vivo*. Zucchi et al. (2011) studied the effects of 7.58E-06 mM and 3.06E-03 mM EHMC on the gene expression of male zebrafish. They concluded that EHMC has potential estrogenic activity in view of the induced transcriptional changes of the genes related to the hormone system or steroidogenesis.

OC showed no estrogenic activity in the BLYES test or in the YES

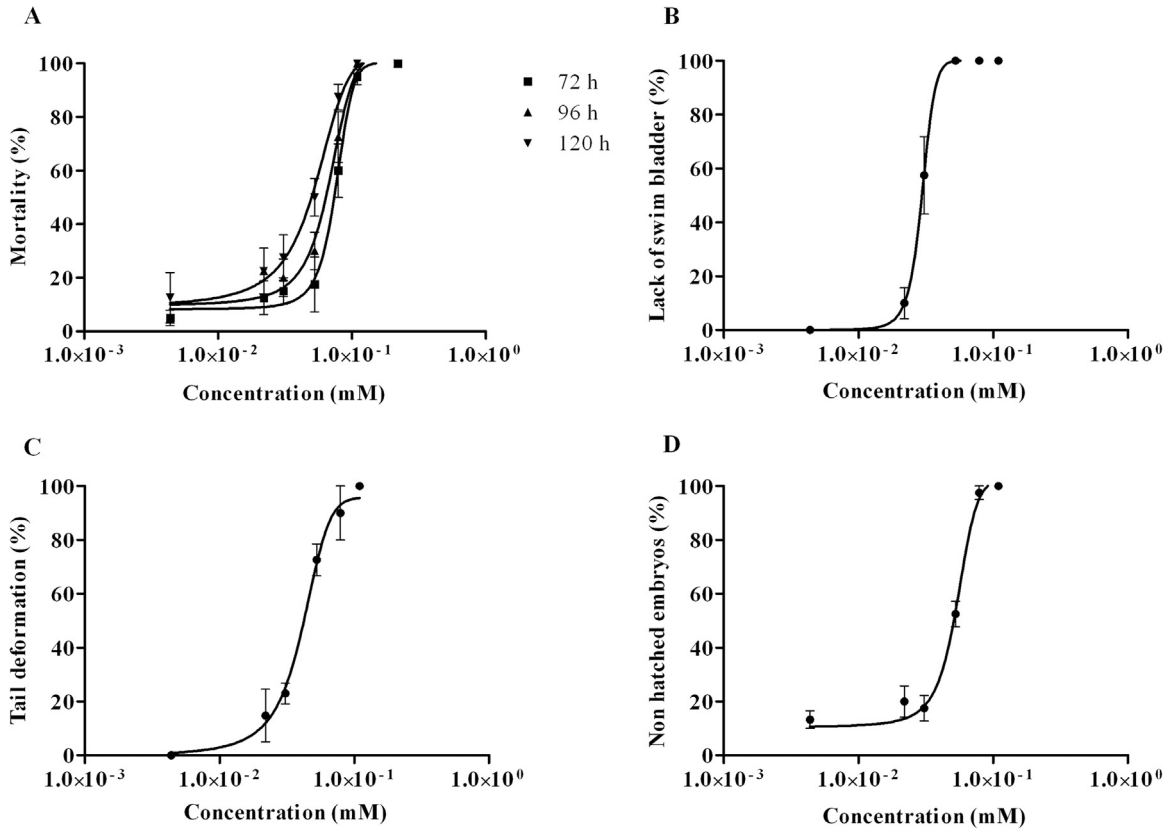

Fig. 5. The concentration-response curves show mortality (A), the lack of swim bladder inflation (B), the tail deformation (C) and the unsuccessful hatching (D) in the percentage of the survived embryos after exposing them to different concentrations of benzophenone-3.

test (Kunz and Fent, 2006).

4.1.2. Androgen assays

Regarding the androgenic activity of the four UV filters, the results are contradictory in the literature. Similarly to the results of the BLYAS test Ma et al. (2003) found no androgenic activity of BP-3, 4MBC and EHMC in the MDA-kb2 cell transcriptional activation assay. Suzuki et al. (2005) also reported no androgenic effect for BP-3 by NIH3T3 cells. However Kunz and Fent (2006) reported the partial androgenic activity of EHMC and OC, the EC_{50} values were 1.01E+01 mM and 6.28E-01 mM, respectively. Zucchi et al. (2011) also found EHMC androgenic in zebrafish.

4.1.3. Antiestrogen assays

Although BP-3 showed sigmoidal concentration-response curve in the antiestrogen assay (Fig. 2A), its antiestrogenic activity can be attributed to cytotoxicity measured by the constitutive control BLYR strain (Fig. 3).

Kunz and Fent (2006) found BP-3 to be the most potent antiestrogen among the tested compounds. BP-3 also showed antiestrogenic effects in a 14-days *in vivo* zebrafish assay at lower concentrations as it was tested in the present assay (Blüthgen et al., 2012), so it is important to test chemicals on organisms belonging to higher developmental levels.

In the present experiment, 4MBC showed antiestrogenic activity but only in high concentrations. In the experiment of Kunz and Fent (2006) 4MBC displayed full concentration-response curve and the calculated EC_{50} value was 8.73E-02 mM.

In comparison to the results of Kunz and Fent (2006) the sensitivity of BLYES strain is lower to EHMC and OC in the antiestrogen assay. According to Beresford et al. (2000) incubation time can significantly influence the sensitivity of an assay. The incubation period is 3 days in the YES assay whilst it only takes 5 h in the BLYES test (Routledge and Sumpter, 1996; Sanseverino et al., 2005). Additionally, there is dissimilarity between the two tests regarding the method of hormonal activity detection, as in the case of the YES assay ß-galactosidase activity (Routledge and Sumpter, 1996), whereas in the case of BLYES assay bioluminescence (Sanseverino et al., 2005) is the indicator of the hormonal activity. This fact can also contribute to the difference of the measured values.

Zucchi et al. (2011) also found EHMC to antiestrogenic in zebrafish on the basis of the induced transcriptional changes of the genes related to the hormone system or steroidogenesis.

In the present study OC showed biphasic concentration-response curve. Endocrine disrupting compounds (EDCs) can often produce nonmonotonic concentration-response curves, due to different mechanisms. In most cases cytotoxicity is responsible for the nonmonotonic relationship. But in this case cytotoxicity was not detected according to the BLYR strain. In addition, receptor ligand selectivity, differential expression of receptors and co-regulators, ligand-induced receptor down-regulation, competition between multiple receptors, endocrine negative feedback loops, receptor effects on multiple endpoints and binding to secondary nuclear receptor site can also be the mode of actions that generate nonmonotonic concentration-response curves (Cookman and Belcher, 2014; Vandenberg et al., 2012). In this case it can be assumed that OC can bind to different binding sites of the receptor in different concentration intervals.

4.1.4. Antiandrogen assays

Similarly to the present experiment, Kunz and Fent (2006) found BP-3 and 4MBC to be antiandrogenic. Their reported EC_{50} values were 3.68E-03 mM and 1.18E-02 mM for BP-3 and 4MBC, respectively. These values did not differ significantly from the EC_{50} values of the present experiment. In addition, in the experiment of

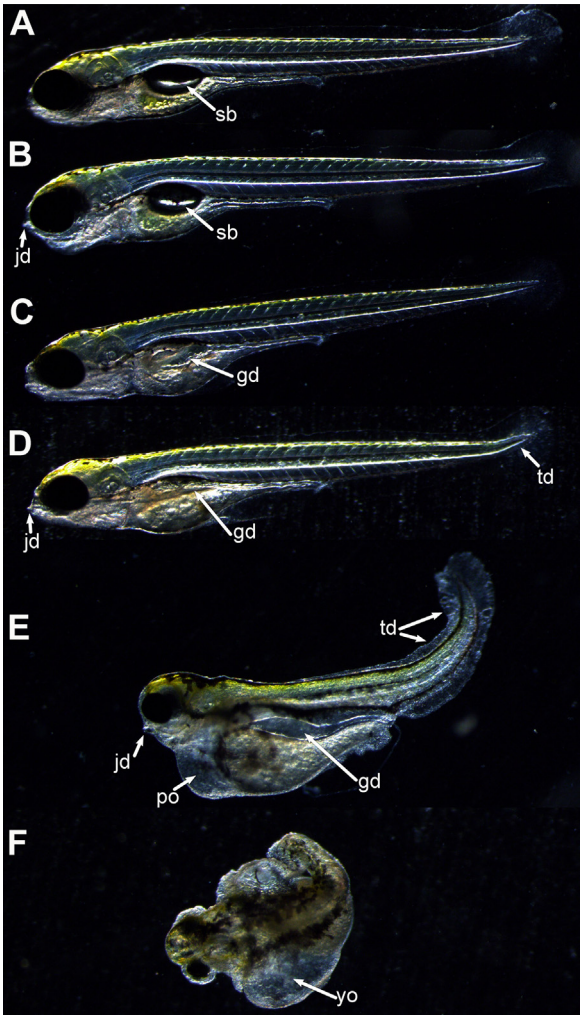

Fig. 6. The different malformations occurred on developing zebrafish embryos after BP-3 exposure. In control (A) and 4.38E-03 mM BP-3 groups all embryos showed inflated swim bladder (sb) at 120 h post fertilization. Jaw deformities (jd) were visible on numerous individuals in the lowest concentration (B) group. Inhibition of swim bladder filling were observed in 2.19E-02 mM and higher BP-3 treated groups (C–F), which showed dose dependence. In the case of embryos treated by 2.19E-02 (C), 3.07E-02 (D) and 5.26E-02 (E) mM gut dilatation (gd) was observed. Further jaw malformations and tail deformities (td) were detected on 3.07E-02 (D), 5.26E-02 (E) and 7.89E-02 mM treated embryos. Tail deformity, accompanied by the malformation of somites in 5.26E-02 and 7.89E-02 mM groups where pericardial (po) and yolk oedema (yo) occurred, too.

Ma et al. (2003) BP-3 could significantly antagonise the activity of DHT in MDA-kb2 cells. BP-3 was found to be antiandrogenic in a 14-days zebrafish assay as it could significantly down-regulate the expression of AR and the 17ß-hydroxysteroid dehydrogenase type 3 genes (Blüthgen et al., 2012).

In spite of the aforementioned results, BP-3 showed only low antiandrogenic activity in NIH3T3 cells measured by Suzuki et al. (2005). 4MBC did not prove to be antiandrogenic at all in MDA-kb2 cells (Ma et al., 2003).

Kunz and Fent (2006) also reported EHMC as an antiandrogen. They found the EC_{50} value of this compound two magnitudes lower as in the present experiment. According to Zucchi et al. (2011) EHMC proved to be antiandrogenic on the basis of down-regulation of genes related to the hormone system or steroidogenesis. Axelstad et al. (2011) exposed Wistar rats to 500, 750 and 1000 mg EHMC/ kg bw/day during the gestational and postnatal period. The testosterone level of male offsprings and the number of sperms of adult offsprings decreased due to a 500 mg EHMC/kg bw/day dose. 750 and 1000 mg EHMC/kg bw/day doses significantly reduced the testes and prostate weight, respectively.

In contrast to the aforementioned results, Ma et al. (2003) found that EHMC was not antiandrogenic in MDA-kb2 cells.

The EC_{50} value of OC was 2.45E-02 mM in the assay of Kunz and Fent (2006) but they did not test the antiandrogenic effect of OC in higher concentrations where OC was found antiandrogenic in the present assay. Similarly to the antiestrogen test OC showed biphasic concentration-response curve in the antiandrogen experiment, the possible reasons of which are discussed in Section 4.1.3.

4.2. Toxicity

4.2.1. Saccharomyces cerevisiae BLYR strain

The results of the BLYR test revealed that BP-3 is cytotoxic to yeasts, while EHMC and OC can enhance the proliferation of the yeasts at higher concentrations. Kunz and Fent (2006) examined the effects of UV filters on yeast growth, which was determined by absorbance at 620 nm, but they found neither of BP-3, 4MBC, EHMC and OC to be cytotoxic or influential to yeast growth.

4.2.2. Aliivibrio fischeri (MicroTox™)

BP-3 proved to be cytotoxic to *A. fischeri* bacteria, as it was found in the BLYR test. BP-3 is registered by the ECHA (European Chemicals Agency) as 100–1000 t are produced annually. A toxicity test was carried out measuring the respiration inhibition of microorganisms in sewage sludge while exposed to different concentrations of BP-3. In this experiment the EC_{50} value was above 100 mg/L concentration after 3 h exposure (http1).

Liu et al., (2015a) examined the toxicological effects of benzophenone-type UV filters on *Photobacterium phosphoreum*, which was formerly used in Microtox test (Wells et al., 1997) as well, and which is, similarly to *A. fischeri*, a marine luminescent bacterium. BP-3 decreased the bioluminescence of *P. phosphoreum* by 50% at 14.27 mg/L concentration. The authors revealed that the toxicity of benzophenones to *P. phosphoreum* is related to the electronic properties of these compounds.

4.2.3. Modified fish embryo acute toxicity test (OECD 236.)

BP-3 is ranked as “Very toxic to aquatic life” and “Toxic to aquatic life with long lasting effects” by the ECHA (http2). For the registration of BP-3, its short-term toxic effects were tested on adult Japanese medaka (*Oryzias latipes*) based on a method equivalent to the OECD 203 guideline. The applied concentrations were 2.27; 2.73; 3.28, 3.93 and 4.72 mg/L. Mortality was determined every day during the 96 h exposure, the LC_{50} value was 3.8 mg/L calculated at the end of the experiment (http3). In the present assay a higher LC_{50} value (6.98E-02 mM = 15.93 mg/L) was found for zebrafish embryos after 96 hpf of exposure than what was calculated for adult Japanese medaka. It can be assumed that there is a difference in the sensitivity of the two fish species and lifestages. Liu et al., (2015b) examined the ability of four benzophenone type UV filters to pose hepatic oxidative stress to *Carassius auratus*. They found that 0.5 mg/L and 5 mg/L BP-3 can induce oxidative stress to the fish after 7, 14 and 28 days of exposure. Moreover, BP-3 causes histological alterations in the liver at 0.5 mg/L concentration after 28 days of exposure.

Blüthgen et al. (2012) examined the toxicity of BP-3 to adult male zebrafish and eleuthero-embryos at lower concentrations than in the present experiment. They exposed the eleuthero-embryos and the adult male zebrafish to 10, 200 and 600 μg/L BP-3 for 120 h and 14 days, respectively. At the end of the experiment, no adult fish or eleuthero-embryo mortality occurred due to BP-3 exposure, and eleuthero-embryos showed normal swimming behaviour. However, BP-3 altered the gene expression in the sex hormone system of adult zebrafish and eleuthero-embryos.

According to the results of the present experiment, BP-3 causes the lack of swim bladder inflation in a concentration dependent way. The lack of swim bladder inflation can lead to the death of the fish as it can adversely influence swimming and feeding. Incardona et al. (2004) found that compounds containing benzene ring can cause swim bladder defects. BP-3 has two benzene rings in its structure. Hagenaars et al. (2011) experienced the uninflation of swim bladder exposing zebrafish embryos to perfluorinated compounds. They found that this kind of malformation resulted in abnormal swimming behaviour.

BP-3 decreases the number of hatched embryos concentration dependently in the present assay (EC_{50}=5.43E-02 mM=12.39 mg/L). Hatching enzymes and the contractile movements of the embryo are necessary for a successful hatching. As the exposure to BP-3 results in the deformation of the tail, it can also contribute to unsuccessful hatching by hindering the movements of the embryo. Coronado et al. (2008) exposed Japanese medaka to nominal concentration of 10, 100 and 1000 μg/L BP-3 for 21 days and monitored the number of produced eggs and the hatching success. They found that BP-3 significantly reduced the percentage of hatched eggs at 620 μg/L measured concentration (Coronado et al., 2008).

In the present experiment, BP-3 causes tail deformation to the zebrafish embryos concentration dependently. Tail deformation was experienced by Incardona et al. (2004) after exposing zebrafish embryos to benzene ring containing fluorene, dibenzothiophene and phenanthrene. In addition to this, dibenzothiophene and phenanthrene caused serious pericardial and yolk sac oedema, and cardiac dysfunction could be observed. They pointed out that cardiac dysfunction can lead to malformations like dorsal and tail curvature. Impaired circulation can adversely affect kidney morphogenesis which can result in yolk sac oedema (Incardona et al., 2004). There can be some other explanations for tail deformation like apoptosis in the tail region and alterations of the muscle fibres in the tail (Huang et al., 2010).

In the present experiment, the impaired development of jaw can be observed at 4.38E-03, 3.07E-02, 5.26E-02 and 7.89E-02 mM (1, 7, 12 and 18 mg/L) concentrations. Teraoka et al. (2002) experienced the growth inhibition of the lower jaw of zebrafish embryos exposing them to 0.3–1 μg/L 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). They assumed that TCDD could adversely affect chondrogenesis in lower jaw primordia via an aryl hydrocarbon receptor dependent mechanism.

In regard to the malformations caused by BP-3 it is necessary to examine whether BP-3 can activate the aryl hydrocarbon receptor. As for hatching, further investigations are needed to reveal whether BP-3 affects the hatching enzyme or the movement of the embryos. Adverse effects on swimming could explain the lack of swim bladder inflation, too. In order to understand the mode of action of BP-3 in the tail, the examination of apoptosis and detailed histological observations are needed. Furthermore, the effects of BP-3 on the heart and circulatory system should be observed.

5. Conclusions

In the present study the hormonal and cytotoxic effects of four frequently used UV filters were investigated using bioluminescent *Saccharomyces cerevisiae* strains. In case of cytotoxicity *Aliivibrio fischeri* and zebrafish were also used to investigate toxicity to aquatic organisms. Modification of the *Saccharomyces* assay procedure gave opportunity for the detection of antiestrogenic and antiandrogenic effects. Multiple hormonal activities were found in the case of the four UV filters. Moreover, BP-3 showed cytotoxic effect, whereas EHMC and OC enhanced yeast growth. BP-3 proved to be estrogenic and antiandrogenic; 4MBC had estrogenic, antiestrogenic and antiandrogenic effects, whilst EHMC and OC proved to be antiestrogenic and antiandrogenic. Regarding BP-3 in the estrogen assay and OC in the antiestrogen and antiandrogen assays, nonmonotonic concentration-response curves occurred. In spite of the fact that the calculated EC_{50} values are much higher than the environmental concentrations of these UV filters, the hormonal activities of these compounds should not be disregarded, since BP-3 and 4MBC proved to have as intense *anti*-androgenicity as the positive control FT has. Regarding the cytotoxicity of BP-3, the chemical was tested by Microtox (ISO11348) on *A. fischeri*, and proved to be cytotoxic to the bacteria. To underpin the toxicity of BP-3 at a higher developmental level a modified fish embryo toxicity test was carried out on zebrafish. Exposure to BP-3 caused mortality, unsuccessful hatching and different malformations to the zebrafish embryos. To our best knowledge this is the first time that lack of swim bladder inflation, tail deformation and impaired development of the jaw was found in connection with BP-3 exposure. These kinds of deformities lead to the death of fish later as they are seriously hindered in their movement and feeding. As information on the toxicological and developmental effects of BP-3 to fish are scarce, the results of the present assay can help to understand the risk of this compound. In conclusion, BLYES and BLYAS test organisms are appropriate to monitor not just agonist but antagonist hormonal effects. According to the results of the present study, alongside the endocrine disruption of the tested UV filters, BP-3 may pose a significant ecotoxicological risk as well, as standard water quality testing assays (ISO11348, OECD 236) detect ecotoxicity.

Acknowledgement

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Multiple hormonal activities of UV filters and comparison of *in vivo* and *in vitro* estrogenic activity of ethyl-4-aminobenzoate in fish

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Abstract

UV filters have been detected in surface water, wastewater and fish, and some of them are estrogenic in fish. At present, little is known about their additional hormonal activities in different hormonal receptor systems despite their increasing use and environmental persistence. Besides estrogenic activity, UV filters may have additional activities, both agonistic and antagonistic in aquatic organisms. In our study, we investigate a series of UV filters for multiple hormonal activities *in vitro* in human receptor systems and evaluate the predictive value of these findings for the activity in fish *in vitro* and *in vivo*. First we systematically analysed the estrogenic, antiestrogenic, androgenic, and antiandrogenic activity of 18 UV filters and one metabolite *in vitro* at non-cytotoxic concentrations with recombinant yeast systems carrying either a human estrogen (hERα) or androgen receptor (hAR). All 19 compounds elicited hormonal activities, surprisingly most of them multiple activities. We found 10 UV-filters having agonistic effects towards the hERα. Surprisingly, we identified for the first time six UV filters with androgenic activities and many of them having pronounced antiestrogenic and antiandrogenic activities. As much as 17 compounds inhibited 4,5-dihydrotestosterone activity in the hAR assay, while 14 compounds inhibited estradiol activity in the hERα assay, indicating antiandrogenic and antiestrogenic activity, respectively. In particular, the antiandrogenic activities of phenyl- and benzyl salicylate, benzophenone-1 and -2, and of 4-hydroxybenzophenone were higher than that of flutamide, a known hAR antagonist.

In a second series of experiments, we investigated the predictive power of the hERα assay for aquatic organisms by further investigating the estrogenic UV filter ethyl 4-aminobenzoate (Et-PABA) *in vitro* and *in vivo* in fish. Et-PABA showed estrogenic activity in a recombinant yeast system carrying the rainbow trout estrogen receptor (rtERα) with higher activity than in the hERα assay. In addition, Et-PABA induced vitellogenin after 14 days of exposure in juvenile fathead minnows at 4394 μg/L. Our study shows estrogenic activity of this UV filter in fish both *in vitro* and *in vivo*. In conjunction with *in vitro* human receptor-based systems our results give a more detailed picture about distinct hormonal activities of UV filters occurring in aquatic systems. We conclude that receptor-based assays are important for *in vitro* assessment of UV-filters prior to or concurrently with *in vivo* assays, which ultimately provide data for the environmental risk assessment of these important personal care products.

Other Papers of Interest:

Toxicol Sci. 2006 Apr;90(2):349-61. Epub 2006 Jan 10.

Comparison of in vitro and in vivo estrogenic activity of UV filters in fish.

Kunz PY[1], Galicia HF, Fent K.

Abstract

In this work, we evaluate whether in vitro systems are good predictors for in vivo estrogenic activity in fish. We focus on UV filters being used in sunscreens and in UV stabilization of materials. First, we determined the estrogenic activity of 23 UV filters and one UV filter metabolite employing a recombinant yeast carrying the estrogen receptor of rainbow trout (rtERalpha) and made comparisons with yeast carrying the human hERalpha for receptor specificity. Benzophenone-1 (BP1), benzophenone-2 (BP2), 4,4-dihydroxybenzophenone, 4-hydroxybenzophenone, 2,4,4-trihydroxy-benzophenone, and phenylsalicylate showed full dose-response curves with maximal responses of 81-115%, whereas 3-benzylidene camphor (3BC), octylsalicylate, benzylsalicylate, benzophenone-3, and benzophenone-4 displayed lower maximal responses of 15-74%. Whereas the activity of 17beta-estradiol was lower in the rtERalpha than the hERalpha assay, the activities of UV filters were similar or relatively higher in rtERalpha, indicating different relative binding activities of both ER. Subsequently, we analyzed whether the in vitro estrogenicity of eight UV filters is also displayed in vivo in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Of the three active compounds in vivo, 3BC induced vitellogenin at lower concentrations (435 microg/l) than BP1 (4919 microg/l) and BP2 (8783 microg/l). The study shows, for the first time, estrogenic activities of UV filters in fish both in vitro and in vivo. Thus we propose that receptor-based assays should be used for in vitro screening prior to in vivo testing, leading to environmental risk assessments based on combined, complementary, and appropriate species-related assays for hormonal activity.

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Estrogenic activity of UV filter mixtures.

Kunz PY[1], Fent K.

Abstract

UV-absorbing chemicals (UV filters) are widely used for protection against UV radiation in sunscreens and in a variety of cosmetic products and materials. Depending on the breadth and factor of UV protection, they are added as single compounds or as a combination thereof. Some UV filters have estrogenic activity, but their activity and interactions in mixtures are largely unknown. In this work, we analyzed 8 commonly used UV filters, which are pure or partial hERalpha agonists, for their estrogenic activity in equieffective mixtures in a recombinant yeast assay carrying the human estrogen receptor alpha (hERalpha). Mixtures of two, four and eight UV filters alone, or in combination with 17 beta estradiol (E2), were assessed at different effect levels and no-observed-effect-concentrations (NOEC). Predictions of the joint effects of these mixtures were calculated by employing the concentration addition (CA) and independent action (IA) model. Most binary mixtures

comprising of pure hERalpha agonists showed a synergistic activity at all mixture combinations. Only in combination with benzophenone-1, antagonistic activity was observed at some effect levels. All mixtures of four or eight, pure or pure and partial hERalpha agonists, alone or including E2, showed synergistic activity at concentrations giving an increase of 10% of basal activity (BC10). This occurred even at concentrations that were at the NOEC level of each single compound. Hence, there were substantial mixture effects even though each UV filter was present at its NOEC level. These results show that significant interactions occur in UV filter mixtures, which is important for the hazard and risk assessments of these personal care products.

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Ultraviolet filters differentially impact the expression of key endocrine and stress genes in embryos and larvae of *Chironomus riparius*

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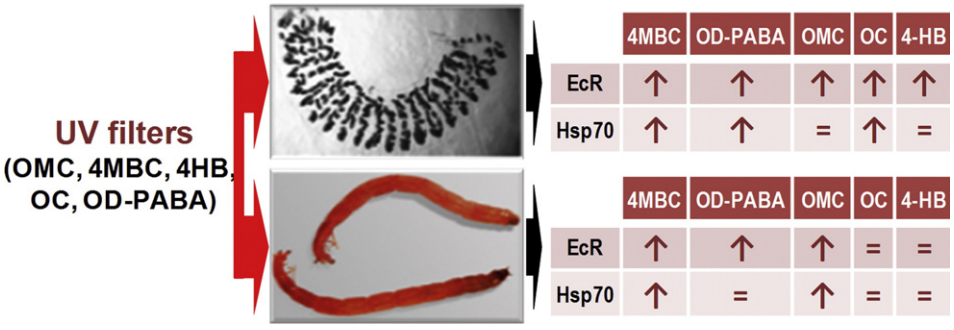
HIGHLIGHTS

- UV filters show scarce effects on the survival of larvae and embryos.
- All the UV filters tested altered *EcR* gene expression in embryos.
- 4MBC, OD-PABA, and OC also alter *hsp70* gene expression in embryos.
- Larvae are less sensitive, only 4MBC and OMC alter *EcR* and *hsp70* genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Several organic UV filters have hormonal activity in vertebrates, as demonstrated in fishes, rodents and human cells. Despite the accumulation of filter contaminants in aquatic systems, research on their effects on the endocrine systems of freshwaters invertebrates is scarce. In this work, the effects of five frequently used UV filters were investigated in embryos and larvae of *Chironomus riparius*, which is a reference organism in ecotoxicology. *LC50* values for larvae as well as the percentage of eclosion of eggs were determined following exposures to: octyl-*p*-methoxycinnamate (OMC) also known as 2-ethylhexyl-4-methoxycinnamate (EHMC); 4-methylbenzylidene camphor (4MBC); 4-hydroxybenzophenone (4HB); octocrylene (OC); and octyldimethyl-*p*-aminobenzoate (OD-PABA). To assess sublethal effects, expression levels of the genes coding for the ecdysone receptor (*EcR*) and heat shock protein HSP70 were investigated as biomarkers for endocrine and stress effects at the cellular level. Life-stage-dependent sensitivity was found. In embryos, all of the UV filters provoked a significant overexpression of *EcR* at 24 h after exposure. OC, 4MBC and OD-PABA also triggered transcriptional activation of the *hsp70* stress gene in embryos. In contrast, in larvae, only 4MBC and OMC/EHMC increased *EcR* and *hsp70* mRNA levels and OD-PABA upregulated only the *EcR* gene. These results revealed that embryos are particularly sensitive to UV filters, which affect endocrine regulation during development. Most UV filters also triggered the cellular stress response, and thus exhibit proteotoxic effects. The differences observed between embryos and larvae and the higher sensitivity of embryos highlight the importance of considering different life stages when evaluating the environmental risks of pollutants, particularly when analyzing endocrine effects.

1. Introduction

Organic, ultra-violet (UV) filters are considered to be emerging contaminants due to their high production volume and increasing use, not

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only in sunscreen but also in many daily use products, such as cosmetics, plastics, and varnish. UV filters are regulated and have to be declared in cosmetics but remain essentially unidentified in technical products (Regulation (EC) No. 1223/2009). Different studies have reported their presence in aquatic environmental samples (Grabicova et al., 2013; Tarazona et al., 2010). Appreciable amounts of UV filters residue has also been detected in tap water, wastewaters and treated sewage sludge (Gago-Ferrero et al., 2011), as well as in continental and coastal waters (Fent et al., 2010a; Tovar-Sánchez et al., 2013). There is also evidence of the presence of these compounds in aquatic biota, such as fishes, aquatic birds and aquatic macroinvertebrates, and in urine and blood samples from humans as well (Buser et al., 2006; Calafat et al., 2008; Fent et al., 2010b; Zhang et al., 2013). Recently, the benefits and dangers of UV filters have come under debate due to suspicion of their potential endocrine disruptive effects (Krause et al., 2012). The first evidence of the hormonal action of certain UV filters came from *in vitro* studies in human cells, as well as from *in vitro* and *in vivo* tests in rodents that demonstrated activation of steroid receptors (Schlumpf et al., 2001). Most UV filters subsequently investigated elicit hormonal activity in vertebrates, in a similar range to that induced by other well-known endocrine disrupting compounds (Díaz-Cruz and Barceló, 2009). Endocrine-Disrupting Compounds (EDCs) include a wide variety of chemicals, both natural and man-made, that may be found in many everyday products, and have the ability to mimic, alter or block the action of hormones. A large number of the tested UV absorbing compounds have shown estrogenic activity both *in vivo* and *in vitro* (Holbech et al., 2002; Klammer et al., 2005; Kunz et al., 2006; Kunz and Fent, 2006; Mueller et al., 2003; Schlumpf et al., 2004; Schreurs et al., 2002). Recent studies in mammals and fishes have demonstrated that estrogen, as well as other hormonal targets are affected by UV filters. Certain UV filters show antiandrogenic activities (Ma et al., 2003; Schreurs et al., 2005; Suzuki et al., 2005), whereas others are suspected of having thyroid-disrupting properties (Axelstad et al., 2011; Klammer et al., 2007; Schmutzler et al., 2007; Seidlová-Wuttke et al., 2006).

Research into the adverse effects of UV filters has mainly concentrated on assessing the potential risk for humans. Studies on the toxicological impact of UV filters in invertebrate species are scarce in comparison to those conducted on vertebrate models. Although these contaminants ultimately pollute aquatic systems, risk assessment for freshwater invertebrates remains widely unexplored. Toxicity data for aquatic invertebrate biota are very limited, and the impact on invertebrate endocrine systems is unknown. Although several alterations in life-cycle parameters have been reported (Fent et al., 2010b; Kaiser et al., 2012; Schmitt et al., 2008; Sieratowicz et al., 2011; Tovar-Sánchez et al., 2013), whether they are based on the effects of UV filters on invertebrate hormonal pathways needs to be determined using molecular assays. We recently published data study indicating that UV filters interfere with insect endocrine pathways, because exposure to 4-methylbenzylidene camphor (4MBC), octyl-*p*-methoxycinnamate (OMC) and octyldimethyl-*p*-aminobenzoate (OD-PABA) activates the ecdysone receptor gene in *Chironomus riparius* larvae (Ozáez et al., 2013). Our results also demonstrated that benzophenone-3 (BP-3) behaves as the hormone ecdysone, regulating a cascade of key endocrine genes in explanted organs. These data represent the first direct evidence of hormonal activity in an invertebrate endocrine system (Ozáez et al., 2014).

Given the limited ecotoxicological information, the purpose of this study was to analyze the toxicity and sublethal effects of five UV filters at different stages of *Chironomus riparius* development: larva and embryo. The larvae of different *Chironomus* species are widely used in aquatic toxicity studies as a reference organism in standardized protocols (EPA, 2000; OECD, 2004) because of their ecological relevance to freshwater environments and their association with benthic sediments where most pollutants accumulate. In contrast, *Chironomus* embryos have not been used to test chemical toxicity. Research shows that endocrine disruptors may pose the greatest risk during embryogenesis and early postnatal development when organ and neural systems are forming. Therefore, we developed a methodology to expose and test the potential endocrine effects of UV filters in embryos. Toxicity of five UV filters: octyl-*p*-methoxycinnamate/2-ethylhexyl-4-methoxycinnamate (OMC/EHMC); 4-methylbenzylidene camphor (4MBC); 4-hydroxybenzophenone (4HB); octocrylene (OC); and octyldimethyl-*p*-aminobenzoate (OD-PABA) were evaluated based upon their lethal effect in larvae and by the percentage of eclosion in embryos. Acute, sublethal effects were also investigated using genes involved in the cellular stress response and endocrine regulation as endpoints. Toxicogenomics is gaining momentum because information concerning genes respond to different chemical compounds is highly powerful for identifying molecular mechanisms and cellular pathways specific to the mode of action of a number of toxicants and drugs (Chen et al., 2012).

2. Materials and methods

2.1. Chemicals

The UV filters 4-methylbenzylidene camphor (4MBC; CAS No. 36861-47-9, purity ≥ 98%), octyldimethyl-*p*-aminobenzoate (OD-PABA, CAS No. 2124502-3; purity ≥ 98%), octyl-*p*-methoxycinnamate/2-ethylhexyl-4-methoxycinnamate (OMC/EHMC, CAS No. 5466-77-3; purity ≥ 98%), octocrylene (OC, 2-ethylhexyl-2-cyano-3,3-diphenylacrylate; CAS No. 619730-4; purity ≥ 97%) and 4-hydroxybenzophenone (4HB; CAS No. 113742-4; purity ≥ 98%) were purchased from Sigma-Aldrich (Germany). Stock solutions were made in ethanol and stored in the dark at 4 °C.

2.2. Animals

The test organism used was the midge *Chironomus riparius*. Stock cultures were originally collected from natural populations in Valencia (Spain), and maintained under standard laboratory conditions for several generations according to toxicity testing guidelines (EPA, 2000; OECD, 2004). Larvae were grown from egg masses in culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) in polyethylene tanks supplemented with nettle leaves, commercial fish food and cellulose tissue, which is used by the larvae to form tubes. Cultures were maintained under constant aeration at 20 ± 1 °C and under a light:dark cycle of 16:8.

2.3. Treatments

Egg masses were taken from the laboratory population < 12 h after oviposition, placed in 6-well culture plates containing 5 mL culture medium and maintained under standard conditions for 24 h prior to testing. Each egg mass was then divided into two; one half was subjected to the experimental treatment in culture medium containing the corresponding UV filter selected (1 mg/L), whereas the other half was maintained as a UV filter-free control. A UV filter nominal concentration of 1 mg/L was selected to ensure that compounds were in contact with the egg, surpassing the gelatinous protective layer around the eggs. The egg masses were exposed for 24 h for expression studies, whereas for developmental studies they were exposed from 24 h after oviposition until hatching (3 days). The percentage of hatching was obtained by counting the number of eggs hatched per total number of eggs. Three experiments were carried out for each treatment and each one was measured three times.

Fourth instar larvae (n = 90 at survival experiments, n = 30 at expression analysis) were maintained in glass vessels (250 mL) with culture medium. For expression analysis, larvae were exposed to the different UV filters for 24 h (1 mg/L, none of them caused larval lethality at this time and concentration), whereas survival experiments were exposed for 24 h, 48 h, 72 h, and 96 h to the following nominal

concentrations of UV filters: 0.1, 1, 10 and 100 mg/L. In the four days treatments the medium was renewed every 24 h and the larvae were fed at 48 h with commercial fish food. Three independent experiments were performed in each analysis using larvae from different egg masses. Non-treated control samples were exposed to the same concentration of solvent (embryos: ethanol 0.1%; larvae: 0.02%) as the corresponding treatment.

2.4. Chemical analysis

The concentration of each compound was measured at *Servicio Interdepartamental de Investigación* (SIdI - UAM) (Spain) by high performance liquid chromatography using a DAD detector at 0 and at 24 h following De Orsi et al. (2006). For each compound the two samples were collected from the vessels for 10 mg/L and 100 mg/L treatments. The first sample was collected at time 0 h and the second sample 24 h later from the same vessel. They were frozen until the analysis. The chromatographic method was as follows. Mobile phase: acetonitrile/water (adjusted at pH 3.0 with 1 M perchloric acid) 10:90 (v/v) then a linear gradient elution up to 90% acetonitrile in 30 min. Flow-rate: 1.0 mL/min; injection volume: 50 μL; column temperature: 35 °C; wavelength: 300 nm. Measured concentrations of 4HB and 4MBC after 24 h were 100% and 92%, respectively, whereas OC was 39% compared to the starting concentration. Although OMC/EHMC and OD-PABA treatments were carried out in the absence of light due to photodegradation, their concentrations decreased to 20% and 16%, respectively, after 24 h.

2.5. RNA isolation and cDNA synthesis

To isolate embryo RNA the egg mass gelatinous cover was removed with 1 × PBS (137 mM ClNa, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and 0.2% sodium hypochlorite. When it disappeared and the eggs reached the bottom of the microcentrifuge tube, several washes were carried out with 1xPBS until the sodium hypochlorite was completely removed. Total RNA was extracted from larvae and embryos using TRIZOL (Invitrogen) following the manufacturer's protocol. Subsequently RNase-free DNase (Roche) treatment for 90 min and an organic extraction with phenol–chloroform was carried out using phase-lock light tubes (5Prime) to optimize aqueous phase recovery. Finally, RNA was precipitated using isopropyl alcohol (0.5 v/v), washed with 70% ethanol, and resuspended in DEPC water. Concentration and quality of the RNA were checked by agarose gel electrophoresis and absorption spectroscopy (Biophotomer Eppendorf). Purified RNA was stored at −80 °C. Reverse transcription was performed using 0.5 μg of the total isolated RNA using 100 units of the M-MLV enzyme (Invitrogen) in the presence of 0.5 μg oligo dT_{20} primer (Sigma) and 0.5 mM dNTPs (Biotools) at 37 °C for 50 min in a reaction volume of 20 μL.

2.6. Real time RT-PCR

Quantitative Real-Time PCR (q-PCR) was used to evaluate the expression profile of the *EcR* and *hsp70* genes in control, as well as treated samples. 25 ng of cDNA, 0.3 μM of forward and reverse primers and SsoFast EvaGreen Supermix (BioRad) were utilized to amplify the sequence of interest using a CFX96 thermocycler (BioRad). *EcR* specific primers were designed from a partial clone obtained by PCR (Planelló et al., 2008). The *hsp70* primers were designed from the *hsp70* gene sequence described previously (Morales et al., 2011). The endogenous reference genes used for larvae experiments were *actin-β*, *ribosomal protein L13* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), whereas *actin-β* and *GAPDH* were used for embryo experiments (Martínez-Guitarte et al., 2012). Primer efficiencies (E%) were determined from a standard curve using template dilutions of 1:2 in five steps and in the same PCR conditions ($R^2 > 0.98$ for all primers). Primers and efficiencies are listed in Table 1. The protocol included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of a 95 °C denaturation for 5 s, 58 °C annealing for 15 s and 65 °C elongation for 10 s. Accuracy of each amplicon was verified using a melting curve analysis performed post run. Each sample was run in duplicate wells and two independent PCR replicates were used for each experiment. BioRad CFX Manager 2.1 software was used to calculate the mRNA levels by the standard $2^{-\Delta\Delta CT}$ method normalized to the reference genes.

Table 1
Primers used for real time PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)	Efficiency (E%)
Actin	GATGAAGATCCTCACCGAACG	CGGAAACGTTCA TTACCG	104.0%
GAPDH	GGTATTTCATTGAATGATCACTTTG	TAATCCTTGGAT TGCATGTACTTG	96.6%
RPL13	AAGCTGCTTTCCCAAGAC	TTGGCATAATTG GTCCAG	107.1%
EcR	CCATCGTCATCTTCTCAG	TGCCCATTGTTC GTAG	106.6%
Hsp70	ACTTGAACCAGTTGAGCGT	TTGCCACAGAAG AAATCTTG	103.8%

2.7. Statistical analyses

Statistical analyses were conducted using SPSS 19 (IBM). Normal distribution and variance homogeneity of data was assessed using Kolmogorov-Smirnov and Levene tests, respectively. For normally distributed data, significant differences were compared using Student's *t*-test. The nonparametric Mann-Whitney *U* test was used for non-normally distributed data. Statistical significance was set at $p \leq 0.05$. All results were expressed as the mean ± standard error of the mean (SEM) of three experiments. The LC50 values and their 95% confidence intervals were calculated by Probit regression analysis using SPSS.

3. Results

3.1. Toxicity of UV filters

The effects of exposure on larval survival and on successful eclosion of eggs were tested. *Chironomus riparius* fourth instar larvae were exposed for 24, 48, 72, and 96 h to a range of concentrations of the different UV filters and larval survival was recorded (Fig. 1). In Table 2, the data are shown for the LC50 values of the five UV filters assessed in this study: octyl-*p*-methoxycinnamate/2-ethylhexyl-4-methoxycinnamate (OMC/EHMC); 4-methylbenzylidene camphor (4MBC); 4-hydroxybenzophenone (4HB); octocrylene (OC); and octyldimethyl-*p*-aminobenzoate (OD-PABA). As observed, 4HB showed the highest toxicity and OC appeared the least toxic for larvae. In all cases, the LC50 values were found to be above concentrations that have been previously reported in environmental samples. To estimate toxicity at other life stages, the lethal effects on embryo development were evaluated under continuous exposure from 24 h after oviposition until hatching. As an endpoint, the percentage of eclosion was recorded as a measure of successful embryo development. Table 3 shows the data of exposed and unexposed (solvent-control) samples. Among the different UV filters tested, only OC affected embryo mortality and significantly decreased the percentage of eclosed eggs. Interestingly, OC was found to be the most toxic for embryos, whereas it appeared as the least toxic for larvae, as shown in Table 2. Compared to other aquatic organic pollutants, for this species, UV filters did not show notable toxic effects, as measured by the potential to cause lethality either in larvae or embryos.

3.2. Sublethal effects of UV filters in embryos and larvae

Despite of the low acute toxicity found for *C. riparius*, subtler, sublethal effects can be exerted on cellular metabolism by UV filters. With the aim of identifying potential target genes of *in vivo* exposures to different

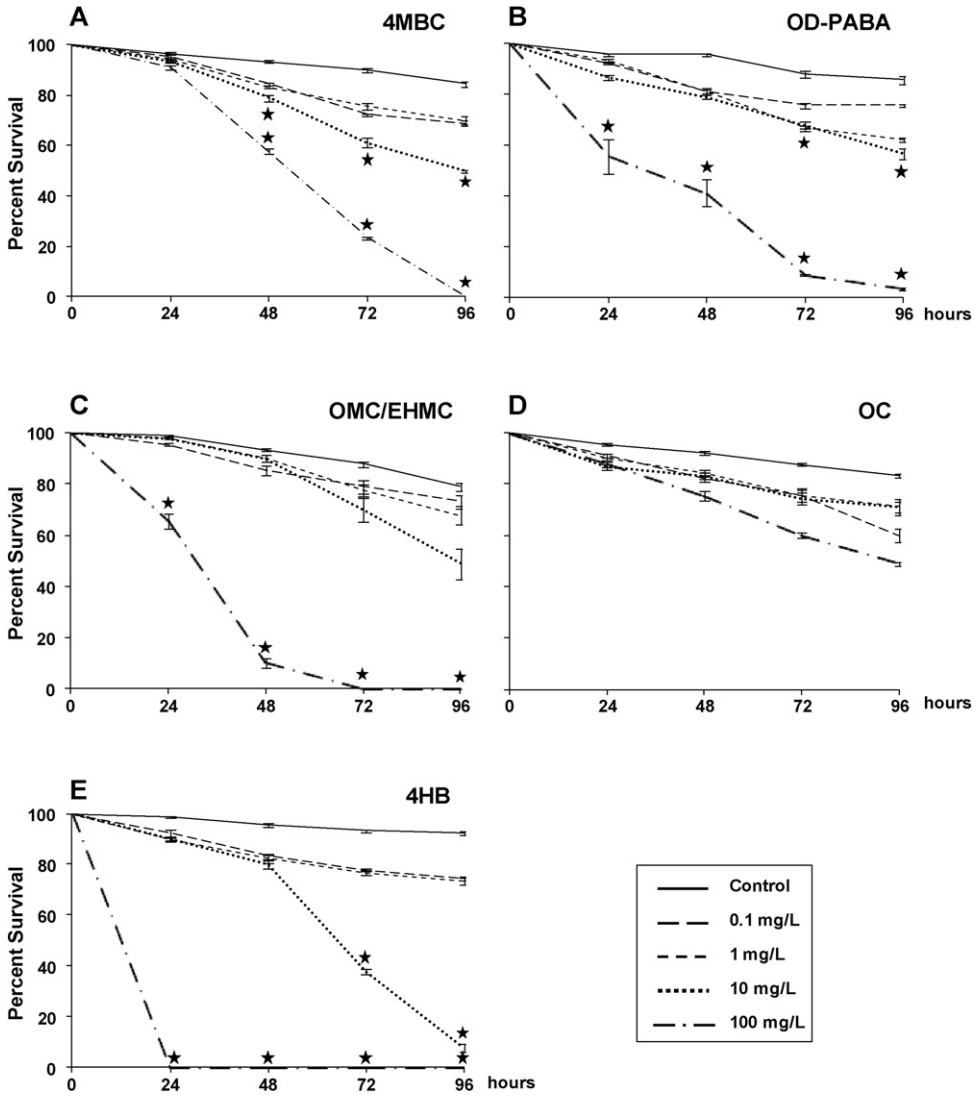

Fig. 1. Dose and time-dependent effects of 4-methylbenzylidene camphor (4MBC), octyldimethyl-*p*-aminobenzoate (OD-PABA), octyl-*p*-methoxycinnamate/2-ethylhexyl-4-methoxycinnamate (OMC/EHMC), octocrylene (OC), and 4-hydroxybenzophenone (4HB) treatments on the survival of fourth instar *Chironomus riparius* larvae. The values represent mean and Standard Error (SE) from data obtained in three independent experiments (n = 90). *Significant differences ($p \leq 0.05$).

UV filters, the *hsp70* stress response gene, the most sensitive and conserved among the family of stress genes in animals (Gupta et al., 2010), was selected as a potential sensor of proteomic damage or metabolic alterations. The ecdysone receptor gene *EcR* was also analyzed to determine if UV filters result in increased transcription of ecdysone-inducible genes in insects. These endpoints were evaluated in larvae and embryos.

Fig. 2 summarizes the results of the different experiments with the five UV filters tested for the fourth instar larvae (L) and embryos (E). Alterations in the expression profiles of *EcR* and *hsp70*, relative to the unexposed control samples, are given for each condition and stage. Important differences among the different UV filters in their capability to alter gene expression patterns were found and, interestingly, there were also notable differences among their effects on larvae and embryos.

The most potent UV filter appears to be 4MBC, which was able to induce transcription of *hsp70* and *EcR* in embryos and larvae (Fig. 2A). *EcR* and *hsp70* mRNA were found to be two to six times higher than in controls after 24 h exposure. Only this compound altered both genes in embryos and larvae.

Table 2
LC50 (mg/L) values and 95% confidence interval in fourth instar larvae of *Chironomus riparius*.

Compound	24 h		48 h		96 h	
4MBC	517.1	n.c.	120.5	(93.2–173.9)	10.9	(7.2–16.1)
OMC/EHMC	116.4	(100.7–142.6)	71.4	(58.8–90.6)	11.1	(6.1–15.4)
OD-PABA	110.9	(76.5–213.5)	81.6	(57.7–134.2)	24.0	(15.8–35.8)
OC	671.3	n.c.	276.9	n.c.	95.9	(53.1–549.2)
4HB	34.7	(23.2–69.4)	30.6	(20.2–65.9)	3.9	(3.0–5.1)

n.c.: non calculated.

Table 3
Eclosion percentage of *Chironomus riparius* eggs (mean and SEM).

Compound	Control	Treatment
4MBC	91.0 ± 4.8	89.5 ± 1.2
OMC	90.3 ± 2.8	85.2 ± 1.7
OD-PABA	90.9 ± 0.5	87.3 ± 4.0
OC	87.0 ± 1.8	74.6 ± 4.0*
4HB	83.7 ± 2.0	85.8 ± 4.2

* Significant differences with control values ($p \leq 0.05$)

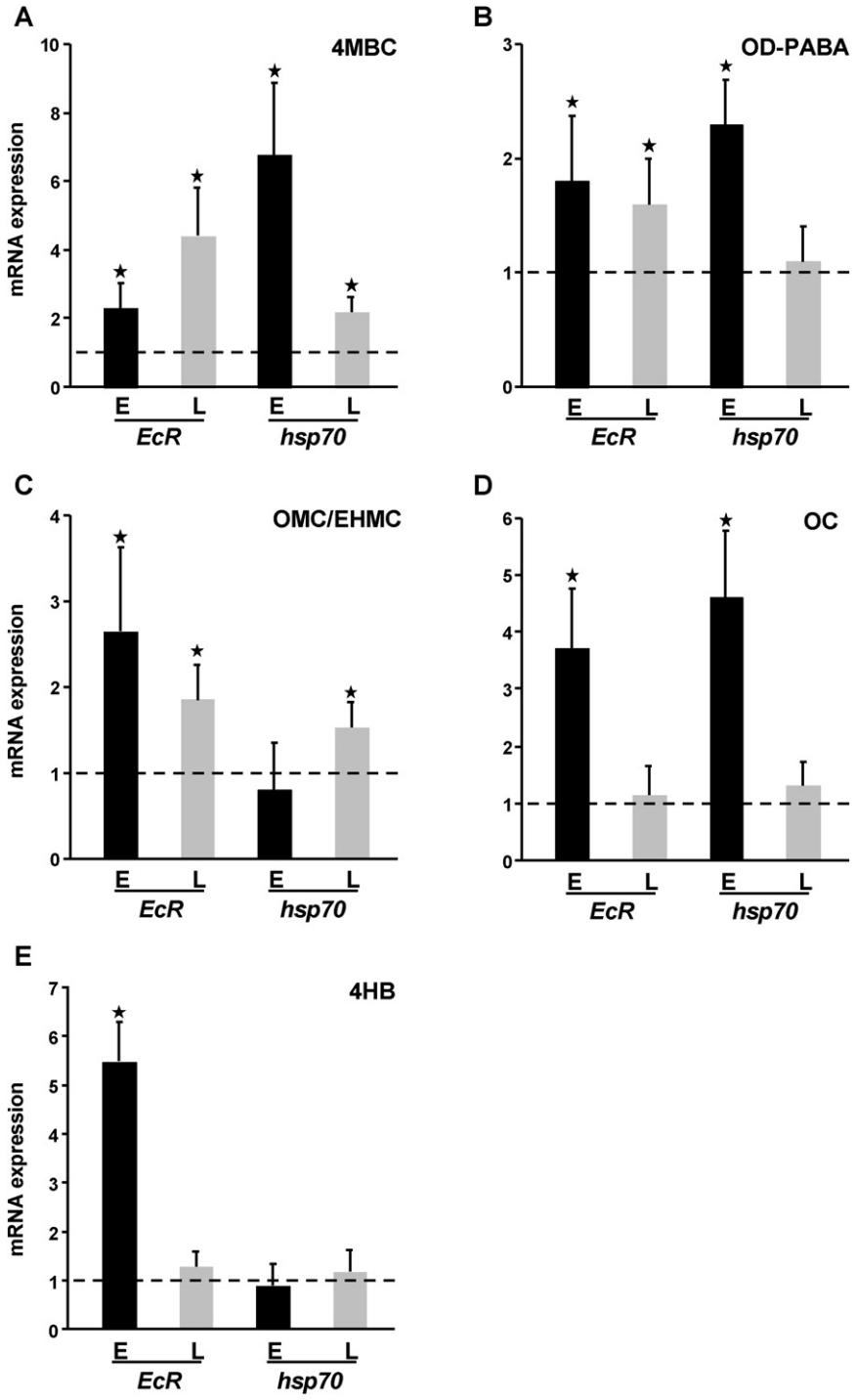

Fig. 2. Expression levels of the ecdysone receptor (*EcR*) and heat shock protein 70 (*hsp70*) genes in embryo (E) and larvae (L) of *Chironomus riparius* larvae exposed to 1 mg/L of (A) 4-methylbenzylidene camphor (4MBC), (B) octyldimethyl-*p*-aminobenzoate (OD-PABA), (C) octyl-*p*-methoxycinnamate/2-ethylhexyl-4-methoxycinnamate (OMC/EHMC), (D) octocrylene (OC), and (E) 4-hydroxybenzophenone (4HB) during 24 h. The expression levels, measured by Real Time qPCR, are presented relative to control (dashed line). The mean and SEM are shown of measurements taken in three independent biological replicates for each experimental condition. *Significant differences ($p \leq 0.05$).

The filters OD-PABA and OMC/EHMC have a significant upregulatory effect on *EcR*, resulting in a two-fold increase in mRNA levels in embryos and larva exposed to these compounds for 24 h (Fig. 2B, C). Both UV filters also activated *hsp70*, but in the case of OD-PABA this effect was only observed in embryos, whereas *hsp70* upregulation was only detected in larvae after exposure to OMC/EHMC. Among all of the conditions studied, only with OMC/EHMC was the effect detected in larvae but not in embryos.

OC exposure also showed that a differential sensitivity exists between embryos and larvae (Fig. 2D). After 24 h of exposure, embryos showed a high overexpression of *EcR*. Specifically, *EcR* expression was found to be four times higher in the treatment group than in the control group, and expressed nearly five-fold higher than *hsp70*. However, this compound did not alter either *EcR* or *hsp70* expression in exposed larvae. It is worthy to note that, as demonstrated previously, OC was the

least toxic filter for larvae, whereas it was the only filter able to induce significant eclosion failure.

Finally, 4HB induced a notable overexpression of *EcR* (over a five-fold increase compared to the controls), however this effect was only detected in embryos (Fig. 2E). The *hsp70* gene was not affected in either embryos or in larvae.

In summary, the analyzed UV filters significantly altered the expression of the nuclear hormonal receptor gene in embryos at 24 h of exposure. Most filters also activate this gene in larvae, as well as the *hsp70* gene in embryos and larvae indicating that exposure to these contaminants induce a cellular response to environmental stress. The filters OC and 4HB only altered these genes in embryos and did not show any effect on larvae. Notably these two filters appear to be the least toxic and most toxic for larvae, respectively, based on the calculated LC50 values.

4. Discussion

There is little available information regarding the toxicity of the emerging contaminants known globally as UV filters, particularly in invertebrates. Survival data, as a function of both concentration and exposure time were analyzed in this study for the first time in an aquatic insect of ecological relevance for freshwater environments. According to the toxicity assays, these aromatic compounds have a low to intermediate toxicity (EC50 among 1–100 mg/L, see Callow, 1998), although it is important to take into account that, in our study, OMC, OC, and OD-PABA showed degradation during exposure, so the LC50 values for these compounds may be lower than those obtained. The 96 h LC50 values for *C. riparius* larvae ranged from 4 to 96 mg/L, with OC being the least toxic. 4HB appear to be the most toxic for larvae, followed by 4MBC and OMC/EHMC which presented a similar toxicity; however, OMC/EHMC was more toxic during the initial exposure times. This ranking of toxicity was similar to that found in previous studies in aquatic invertebrates (Table 4), such as the crustacean *Daphnia*, where the 48 h LC50 values of 4MBC, EHMC, benzophenone-3 (BP3) and benzophenone-4 (BP4) were 0.56, 0.29, 1.9 and 50 mg/L, respectively (Fent et al., 2010b). Growth inhibition and effects on cell viability were described in the protozoan *Tetrahymena*, with 24 h EC50 values that ranged from 5.1 to 7.5 mg/L for BP3 and 4MBC, whereas in our experiment other UV filters such as OMC/EHMC or OC did not inhibit growth (Gao et al., 2013). In a recent ecotoxicological evaluation of UV filters in four marine species from different trophic levels, 4MBC and OMC/EHMC were determined to be the most toxic, with microalgae being the most affected by these compounds (Paredes et al., 2014). Benzophenone-1 (BP1) was also found to be acutely toxic for a marine copepod with a 48 h LC50 of 2.6 mg/L (Kusk et al., 2011). High toxicity of UV filters and cell death has also been detected in corals with a 24 h LC50 ranging from 165 to 548 ppb (μg/L) for benzophenone-2 (BP2) (Downs et al., 2014). In any case, concentrations that compromise viability are relatively high compared to estimated environmental concentrations of UV filters. Nevertheless, it should be taken into account that analytical studies are still scarce and limited to a very few locations. The concentrations reported in wastewater treatment plant influents and effluents are 19 μg/L for EHMC, 2.7 μg/L for 4MBC, 3.3 μg/L for BP3 and 4.3 μg/L for OC (Tarazona et al., 2010); however, higher concentrations may occur at specific points in hotspot areas or due to occasional discharges.

Regarding the viability of embryos, only OC showed a low but significant effect on egg eclosion suggesting a lethal impairment in embryo development. Interestingly, OC was the compound that showed the lowest toxicity for larvae. This is the first evidence that demonstrates a differential sensitivity dependent on the stage of development in insects. This is relevant for ecotoxicological studies, and suggests that studies should consider effects at different developmental phases in the assessment of the environmental risk of toxicants.

The five UV filters tested in *C. riparius* showed little effect on the viability of embryos or survival of the larvae, however notable sublethal effects were detected in this study which confirms the existence of subtler alterations on cellular physiology. Two genes representing the endocrine pathway and cellular stress response were sensitive end points rapidly altered by exposure to the UV filters analyzed.

The presence of UV filters in the aquatic medium rapidly induces overexpression of the *EcR* gene. This effect is particularly strong during the first stages of development as demonstrated in embryos. EcR is a nuclear receptor that responds to ecdysone, an insect steroid hormone, and acts as a transcription factor that activates a cascade of hormonal effector genes (Spindler et al., 2009). The effect of UV filter exposure on a key transcription factor for the ecdysone-genomic response in arthropods suggests the possibility of a broad and long-term effect on this endocrine pathway. Altered transcription rates or timing in larvae or embryos due to the presence of UV filters in the aquatic medium, can also affect the timing of egg hatching or metamorphosis in insects and might be sufficient to alter developmental transitions. Although embryos that develop in an aquatic medium are usually exposed to adverse conditions and often possess protecting structures, this is the first study proving that embryos are highly susceptible to environmental pollutants, such as UV filters, as compared to other developmental stages, such as larvae.

Most ecotoxicological studies on the effect of UV filters have been conducted in fishes. In particular, 4MBC and OMC/EHMC, which are extensively used, have shown estrogenic activity in fish, whereas others such as BP3 have been shown to disrupt multiple types of endocrine activity (Coronado et al., 2008; Kim and Choi, 2014). Effects on sex steroid hormone levels and down regulation of transcription of gonadal steroidogenic genes were also observed (Kim et al., 2014). 4MBC had highest estrogenic potency than OMC/EHMC, and in medaka, both were found to increase the expression of estrogen receptor (ERa) mRNA, as well as the production of vitellogenin and choriogenin, which are known to be estrogen-responsive gene products (Inui et al., 2003). There was also evidence regarding the effects of some UV filters on endocrine regulated processes such as growth and reproduction in some invertebrate species (Fent et al., 2010b; Kaiser et al., 2012; Schmitt et al., 2008; Sieratowicz et al., 2011). Moreover, ecotoxic effects in some invertebrates had also been reported for 3BC (3-benzylidene camphor), and OMC/EHMC was previously found to increase unshelled embryos. Our results confirm that UV filters directly affect hormonal systems in invertebrates and are consistent with the known effects of UV filters on vertebrate hormonal receptor genes. Previous *in vitro* studies have shown that 4MBC, OMC/EHMC and OD-PABA can induce transcriptional activation of human estrogen receptor gene (hERa,

Table 4
Concentrations of UV filters detected in invertebrates.

	4MBC	EHMC	BP3	OC	BP4	BP1	BP2	
WWTP	2.7 μg/L	19 μg/L	3.3 μg/L	4.3 μg/L	–	–	–	Tarazona et al. (2010)
Daphnia 48 h LC50	0.56 mg/L	0.29 mg/L	1.9 mg/L	–	50 mg/L	–	–	Fent et al. (2010b)
Tetrahymena 24 h EC50	5.1 mg/L	–	7.5 mg/L	–	–	–	–	Gao et al. (2013)
Acartia tonsa 48 h LC50	–	–	–	–	–	2.6 mg/L	–	Kusk et al. (2011)
Corals 24 h LC50	–	–	–	–	–	–	165 to 548 ppb	Downs et al. (2014)

b) (Schreurs et al., 2002). The ER is also functionally activated at the protein level by 4MBC (Mueller et al., 2003). More recently, it has also been confirmed in rats that 4MBC exposure not only affected the mRNA levels of ERa, but also the progesterone receptor, preproenkephalin, and insulin-like growth factor-I (Maerkel et al., 2007). Overall, these data show that UV filters are capable of activating hormonal genes in different species and affecting the endocrine system in multiple ways.

In addition to the study of an endocrine-related gene, the possible effect on *hsp70* was also examined because it is a stress response gene that might be a sensitive biomarker of the proteotoxic effects of UV filters. HSPs play a primary role in cellular recovery from stress by acting as chaperones to maintain correct protein folding (Morris et al., 2013). In addition, *hsp70* has also been related to the endocrine pathway (Arbeitman and Hogness, 2000; Nolen and Morimoto, 2002). All the UV filters analyzed were able to induce *hsp70* expression with the exception 4HB, despite the fact that it appeared to be the most toxic for larvae. In addition, OC, which was found to be the least toxic, failed to alter the expression of this gene in larvae. Therefore, levels of *hsp70* expression do not seem to be directly related with the toxic response to these compounds. In contrast, in embryos, exposure to 4MBC and OC resulted in the overexpression of *hsp70* up to six-fold as compared to control, unexposed samples. As far as we know, there are no studies regarding the effects of UV filters on the cellular stress response in other organisms. Regarding *Chironomus*, many environmental stressors and toxicants induced a rapid upregulation of heat shock genes, particularly *hsp70* (Herrero et al., 2015; Martínez-Paz et al., 2013; Morales et al., 2011, 2013, 2014; Planelló et al., 2011).

5. Conclusions

Knowledge of target genes that provide information regarding altered endocrine or metabolic activity or toxicity are central for understanding the risk of anthropogenic chemicals. The alterations in *EcR* transcriptional activity observed in embryos and larvae of provide evidence that environmental exposure to UV filters may significantly impact expression of endocrine-related genes in aquatic invertebrates, and might be especially dangerous during critical windows of development. We show for the first time that these five UV filters impact a hormonal gene in embryos. This finding is environmentally relevant, because most insect embryos develop in water systems, where these emerging contaminants accumulate. Interestingly, embryos appeared to be more sensitive to UV filters than larvae, and show a stronger activation of the analyzed genes when exposed to the UV filters tested. The differences found between different life stages, including embryo and larvae, should be considered in the risk assessment of these compounds particularly when analyzing endocrine effects. *Hsp70* upregulation demonstrates that UV filters also induce the cellular stress response, indicating proteotoxic effects. Finally, the analyzed UV filters share common effects, but there are subtle differences among them that suggest specificities in their modes of action. In conclusion, these results reinforce previous evidence of the endocrine effects of UV filters in vertebrate systems and extend these effects to invertebrates, which supports the need for the evaluation of these compounds in aquatic biota under environmentally relevant conditions.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Ecotoxicological effect characterisation of widely used organic UV filters
Panel
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Abstract
Chemical UV filters are used in sun protection and personal care products in order to protect consumers from skin cancer induced by ultraviolet (UV) radiation. The present study aims to evaluate the effects of three common UV filters butyl-methoxydibenzoylmethane (B-MDM) ethylhexyl-methoxycinnamate (EHMC) and octocrylene (OCR) on aquatic organism, focussing particularly on infaunal and epibentic invertebrates (*Chironomus riparius, Lumbriculus variegatus, Melanoides tuberculata* and *Potamopyrgus antipodarum*). Due to their life habits, these organism are especially affected by lipophilic substances. Additionally, two direct sediment contact assays utilising zebra fish (*Danio rerio*) embryos and bacteria (*Arthrobacter globiformis*) were conducted.
EHMC caused a toxic effect on reproduction in both snails with lowest observed effect concentrations (LOEC) of 0.4 mg/kg (*Potamopyrgus antipodarum*) and 10 mg/kg (*Melanoides tuberculata*). At high concentrations sublethal effects could be observed for *D. rerio* after exposure to EHMC (NOEC 100 mg/kg). B-MDM and OCR showed no effects on any of the tested organism.
Highlights
► Ecotoxicological effects of common used UV filters on aquatic invertebrates. ► Butyl-methoxydibenzoylmethane, ethylhexyl-methoxycinnamate, and octocrylene used. ► Sediment based test systems. ► Ethylhexyl-methoxycinnamate caused a toxic effect on reproduction in both snails. ► Other substances showed no effects on any of the tested organism.

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Global gene expression profile induced by the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) in zebrafish (*Danio rerio*)

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ABSTRACT

Residues of the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) are ubiquitously found in aquatic biota but potential adverse effects in fish are fairly unknown. To identify molecular effects and modes of action of EHMC we applied a gene expression profiling in zebrafish using whole genome microarrays. Transcriptome analysis and validation of targeted genes were performed after 14 days of exposure of male zebrafish. Concentrations of 2.2 μg/L and 890 μg/L EHMC lead to alteration of 1096 and 1137 transcripts, respectively, belonging to many pathways. Genes involved in lipid metabolism and estrogenic pathway (*vtg1*), lipid biosynthesis (*ptgds*), vitamin A metabolic process (*rbp2a*), DNA damage and apoptosis (*gadd45b*), and regulation of cell growth (*igfbp1a*) were investigated by qRT-PCR analysis in whole body, liver, brain and testis. The analysis showed tissue-specific gene profiles and revealed that EHMC slightly affects the transcription of genes involved in hormonal pathways including *vtg1*, *esr1*, *esr2b*, *ar*, *cyp19b* and *hsd17β3*.

1. Introduction

Personal care products are increasingly used in daily life and chemical residues enter aquatic systems, where they may lead to adverse effects in biota. UV-absorbing chemicals (UV-filters) are widely used in sunscreens and in a wide variety of cosmetics (lipsticks, shampoos, creams, fragrances, skin lotions, hair sprays). In addition, UV-protection is also applied in numerous materials and products, where UV-filters (Balmer et al., 2005; Fent et al., 2010a,b) or benzotriazole UV-stabilizers (Nakata et al., 2009) are used. UV-filters are organic (e.g. 2-ethyl-hexyl-4-trimethoxycinnamate, EHMC) or inorganic (TiO_2, ZnO) ingredients of personal care products, whose purpose is to filter UV-A and/or UV-B radiation from sunlight in order to protect the human skin and products, respectively, from negative effects.

Currently, 28 UV-filters are registered in the European Union (Zenker et al., 2008; Schlumpf et al., 2008). As a result of their various applications, UV-filters enter the aquatic environment either directly via wash-off from skin and clothes, or via effluents of wastewater treatment plants or swimming pool waters, where rather high levels of different UV-filters were measured (Balmer et al., 2005; Zwiener et al., 2007; Rodil and Moeder, 2008). Further sources of UV-filter residues are landfill leachates, sewage sludge, as well as deposition from building parts, which are protected with coatings (Plagellat et al., 2006).

Many organic UV-filters are lipophilic, photostable and relatively stable in the aquatic environment. They adsorb into sewage sludge (Plagellat et al., 2006), and some of them bioaccumulate in aquatic biota. 2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC), benzophenone-3 and 4-methoxycinnamate were demonstrated to accumulate in fish (Buser et al., 2006; Fent et al., 2010a), and in human breast milk (Schlumpf et al., 2008). The acute toxicity of some UV-filter increases with the log *Pow* (lipophilicity) of the compound in *D. magna* showing an LC_{50} value of 0.28 mg/L for EHMC (Fent et al., 2010b). Since significant amounts of these chemicals are used today, there are health concerns, and therefore, more knowledge is needed for a better understanding of potential toxicological effects and on the modes of action of these compounds.

Some UV-filters interfere with the sex hormone system and may act as endocrine disruptors. Hormonal activity was documented *in vitro* (Schlumpf et al., 2001; Kunz and Fent, 2006a) as well as *in vivo* (Kunz et al., 2006a). In fathead minnows (*Pimephales promelas*), the UV-filters EHMC displays multiple hormonal activities (Christen et al., 2011) and 3-benzylidene camphor and benzophenone-2 exhibit estrogenic activity and adverse effects on

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fertility and reproduction (Kunz et al., 2006a, b; Weisbrod et al., 2007).

Benzophenone-3 (BP-3) led to induction of vitellogenin in rainbow trout and Japanese medaka (Coronado et al., 2008), and benzophenone-4 (BP-4) was demonstrated to interfere with the sex hormone system displaying multiple hormonal activities in zebrafish (*Danio rerio*) (Zucchi et al., 2011).

EHMC is one of the most widely used UV-filters and included in more that 90% of commercial topically applied sunscreens formulation (Díaz-Cruz et al., 2008). The environmental concentration of EHMC ranges between 0.01 and 0.1 μg/L in treated wastewater, and up to 19 μg/L in untreated municipal wastewater (Balmer et al., 2005). EHMC residues were detected in lakes and rivers (Balmer et al., 2005; Fent et al., 2010a) and in coastal seawater up to 390 ng/L were reported (Langford and Thomas, 2008). Recently up to 3 μg/L EHMC were found in a lake (Rodil et al., 2009), and 0.26–5.61 μg/L were reported in drinking water (Loraine and Pettigrove, 2006). Unexpectedly, even at very remote environments such as the Pacific Ocean (Polynesia) this compound was detected (Goksoyr et al., 2009). EHMC is lipophilic (log $P = 5.66$; Zenker et al., 2008) and accumulates in aquatic biota (Fent et al., 2010a). Residues were found in different trophic levels showing a tendency for bioaccumulation along the food-chain, with concentrations up to 340 ng/g lipids in cormorants (Fent et al., 2010a).

Despite its widespread presence in the environment, little is known about the potential risk posed by EHMC to aquatic life. In recombinant yeast systems EHMC showed anti-estrogenic and anti-androgenic activities, combined with weak androgenic activity (Kunz and Fent, 2006b). Injection of high concentrations of EHMC in male medaka led to induction of vitellogenin (VTG) (Inui et al., 2003), while no significant VTG induction was observed in juvenile fathead minnows exposed to lower aqueous concentrations (Kunz and Fent, 2006b). Despite these studies the potential endocrine activity and molecular modes of action of EHMC in fish remain elusive and need further investigation. In light of the importance and increasing use of this UV-filter, and considering that potential hormonal activities and adverse effects on fertility are of concern, there is a need for a better understanding of potential environmental risks associated with EHMC contamination.

Since changes in gene expression often precede cellular, physiological and toxicological responses, analysis of gene expression profiles upon exposure may be a sensitive tool for investigating adverse effects of pollutants including their molecular mode of action (Oggier et al., 2010, 2011). Thus far, toxicogenomics was applied mainly with compounds whose modes of action are known (e.g. Lettieri, 2006; Hoffmann et al., 2006, 2008). However, microarray analysis focusing on thousands of genes also offers the potential to analyse for the molecular effects of chemicals with unknown modes of action. By employing global gene expression analysis, in the present study, we focus on EHMC to elucidate its unknown mode of action in order to shed a new light on its potential effects in fish. Whole-adult microarray analysis performed on a small vertebrate such as zebrafish might represent a strategy to potentially obtain a large amount of *in vivo* data on the transcriptome of the entire organism (Lam et al., 2008), and thus to obtain insight into the mode of action of EHMC.

To date, very little is known about the toxicological effects of EHMC to fish. Potential target organs of EHMC are unknown, although EHMC was recently shown to interfere with the sex hormone system (Christen et al., 2011). Therefore, our aim was to evaluate the overall molecular effects of EHMC by analysing the whole body of adult male zebrafish. This allows an overall fingerprint of EHMC as a sum effect in all tissues, which is in contrast to analysis of only one tissue alone, hard to select without knowledge about the target tissues of the compound. We analyzed the effects at nominal low (3 μg/L) and high (3000 μg/L) EHMC concentrations. The lower concentration represents an environmental relevant concentration, while the higher was chosen to stimulate mRNA expression of target genes to elucidate potential molecular modes of action of EHMC. Data from previous studies suggest that EHMC interferes with the sex hormone system (Inui et al., 2003; Kunz and Fent, 2006b; Christen et al., 2011). Additionally, by analyzing transcriptional changes of selected target genes belonging to different pathways including vitellogenin 1 (*vtg1*), prostaglandin D2 synthase (*ptgds*), insulin growth factor binding protein 1a (*igfbp1a*), retinol-binding protein type II (*rbp2a*), and growth arrest and DNA-damage-inducible beta (*gadd45b*), we aimed at comparing expressional changes in the whole body to those in the liver, brain and testis. To analyse for potential hormonal effects, we focused on expressional changes of more classical genes involved in hormonal pathways and steroidogenesis including vitellogenin 3 (*vtg3*), ERalpha (*esr1*), ERbeta1 (*esr2b*), androgen receptor (ar) hydroxysteroid 17-β dehydrogenase-3 (*hsd17ß3*), P450aromB (*cyp19b*) and P450aromA (*cyp19a*).

By following an approach to analyse for unknown molecular effects of EHMC by means of global expression analysis (microarrays), and by following a targeted gene expression approach (qRT-PCR) focusing on mainly hormonal pathways in multiple tissues, we demonstrate the involvement of several pathways in response to EHMC exposure.

2. Materials and methods

2.1. Chemicals

2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC, $C_{18}H_{26}O_3$, CAS No. 5466-77-3, mass 290.4 g/mol) and 3-(4-methylbenzylidene)-camphor (4MBC; CAS No. 36861-47-9) were purchased from Merck (Glattbrugg, Switzerland), ethanol (EtOH), methanol and dichloromethane in HPLC grade from J.T. Baker (Stehelin AG, Basel, Switzerland). Formic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Fluka AG, Buchs, Switzerland). All compounds were >99% pure. The water solubility of EHMC is 0.2 mg/L at 20 °C (http://www.merck-chemicals.com), and the log *P* is 5.66 (Zenker et al., 2008). Master concentrated stock solution of 30 and 0.3 g/L EHMC were made up in DMSO and stored in the dark at 4 °C between uses. After dilution with tank water (10 L), DMSO concentrations did not exceed 0.01% in each tanks used in the experiment.

2.2. Analytical chemistry

During the experiment aliquots of exposure water were taken three times randomly from the tanks to determine the actual exposure concentration of EHMC. Water samples of each replicate were taken at the beginning (0 h), after 24 h and prior to water renewal (48 h). This was done on days 1–3, 7 to 9 and 11 to 13, respectively, from different replicate tanks. The water samples were stored in the dark at −20 °C until analysis by HPLC-DAD. Extraction of water samples and chemical analysis was performed according to Kunz et al. (2006b).

2.3. Maintenance of adult zebrafish

Adult zebrafish (*Danio rerio*) (>121 days) were obtained from a local dealer (Swiss tropical fish, Niederönz, Switzerland), transferred to culture tanks (300 L) and acclimatized one month in our laboratory prior to commencing the experiments. Fish of both sexes were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 μS/cm. The water temperature was held constant at 27 ± 1 °C with the photoperiod set at 16:8 h light/dark. Fish were fed twice daily with TetraMin flakes (Tetra GmbH, Melle, Germany) and once a day with a combination of brine shrimp (*Artemia salina*) and white mosquito larvae.

2.4. Exposure of adult male zebrafish

Adult male zebrafish were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated water (12 fish/tank). The experimental setup consisted of 4 groups: 3 μg EHMC/L, 3000 μg EHMC/L, solvent control (SC, 1 ml of DMSO in 10 L of reconstituted water) and water control (reconstituted water). Each dose group and the control consisted of four replicates. The quality of the exposure water was continuously monitored by measuring the oxygen concentration (>70%), pH value (6.7–7.2) and temperature (27 ± 1 °C). During the

experiment, appearance, mortality and abnormal behaviour of fish were recorded daily.

Fish were exposed for 14 days to EHMC in a semi-static renewal procedure; every 48 h fish were transferred to new tanks containing the appropriate EHMC concentrations. During the exposure period fish were fed daily as previously described. At the end of the experiment, fish were euthanized in a clove oil solution (Fluka AG, Buchs, Switzerland), the total body length and weight were measured. For whole-body microarray analyses and subsequent quantitative real-time polymerase chain reaction (qRT-PCR) confirmation, two fish per replicate of every group were snap-frozen in liquid nitrogen after sex determination.

Zebrafish sex determination was carried out opening the belly to ensure testis presence before collecting fish for further molecular analysis.

A number of 10 fish of each replicate of each dose group were used for qRT-PCR analysis, and liver, brain and testis were immediately excised. Pools of tissues of ten fish per replicate were collected, placed in RNA*later* and stored at −80 °C for subsequent total RNA extraction.

2.5. RNA isolation, array hybridization and sample selection

For microarray analysis one whole fish per replicate of each dose group was pounded in a ceramic mortar to powder with liquid nitrogen, and subsequently equally transferred into two Eppendorf tubes for subsequent total RNA extraction using RNeasy Mini Kit (Qiagen, Basel, Switzerland).

For qRT-PCR analysis of tissues, total RNA was extracted from pools of 10 fish of liver, brain and testis using RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 UV–VIS spectrophotometer at 260 nm. The integrity of each RNA sample was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing a 260/280 nm ratio between 1.8–2.1, a 28S/18S ratio between 1.5–2 and an RNA integrity number (RIN) > 8 were further processed.

For transcriptom analysis, a total of 16 arrays (Agilent 4 × 44 K Zebrafish microarray) were used, including four for the 3 μg/L, four for the 3000 μg/L EHMC dose group, four for the solvent control group (DMSO) and four for the water control group. Total RNA samples (600 ng) were reverse-transcribed into double-strand cDNA in the presence of RNA poly-A controls with the Agilent One-Color RNA Spike-In Kit. Cy3 labeling and hybridization were performed according to the manufacturer's manual.

After reverse transcription of RNA into double-stranded cDNA, double-strand cDNA was *in vitro* transcribed into cRNA in the presence of Cy3 labeled nucleotides using a Low RNA Input Linear Amp Kit + Cy dye (Agilent Technologies, Basel, Switzerland), performed at the Functional Genomic Centre (ETHZ and University of Zürich, Switzerland). The Cy3-labeled cRNA was purified using an RNeasy mini kit (Qiagen, Basel, Switzerland), and quality and quantity was determined using a NanoDrop ND-1000 UV–VIS Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Only cRNA samples with a total cRNA yield higher than 2 μg and a dye incorporation rate between 9 pmol/μg and 20 pmol/μg were used for hybridization. Cy-3-labeled cRNA samples (1.65 μg) were mixed with Agilent blocking solution, subsequently fragmented randomly to 100–200 bp at 65 °C with fragmentation buffer and resuspended in hybridization buffer as provided by the gene expression hybridization Kit (Agilent Technologies). Target cRNA samples (100 μL) were hybridized to the Agilent Zebrafish 4 × 44 K Gene Expression Microarray for 17 h at 65 °C. The hybridized arrays were then washed using Agilent GE wash buffers 1 and 2 according to the manufacturer's instructions and scanned by an Agilent Microarray Scanner (Agilent *p*/n G2565BA) at 5 μm resolution with the green photomultiplier tube set to 100% and a scan area of 61 × 21.6 mm. Image generation and feature extraction was performed using the Agilent Feature Extraction (FE) software version 9.5.3. Quality control was additionally considered before performing the statistical analysis. These included array hybridization pattern inspection: absence of scratches, bubbles, areas of non-hybridization, proper grid alignment, spike performance in controls with a linear dynamic range of 5 orders of magnitude and the number of green-feature non-uniformity outliers which should be below 100 for all samples.

2.6. qRT-PCR analysis

Changes in the expression of selected genes identified as being different following microarray analysis were validated using real-time reverse transcription-polymerase chain reaction (qRT-PCR). The selected genes included, vitellogenin 1 (*vtg1*), insulin-like growth factor binding protein 1a (*igfbp1a*), prostaglandin D2 synthase (*ptgds*), growth arrest and DNA-damage-inducible beta (*gadd45b*) and cellular retinol-binding protein type II (*rbp2a*) (Table 2). In addition, seven target transcripts were chosen for qRT-PCR analysis, because of their involvement in hormonal pathways and steroidogenesis. They included vitellogenin 3 (*vtg3*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), hydroxysteroid 17-β dehydrogenase-3 (*hsd17ß3*), androgen receptor (*ar*), P450aromB (*cyp19b*) and P450aromA (*cyp19a*).

Gene-specific primers of all the genes were obtained either from published zebrafish primers sequences, or designed based on zebrafish sequences available at NCBI (http://www.ncbi.nlm.nih.gov/) (Table 2). Primer sequences not previously published were designed using IDTDNA software (www.idtdna.com).

Amplicons were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide, in order to confirm correct product size and primers specificity. Total RNA from individual whole body fish and pools of 10, each of zebrafish liver, brain and testis were isolated as described above ($n = 4$ replicates for adults).

RNA samples used for qRT-PCR analysis were further treated with RNase free DNase set (Qiagen, Basel, Switzerland) used to purify the RNA preparations from DNA contamination and to subsequently remove DNase and divalent cations from the samples.

Subsequently 1 μg of total RNA template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and desoxynucleoside triphosphate (Sigma–Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR-Green Master, Roche Diagnostics, Basel, Switzerland).

The real-time PCR program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 57–60 °C, depending on transcript target as shown in Table 2 (30 s) and 72 °C (30 s), followed by a melting curve analysis post run. All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland).

For determining the efficiencies of the PCR reactions, reaction mixtures with template diluted 1:10 in five steps were also run and the slopes of the regression curves were calculated. For calculating expression levels of selected genes, mRNA normalization was performed against the housekeeping gene (*RpL13α*). The ΔC_T values were calibrated against the control ΔC_T values for both target genes.

The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), all gene expression data are reported as log2-transformed.

2.7. Data analysis and statistics

To identify lists of differentially-expressed genes (DEGs) in this study, the text outputs (raw microarray data) obtained from Functional Genomic Centre Zurich (FGCZ) were imported into GeneSpring GX 11 (Agilent Technologies). In a first step, the Agilent Feature Extraction software output was filtered on the basis of feature saturation, non-uniformity, pixel population consistency and signal strength relative to back ground level (Agilent Feature Extraction Manual). Only positively marked entities, in which at least 50% of the values for any out of the three conditions were accepted for further evaluation. All data were quantile normalized. In a second step, several quality control steps (e.g. correlation plots and correlation coefficients, quality metric plots and PCA) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality.

Differentially expressed genes from the microarray were determined using a Benjamini-Hochberg multiple correction-ANOVA test ($p < 0.05$), followed by a TukeyHSD post-hoc test. The genes were considered differentially expressed when $p < 0.05$ and the fold change (FC) was ≥2.

To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneGo (GeneGo, San Diego, CA, Version 6.3, http://www.genego.com) was used. Enrichment was examined in all three major GO categories (e.g., biological process, cellular component, molecular function), but only biological process results are reported here, as they were the most relevant category for the purposes of this study. Only those categories where $p < 0.05$ are considered differentially altered.

MetaCore™ (GeneGo, San Diego, CA, Version 6.3) from GeneGo Inc.http://www.genego.com was used to identify and to visualize the involvement of the differentially expressed genes in specific pathways (FDR< 0.05).

Data from qRT-PCR were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov–Smirnov test and the variance homogeneity with

Table 1
Nominal and median of actual concentrations of EHMC in exposure waters at different time points (0 h, 24 h and 48 h).

Exposure Concentrations				
Nominal	Measured			
[μg/L]	0 h [μg/L]	24 h [μg/L]	48 h [μg/L]	Median[b] [μg/L]
0 ($n = 3$)	0[a]	0[a]	0[a]	**0**
3 ($n = 1$)[c]	2.2	3.6	0	**2.2**
3000 ($n = 3$)	1741 ± 23[a]	890 ± 9[a]	140 ± 2[a]	**890**

[a] Median ± standard deviations of replicates.
[b] Median of actual exposure concentration after 0, 24 and 48 h.
[c] Only one replicate could be measured due to lack of sampling water evaluated.

Table 2
Primer sequences for quantitative real-time PCR analysis and sources: vitellogenin 1 (*vtg1*), vitellogenin 3 (*vtg3*), ERalpha (*esr1*), ERbeta1 (*esr2b*), androgen receptor (ar) hydroxysteroid 17-β dehydrogenase-3 (*hsd17ß3*), P450aromB (*cyp19b*), P450aromA (*cyp19a*), growth arrest and DNA-damage-inducible beta (*gadd45b*), retinol-binding protein type II (*rbp2a*), prostaglandin D2 synthase (*ptgds*), insulin growth factor binding protein 1a (*igfbp1a*) and *RpL13α*.

Target gene	GenBank number	Sense primer (5′–3′)	Antisense primer (5′–3′)	Product size (bp)
Vtg1[a]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94
Vtg3[b]	AF254638	TTAGAACCAGCAAAGGATGC	CATCTCTTTTCTCCTTAAATAC	208
esr1[c]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163
esr2b[d]	NM_174862.3	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT	80
ar[e]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237
hsd17ß3[f]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121
cyp19b[g]	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94
cyp19a[g]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92
gadd45b	NM_213031	GGGACGAACATTTTGAAGGA	AACACGGTCCTTTTCAGTGC	131
rbp2a	AF363957	GGAGATGCTCAGCAATGACA	TCTGCACAATGACCTTCGTC	110
Ptgds	NM_213634	CCATCAAGACCAAAGGAGGA	TCCATTTTGTGGAAGCATGA	152
Igfbp1a[a]	NM_173283	GTCATCCTGGAATGGGAAGA	TGTGTGACGGATCAGTGGTT	93
RpL13α[h]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100

Data sources.
[a] Hoffmann et al., 2006. Aquat. Toxicol. 79, 233–246.
[b] Meng et al., 2010. Anal. Bioanal. Chem. 396, 625–630.
[c] Martyniuk et al., 2007. Aquat. Toxic. 84, 38–49.
[d] Chandrasekar et al., 2010. Plos One 5, e9678.
[e] Hossain et al., 2008. Biol. Reprod., 78,361–369.
[f] Hoffmann et al., 2008. Aquat. Toxicol. 87, 69–80.
[g] Arukwe et al., 2008. Environ. Res. 107, 362–370.
[h] Oggier et al., 2010. Environ. Sci. Technol. 44, 7685–7691.

the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p < 0.05$) to compare treatment means with respective controls. Results are given as mean ± standard error of mean. Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. EHMC exposure concentrations and gross toxicological parameters

Concentrations of EHMC in experimental water were determined by HPLC-DAD after 0, 24, and 48 h of exposure for each treatment group. At the nominal EHMC concentration of 3 μg/L, measured concentrations were 2.2 μg/L at 0 h, 3.6 μg/L at 24 h, and not detectable (*n.d.*) at 48 h. At the nominal EHMC concentration of 3000 μg/L measured concentrations (median ± SD) were 1741 ± 23 μg/L ($n = 3$) at 0 h, and decreased to 890 ± 9 μg/L ($n = 3$) at 24 h, and to 140 ± 2 μg/L ($n = 3$) at 48 h. This gives a median of the actual exposure concentration over this time period of 2.2 μg/L (Table 1). The data demonstrate that actual concentrations of EHMC (expressed as median concentrations) were lower than nominal and further decreased during exposure.

During the 14-d experiment, no mortality occurred even in the highest group dose and there were no indications of abnormal behaviour during the exposure. No significant alteration of condition factors (CF) was noted (Fig. S1).

3.1.1. Differential expression of genes in whole body determined by microarrays

All microarray hybridizations in this experiment met our quality requirements and were included in the analysis. For gene expression profiles determined by microarrays, four individual whole body samples, each of control, DMSO-solvent control and EHMC-exposed male fish, were analysed. Exposure to 2.2 μg/L and 890 μg/L EHMC resulted in a differential expression of 1096 and 1137 genes (fold change ≥2, $p < 0.05$), respectively, as listed in Table S1. At 2.2 μg/L EHMC, 594 (54.2%) genes were down-regulated, and 502 (45.8%) up-regulated. Of the 1137 genes differentially expressed at 890 μg/L EHMC 699 (61.48%) were down-regulated, and 438 (38.52%) up-regulated. A total of 708 of the significantly altered genes were regulated at both EHMC concentrations, and they were always regulated in the same direction (up or down) (Table S1). The Venn Diagram (Fig. 1) indicates that the expression of 388 and 429 genes were differently altered (fold change ≥2, $p < 0.05$) by 2.2 and 890 μg/L EHMC, respectively.

The Gene Ontology (GO) provides a controlled way to describe gene products in three categories, namely cellular components, molecular functions and biological processes (Ashburner et al., 2000). GO terms for biological processes were examined to determine the function of genes with altered patterns of expression. At both concentrations, functionally identified genes fell into 1650 different categories. 'Cellular Process' was the most significantly overrepresented Biological Process GO term (Table S2). The top 50 GO processes are listed in Table S2. GO-categories show that mainly cellular processes, development process, system development, multicellular organismal development, anatomical structure development, muscle system process, muscle contraction and response to hormone stimulus are affected at both EHMC concentrations (Table S2).

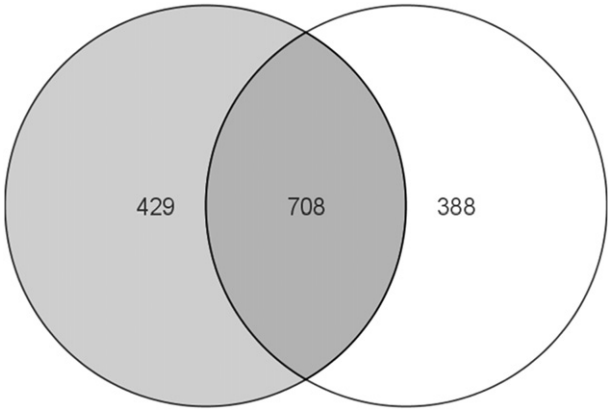

Fig. 1. Venn diagram illustrating the number of altered genes that are in common between the two EHMC exposures (708 genes), and number of genes that are only regulated by 2.2 (388) and 890 (429) μg/L EHMC, respectively.

Additionally to the GO analysis, we performed a pathway analysis (GeneGo Pathway Maps) with MetaCore TM. The different treatments showed 33 maps (2.2 μg/L EHMC: 33 maps; 890 μg/L: 36 maps) with their corresponding pathways in common (Table S3). The 890 μg/L EHMC treatment displayed 3 additional maps, namely, retinoid signalling, hypoxia response regulation and visual perception.

The 15 most relevant maps shown in Table S3 include pathways involved in tissue remodeling and wound repair, immune system response, inflammatory response, cell differentiation, DNA-damage response, cell cycle and its regulation, apoptosis, blood clotting, protein synthesis, calcium signalling, vasoconstriction, vascular development (angiogenesis), mitogenic signalling, protein degradation and androgen signalling.

3.1.2. Validation of microarray data by real-time qRT-PCR by assessment of target genes

To validate gene expression changes determined by microarrays, five genes involved in different pathways were selected. They include genes belonging to hormonal pathways such as vitellogenin 1 (*vtg1*), lipid metabolism such as the prostaglandin D2 synthase (*ptgds*), a gene that mediates cell growth, and known to be induced by estrogenic compounds (Riley et al., 2004; Hoffmann et al., 2006; Martyniuk et al., 2007; Baker et al., 2009) such as the insulin growth factor binding protein 1a (*igfbp1a*), a gene involved in vitamin A metabolism, the retinol-binding protein type II (*rbp2a*), and a gene involved in apoptosis, DNA damage and immune response such as the growth arrest and DNA-damage-inducible beta (*gadd45b*). The latter was previously found to be modulated by EE2 (Hoffmann et al., 2006).

These genes, *ptgds*, *igfbp1a*, *rbp2a* and *gadd45b* (except for *vtg1*, MetaCore software visualize mouse, rat, worm, fly, yeast and dog data on networks) were then used to build corresponding networks using MetaCore, as illustrated in Fig. S4 A, B, C and D.

These selected genes were validated by qRT-PCR in whole-body samples. In addition, these five genes were analysed in different tissues including liver, brain and testis to compare their expression in individual organs with those in the whole body (Table 3).

The observed mRNA alterations occurred always in the same direction in the whole body in both the microarrays and qRT-PCR measurements. This demonstrates that the transcriptional changes determined by microarrays are paralleled by qRT-PCR measurements, but the magnitude of the fold increase of these transcripts was more pronounced in the microarray analysis.

Different trends in expressional changes were observed in whole-body samples, and different tissues (Table 3). The *vtg1* mRNA was significantly down-regulated in the whole body at both EHMC concentrations, and this was also noted in the brain and testis. Conversely, a dose-related *vtg1* up-regulation occurred in the liver (Table 3). It is important to highlight that for the *vtg1* transcript the microarray data demonstrate a down regulation for 3 *vtg1* partial sequences (as for example agilent probe ID: A_15_P110740; Supplementary data S1). Based on BLAST (http://blast.ncbi.nlm.nih.gov/) comparisons and ClustalW multiple sequence alignment (http://www.ebi.ac.uk/Tools/clustalw/) we confirmed that the 3 partial *vtg1* sequences correspond to the *Danio rerio* gene *vtg1* with accession number NM_001044897.

Similar to the *vtg1* transcript, the *ptgds* transcript showed a different expression in the liver as compared to the whole body and the other tissues. This transcript was up-regulated in the whole body, brain and testis at both concentrations, while in the liver, it was down-regulated (Table 3). In the brain we observed a tendency for induction, whereas in the testis a significant up-regulation occurred, especially at 890 μg/L EHMC.

A dose-related and parallel increase occurred for the *igfbp1a* transcript in the whole body, and in the liver, brain and testis (Table 3). In brain and testis a considerable up-regulation is noted at 890 μg/L EHMC. The *rbp2* mRNA was down-regulated in the whole body and in all of the investigated tissues. The microarray data show that *rbp2* mRNA was strongly down-regulated in the whole body at 2.2 μg/L EHMC (Table 3). A significant induction was noted at both EHMC concentrations for the *gadd45b* transcript in the whole body, whereas the alteration was not significant in individual tissues (Table 3).

3.1.3. Transcriptional changes of selected target genes in tissues

Based on our previous study (Zucchi et al., 2011) seven additional candidate genes were chosen for the evaluation of molecular effects of EHMC with a focus on hormonal activity and steroidogenesis to test the hypothesis that this UV-filter acts on these processes. The selected genes included vitellogenin 3 (*vtg3*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), androgen receptor (*ar*) and hydroxysteroid 17-β dehydrogenase-3 (*hsd17ß3*). Their expression levels were evaluated by qRT-PCR in the whole body, liver, brain and testis (Fig. S2; Fig. 2 A, B, C; Fig. 3). Additionally, differential expressions of P450aromB (*cyp19b*) and P450aromA (*cyp19a*) were determined in brain and testis, respectively. The selected genes belong to three categories including an estrogen-responsive gene (*vtg3*), nuclear receptors (*esr1*, *esr2b*, *ar*) and steroid metabolism (*hsd17ß3*, *cyp19b* and *cyp19a*).

The modulation of *esr1* and *esr2b* transcripts in whole body and tissue samples show an almost identical trend and similar expression pattern. An induction occurred in the whole body and liver, whereas the transcripts were down-regulated in brain and testis (Fig. 2 A and B). The alterations were mostly significant at 890 μg/L EHMC, except for the brain, were the down-regulation was

Table 3
Fold changes (Log2) of selected genes differentially regulated in zebrafish adult male determined by microarray (GeneSpring normalization) and qRT-PCR ($2^{-\Delta\Delta Ct}$ normalization) after exposure to 2.2 μg/L and 890 μg/L EHMC. Values are expressed as average fold change. Asterisks show statistically significant difference to control ($p < 0.05$).

		Fold Change (Log2)									
		2.2 μg/L EHMC					890 μg/L EHMC				
		Whole fish	Whole fish	Liver	Brain	Testis	Whole fish	Whole fish	Liver	Brain	Testis
GenBank Acc	Description	Array	qRT-PCR	qRT-PCR	qRT-PCR	qRT-PCR	Array	qRT-PCR	qRT-PCR	qRT-PCR	qRT-PCR
NM_001044897	Vitellogenin 1 (***vtg1***)	−5.03*	−1.66*	1.05*	−0.60*	−0.57*	−2.96*	−3.31*	1.39*	−0.63*	−0.69*
NM_213634	Prostaglandin D2 synthase (***ptgds***)	1.75*	1.35*	−0.90	0.51	1.58*	2.54*	1.93*	−0.57	0.70	2.38*
NM_173283	Insulin-like growth factor binding protein 1a (***igfbp1a***)	1.46*	0.71	1.03*	0.83*	1.66*	1.18*	1.82*	1.48*	3.24*	3.62*
AF363957	Retinol-binding protein type II (***rbp2a***)	−5.08*	−0.51	−0.60	−2.53*	−0.23	−3.69*	−0.97*	−0.55	−1.60*	−0.59
NM_213031	Growth arrest and DNA-damage-inducible, beta (***gadd45b***)	2.80*	2.26*	−1.17	−0.74	−0.83	1.61*	0.98*	−0.42	0.61	−0.42

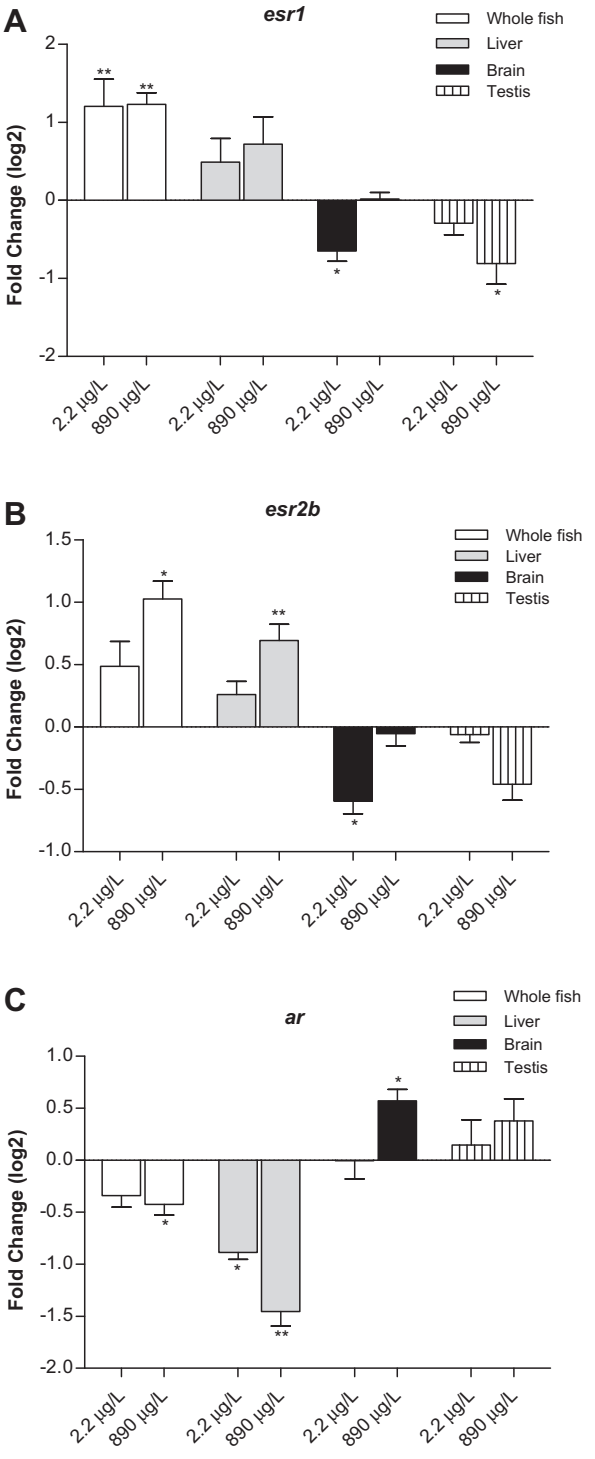

Fig. 2. Relative gene expression of *esr1* (A), *esr2b* (B) and *ar* (C) in whole body, liver, brain and testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes (log2) in *esr1*, *esr2b* and *ar* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation ($n = 4$ replicates for adults). Asterisks indicate significantly higher expression than control ($^{*}p < 0.05$), and ($^{**}p < 0.01$).

significant at 2.2 µg/L. The *vtg3* transcript was not significantly altered in all the investigated tissues (Fig. S2).

The *ar* transcript was significantly down-regulated in the whole body at 890 µg/L (Fig. 2C). A dose-related down-regulation was also

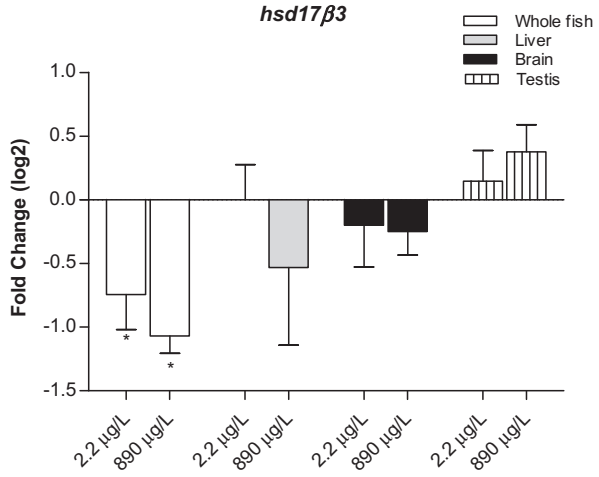

Fig. 3. Relative gene expression of *hsd17β3* in whole body, liver, brain and testis of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in *hsd17β3* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation ($n = 4$ replicates for adults). Asterisks indicate significantly higher expression than control ($^{*}p < 0.05$), and ($^{**}p < 0.01$).

noted in the liver (Fig. 2C), whereas in the brain, a slight but significant up-regulation occurred at 890 µg/L EHMC. The *hsd17β3* transcript was significantly down-regulated in whole-body samples in a dose-dependent manner (Fig. 3).

In the brain the transcription of *cyp19b,* encoding the aromatase B, was significantly induced at 890 µg/L EHMC (Fig. 4). However, the amount of *cyp19a* transcripts, encoding aromatase A, did not significantly change after EHMC treatment (Fig. S3).

4. Discussion

In the present study we examined molecular effects of EHMC in zebrafish by applying gene expression profiling and a targeted gene expression approach. The high lipophilicity of EHMC (log P =5.66) made it indispensable to use of DMSO (concentration 0.01%) as a solvent in agreement with OECD guidelines. DMSO readily crosses the skin of humans and acts as a skin penetration enhancer for drugs and other substances in man, but its influence on fish body surfaces and gills are not understood. Although DMSO shows no toxicity at the concentrations used, it cannot be ruled out that the uptake of EHMC may have been facilitated. However, we do not expect that DMSO had an effect on the nature of expressional changes determined and therefore induced by EHMC.

Global gene expression profiling was applied in the whole body and alterations of selected microarray-detected target genes were validated by means of qRT-PCR. Therefore, we determined the expression of a suite of target genes to compare the response in the whole body to that in individual tissues. Based on previous *in vitro* and *in vivo* studies (Kunz and Fent, 2006b; Inui et al., 2003) indicating a hormonal activity, we additionally chose target genes to test the hypothesis that EHMC interferes with genes involved in sex hormonal signalling and steroidogenesis, as indicated in fathead minnows (Christen et al., 2011).

We analyzed the response in whole organisms, because the target tissues of EHMC are currently unknown. By this approach we capture the total sum of the transcriptional changes in the entire organism, and thus the overall molecular effect giving indications on the mode of action of EHMC on the transcriptome. However, at the same time, this approach has its limitations, such as loss of

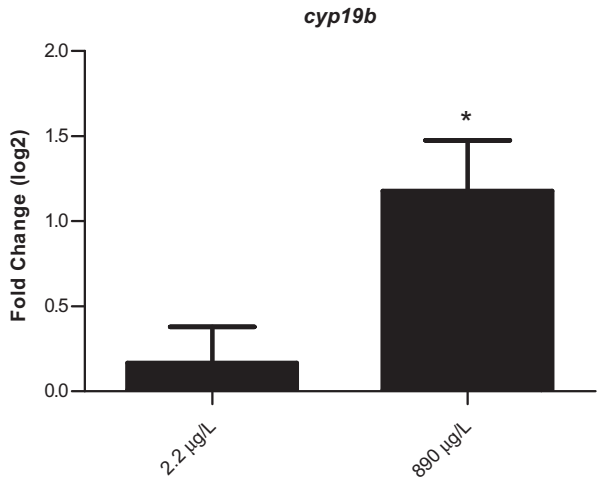

Fig. 4. Relative gene expression of *cyp19b* in brain of adult male zebrafish after exposure to 2.2 and 890 µg/L of EHMC. Relative transcript abundance was quantified by real-time reverse transcription PCR (qRT-PCR); the fold changes in *cyp19b* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation ($n = 4$ replicates for adults). Asterisks indicate significantly higher expression than control (*$p < 0.05$), and (**$p < 0.01$).

weak signals or signals from small tissues, and the tissue-specific contributions cannot be unravelled. The microarray data demonstrate a high number of expressional changes in the whole-body samples and associated biological pathways. As in the case of our present study, they do not necessarily provide a defined, clear and simple picture of affected processes, making data interpretation challenging. Despite these limitations, we have chosen this promising approach as the predictive power of whole-organism genomics has been demonstrated in zebrafish exposed to polycyclic aromatic hydrocarbons and estrogenic compounds (Lam et al., 2008). This approach also provides a fingerprint of all expressional changes within the organism, as the specific target organs of EHMC are unknown. This is in contrast to our previous studies on pharmaceuticals having a known mode of action (Oggier et al., 2010, 2011). In addition, we chose a targeted gene approach to scrutinize potential mode of actions, with emphasis on hormonal pathways.

4.1. Microarray analysis and gene categorization in whole-organism

Exposure to EHMC altered the expression of a considerable number of genes in the entire organism. A comparison of the transcriptional profiles in whole bodies and different tissues demonstrated that the overall expression pattern and tissue-specific profiles do not necessarily match.

A total of 1096 and 1137 genes were altered at 2.2 µg/L and 890 µg/L EHMC, respectively, which is in the same range of genes affected in the liver of zebrafish after exposure to 17α-ethynylestradiol (EE2) (Hoffmann et al., 2006). Complex interrelationships in expressional changes with most genes showing differential responses between tissues (and sexes) were also reported upon exposure of fathead minnows to 17β-estradiol (E2) (Filby et al., 2006).

EHMC influences mainly the biological process (GO:008150) and molecular function (GO:003674) as highlighted by GO terms classification. At both EHMC concentrations differentially altered genes belong to many biological processes indicating a diverse effects pattern. The top 50 GO processes include cell processes (*e.g.*: cell death, cellular metabolic process, cell cycle and transmembrane transport), developmental process (*e.g.*: multicellular organismal development, anatomical structure development), muscle system process and contraction (*e.g.*: muscle cell differentiation, smooth muscle contraction), response to hormone stimulus (*e.g.*: response to steroid hormone stimulus, response to chemical stimulus), response to endogenous stimulus (*e.g.*: response to epidermal growth factor stimulus, response to hormone stimulus), regulation of multicellular organismal process (*e.g.*: regulation of angiogenesis, regulation of nervous system development, regulation of cytokine production), tissue development (*e.g.*: multicellular organismal development, organ development), among others (Table S2). According to the GO processes, the major altered pathways by EHMC are mainly involved in tissue remodeling, wound repair, immune system response, inflammatory response, cell differentiation, DNA-damage response, cell cycle and its regulations and apoptosis.

Often GO analysis can be useful in providing information about the modes of action of a chemical, but the many GO terms affected by EHMC exposure are general descriptions, which based on the results of this experiment alone without phenotypic anchoring, makes it difficult to link them to a specific mode of action. The differentially expressed genes involve many pathways indicating that EHMC rather than affecting just a few key pathways exhibits distributed effects across several biological processes and pathways. This is in contrast to our previous study on the neuropharmaceutical diazepam, which selectively affects few processes related to its known mode of action in zebrafish brain (Oggier et al., 2010).

We critically screened the genes involved in the most altered pathways by EHMC. Interestingly, we found genes such as talin 1 (*tln1*; NM_001009560) and transcripts of many homeobox genes to be affected by EHMC. In particular, *hoxa13b* (NM_131194), *hoxb6b* (NM_131538), *hoxa10b* (NM_131155) were increased at both EHMC concentrations, while the expression of *hoxb6a* (NM_131119) and *hoxc6b* (NM_131119) was reduced. EHMC lead to down-regulation of *tln1*; this gene encodes a cytoskeletal protein, which is concentrated in areas of cell-substratum and cell–cell contacts. The down-regulation by EHMC is in agreement with the observed affected tissue remodelling and wound repair pathways. In contrast, EE2 was found to induce *tln1* in stickleback (Katsiadaki et al., 2010), and estrogenic compounds are effective regulators of cell migration via cytoskeleton alteration (Acconcia et al., 2006; Giretti and Simoncini, 2008).

Hox genes play an important role in controlling development processes and patterning of the body axis during embryogenesis but *hox* genes are also expressed in the adult organism (Morgan, 2006). Expressional changes of *hox* genes suggest effects of EHMC on developmental processes, but functions and consequences of *hox* genes in the adult fish remain unclear. A similar pattern (although not involving all the same genes) occurred in the zebrafish telencephalon exposed to EE2 (Martyniuk et al., 2007). Moreover, alteration of *hox* gene expression in whole-body homogenates occurred in zebrafish after exposure to E2 (Cohen et al., 2008). Several hormones regulate *hox* gene expression (Daftary and Taylor, 2006), and estrogens including diethylstilbestrol (Akbas et al., 2004) and methoxychlor (Fei et al., 2005) alter *hox* expression. These data suggest that EHMC also acts similarly as hormonally active compounds on these genes. In addition, we found xeroderma pigmentosum, complementation group C, (*xpc*; NM_001045210), excision repair cross-complementing rodent repair (*ercc1*; NM_001103138), proliferating cell nuclear antigen (*pcna*; NM_131404), fanconi anemia complementation group L (*fancl*; NM_212982), replication protein A2 (rpa2; NM_131711), ezrin like (*ezrl*; NM_001020490), wingless-type MMTV integration site family, member 7Aa (*wnt7a*; NM_001025540), phosphatase and tensin homolog B (*ptenb*; NM_001001822), mitogen-activated

protein kinase 1 (*map2k1*; NM_213419) and glutathione S-transferase pi (*gstp1*; BC083467) being altered in both EHMC dose groups. The alteration of these genes, in particular those involved in nucleotide excision repair (NER) pathway, such as *xpc, ercc1*, *fancl*, *rpa2*, *pcna* and *gadd45b* are in agreement with the observed modulated pathways such as DNA-damage response, apoptosis and inflammatory response. This is in accordance to data in human cell lines, where *xpc* and *ercc1* involved in cellular response to DNA-damage repair was altered after exposure to EHMC (Duale et al., 2010). NER is an important DNA repair system that eliminates a wide variety of helix-distorting DNA base lesions. EE2 led to a decrease in hepatic NER gene expression in adult male zebrafish including *xpc* (Notch et al., 2007), similarly to the effects displayed by EHMC in the present study.

4.2. Validation of microarray data

Based on the results obtained from GeneSpring normalization, canonical pathways analysis, and based on literature we choose 5 target genes including *vtg1*, *ptgds*, *igfbp1a*, *rbp2a* and *gadd45b* to validate the microarray results by means of qRT-PCR. We found that the mRNA alterations occurred always in the same direction in the whole body, both in microarrays and qRT-PCR. This demonstrates the validity of the microarray data.

4.3. Differentially regulated genes in whole body, liver, brain and testes

The differential expression profile of the chosen genes upon exposure to EHMC is often tissue-specific. For some genes including *igfbp1a* and *rbp2a*, similar changes were observed in the whole body and different tissues, for the other chosen genes, however, a differential expression occurred (Table 3; Figs. 2 and 3 A, B, C). As for the selected genes, the data obtained by microarrays seem more similar to the data obtained by qRT-PCR in the brain than those in the liver. To a lesser extent this also holds for the testis, where we observed the highest variability in terms of Ct value and the lowest responsiveness among the selected genes. The expressional changes were, however, not paralleled in the liver, which is often analysed (Sumpter and Jobling, 1995; Hoffmann et al., 2006; Filby et al., 2007). This indicates that the molecular effects and modes of action of a compound cannot sufficiently be described in one organ alone, but rather needs a multi-organ approach for a more complete understanding. This has also been shown in fathead minnows (*Pimephales promelas*) experimentally exposed to E2 (Filby et al., 2006).

A similar responsiveness of some transcripts selected from microarrays was observed between whole body and the tissues. This holds for the expression of genes involved in regulation of growth and proliferation (*igfbp1a*), and those involved in vitamin A biosynthesis (*rbp2a*), which are also modulated by estrogens (Hoffmann et al., 2006; Martyniuk et al., 2007). The alterations by EHMC (up-regulation of *igfbp1a*, down-regulation of *rbp2a*, Table 3) are similar in the whole body and in all the investigated tissues (liver, brain and testis), although not to the same extent. A similar pattern, up-regulation of *igfbp1a* and down-regulation of *rbp2a,* was found after exposure of zebrafish to EE2 (Hoffmann et al., 2006), which suggests an estrogenic activity of EHMC towards these genes and processes.

A well established biomarker for estrogenic activity is the induction of *vtg1* and its protein, which is tissue-, stage- and sex-specific (Sumpter and Jobling, 1995; Tyler and Sumpter, 1998). Whole-body microarray data in zebrafish show that among other genes *vtg1* expression occurs after exposure to estradiol, diethylstilbestrol and bisphenol A (Lam et al., 2008). In our study, we observed a down-regulation of *vtg1* transcripts in the whole body by EHMC. However, the differential expression profile was not equal in all the tissues. Similar to the whole body, *vtg1* expression was down-regulated in brain and testis, whereas a dose-related up-regulation occurred in the liver. Thus far, induction of *vtg1* by estrogens has mainly been investigated in the liver (Islinger et al., 2003; Wang et al., 2005; Hoffmann et al., 2006; Sun et al., 2010; Levi et al., 2009). Consequently, our data suggest an estrogenic activity of EHMC in the liver, which is supported by the parallel induction of *esr2b*. On the other hand, down-regulation of *vtg1* in the whole body, brain and testis suggests an anti-estrogenic activity of EHMC in these extrahepatic tissues. This finding is supported by recent findings in fish, where estradiol and BP-4 induced *vtg1* not only in liver, but also in heart and brain (Yin et al., 2009; Ma et al., 2009; Zucchi et al., 2011). An alteration of *vtg* expression cannot univocally be ascribed to an estrogenic response as its induction occurs by hypoxia (Wu et al., 2003), and recently Zhang et al. (2010) demonstrated that vitellogenin plays an important role in innate immune responses.

In general in the whole body, liver, brain and testis EHMC led to a dose-dependent up-regulation of the insulin-like growth factor binding protein 1a (*igfbp1a*). This transcript was selected in our study, because of its alteration by estrogens in fish (Riley et al., 2004; Hoffmann et al., 2006; Martyniuk et al., 2007; Baker et al., 2009). Our data on EHMC are in agreement with previous studies in fish that showed alteration of *igfbp1a*, a transcript involved in cell growth. However, the *igfbp1a* up-regulation cannot univocally be ascribed as a pure estrogenic response, since other physiological factors are also inducers (Kelley et al., 2002; Riley et al., 2004). Further studies should evaluate, whether the alteration of genes involved in growth and proliferation will result in effects on these processes.

A gene involved in vitamin A biosynthesis, the retinol-binding protein type II (*rbp2a*), was down-regulated by EHMC in the whole body and brain (and in liver and testis, although not significantly). The expression of retinol-binding protein (*rbp*) has been proposed as a possible biomarker for exposure to endocrine disrupting compounds (Levy et al., 2004). Similar to our observation with EHMC, the expression of *rbp2a* was down-regulated by EE2 in zebrafish (Hoffmann et al., 2006), and rainbow trout (Sammar et al., 2001). The effect of EHMC on *rbp2a* and thus vitamin A biosynthesis, metabolism and transport seems to represent an estrogenic response, but this needs to be further evaluated on a physiological level.

The prostaglandin D_2 synthase catalyzes the conversion of prostaglandin H_2 (PGH_2) to prostaglandin D_2 (PGD_2) and the expression of *ptgds* is androgen-regulated (Love et al., 2009). This transcript is down-regulated after exposure to (EE2) (Hoffmann et al., 2006), but also by the androgen 17β-trenbolone (Dorts et al., 2009). In contrast, in our study EHMC led to up-regulation of *ptgds* in the whole body and testis. Similarly, an up-regulation was observed in flounder (*P. americanus*) experiencing multiple stress (Straub et al., 2004).

EHMC led to significant induction of DNA-damage-inducible beta (*gadd45b*) in the whole body, which is in agreement with the pathways identified including apoptosis and growth arrest. Similarly, EE2 led to a significant up-regulation of this transcript in zebrafish liver (Hoffmann et al., 2006). No significant differential expression of *gadd45b* occurred in the tissues analysed. Consequently, its transcription is induced in tissues other than liver, testis and brain.

4.4. Effects of EHMC on genes involved in hormonal pathways and steroidogenesis

EHMC led to a significant down-regulation of *vtg1*, which was not the case for *vtg3*, although the same trend was observed.

Estrogens alter the expression of both *vtg1* and *vtg3* in fish (Miracle et al., 2006), although the responsiveness may differ (Islinger et al., 2003; Martyniuk et al., 2007).

The estrogen receptor genes *esr1* and *esr2b* were significantly up-regulated by EHMC in the whole body, which seems in contrast to the down-regulation of the *vtg1* transcript (as parallel induction occurs with estrogens). In contrast, down-regulation of *vtg1* was paralleled with *esr1* and *esr2b* down-regulation in the brain. In the liver, however, *esr2b* up-regulation would support the observed hepatic *vtg1* induction. The differential effect of EHMC on *vtg1* expression in different tissues highlights the importance for analyzing transcription profiles in multiple tissues

In the whole body and liver, we observed a down-regulation of *ar* expression, while a slight up-regulation occurred at 890 μg/L EHMC in the brain. Filby et al. (2007) found no modulation of the *ar* transcript after EE2 exposure in male fathead minnows, while a strong down-regulation in hepatic *ar* mRNA was observed after exposure to the antiandrogen flutamide. This suggests an anti-androgenic activity of EHMC in the liver, and possibly in other organs.

EHMC-exposure slightly affected the expression of genes involved in steroidogenesis. A significant down-regulation of *hsd17ß3* was observed in the whole body. This may suggest a reduced formation of the enzyme, and in turn, reduction of testosterone synthesis. Additionally, expression of *cyp19b* was significantly up-regulated in the brain. Estrogens enhance the expression of *cyp19* in fish, in particular *cyp19b* in the brain (Callard et al., 2001), but also androgens differentially affect *cyp19* expression (Govoroun et al., 2001; Mouriec et al., 2009). This infers that EHMC acts on the expression of genes important for sex hormone synthesis. EHMC also displayed multiple hormonal activities in fathead minnows (Christen et al., 2011). The expressional changes in both fish species are supported by previous *in vitro* data (Kunz and Fent, 2006a). Forthcoming studies are needed to evaluate the consequences of observed transcriptional changes on plasma sex steroid levels, and on fertility and reproduction.

5. Conclusions

Our microarray study demonstrates that the expression profile in whole fish after EHMC exposure affects many biological processes with pathways mainly involved in tissue remodelling, immune system response, inflammatory response, DNA-damage and apoptosis. The high abundance of significantly altered transcripts by EHMC makes a pathway analysis interpretation challenging. No single pathway or a simply defined pattern of affected cellular mechanisms could be identified. The whole-body microarray analysis approach captures the total sum of the transcriptional changes, hence providing an overall expression profile. As it is less sensitive than the organ-specific analysis, data interpretation is more challenging. For obtaining a more complete toxicological profile of a compound, we conclude that the analysis should cover multiple tissues.

Based on previous knowledge about hormonal activities of EHMC (Inui et al., 2003; Kunz and Fent, 2006b) we focused on hormone and related pathways (lipid and cholesterol metabolism, thyroid hormone biosynthesis or metabolism). We found that EHMC induces transcriptional chances of genes involved in hormone pathways and steroidogenesis. A down-regulation of *vtg1* in whole body, brain and testis, but a dose-related up-regulation was observed in the liver. The induction of estrogen receptors *esr1* and *esr2b* in the whole body and of *vtg1*, *igfbp1*, and *esr2b* in the liver suggests an estrogenic activity of EHMC. The associated down-regulation of *ar* in the liver seems to reflect an additional anti-androgenic activity. Differently, in the brain, a down-regulation of *vtg1*, *esr1, esr2b* and *rbp2a* suggests an anti-estrogenic, and the up-regulation of *ar* and *cyp19b* a potential androgenic activity. Therefore, the observed multiple hormonal activities of EHMC on gene expression in different tissues coincide with the activities *in vitro* (Kunz and Fent, 2006a). Differential expression profiles in the whole body and analysed tissues (brain, liver, testis) for several genes underlines the need for analysing multi-tissue gene profiling for understanding potential effects of this UV-filter. The data lead to the conclusion that EHMC has an overall low, but multiple hormonal activity. Furthermore, additional biological processes are modulated by EHMC, including tissue remodelling, cell differentiation, immune system response, DNA-damage response and apoptosis.

The transcriptional changes were observed at environmentally realistic concentrations of 2.2 μg/L EHMC. Up to 19 and 3 μg/L EHMC were reported in raw wastewater (Balmer et al., 2005) and in a lake (Rodil et al., 2009), respectively. More data are needed on dose-response relationships and on the time course of expressional changes to describe the toxicological implications of EHMC exposure. Forthcoming studies should investigate the toxicological consequences and environmental risks of EHMC, in particular by linking expressional changes with physiological outcomes including fertility and reproduction.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2011.04.013.

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https://link.springer.com/article/10.1007%2Fs00204-002-0348-4 (OMC one of 5 UV filters that caused an increase in MCF-7 cell proliferation and an increased secreation of the estrogenic-regulated pS2 protein. And increase rat uterin weight)

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Estrogenic activity of UV filters determined by an in vitro reporter gene assay and an in vivo transgenic zebrafish assay.

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Abstract
In the past decade the list of chemicals in the environment that are able to mimic the natural hormone estrogen, thereby disrupting endocrine function, has grown rapidly. These chemicals are able to bind to estrogen receptors (ERs) and influence estrogen signalling pathways, although several of them have structures that differ substantially from the endogenous hormone 17beta-estradiol. In this study, six extensively used ultraviolet (UV) filters were assessed for transcriptional activation of estrogen receptors. Because of their high lipophilicity, these UV filters tend to bioaccumulate in the environment. They have been found in surface waters, fish, and in human milk fat. Using a sensitive in vitro reporter gene assay, we found that all six compounds induce estrogenic activity towards ERalpha, while four out of six compounds induced transcriptional activity of ERbeta. Zebrafish, in which an estrogen responsive luciferase reporter gene has been stably introduced, were used for in vivo testing. In this transgenic zebrafish assay none of the compounds showed estrogenic activity. Our findings suggest that one should be aware of over-interpretation when predicting in vivo effects from weak in vitro data. However, it can not be ruled out that these UV filters have long-term effects in the environment.

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Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*)

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Abstract

Ultra violet (UV) screens and preservatives are widely and increasingly used in cosmetics and pharmaceuticals. In the present study, we examined the estrogenicity of 4-methyl-benzylidene camphor (4-MBC), octyl-methoxycinnamate (OMC), and propyl paraben (*n*-propyl-*p*-hydroxy-benzoate; PP), among UV screens and preservatives, using male medaka (*Oryzias latipes*), in regard to production of vitellogenin (VTG) and choriogenin (CHG) which are known to be estrogen-responsive gene products. First, using a VTG enzyme-linked immunosorbent assay (ELISA) system, we determined the increase in VTG plasma concentration in medaka due to exposure to 4-MBC, OMC, and PP, and compared this concentration to the non-treated control. Next, we found increases in mRNA expression levels of VTG subtypes VTG-1 and VTG-2, and CHG subtypes CHG-L and CHG-H, in liver due to exposure to 4-MBC, OMC, and PP compared to the non-treated control. In addition, we also found increased mRNA expression levels of estrogen receptor (ER) α, among sex hormone receptors in the liver, due to exposure to 4-MBC, OMC, and PP compared to the non-treated control. In this study, we showed that 4-MBC, OMC, and PP have estrogenic activity in fish.

Keywords: UV screens; Preservatives; ELISA; Real-time RT-PCR; Medaka

Abbreviations: AR, androgen receptor; CHG, choriogenin; E_2, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; 4-MBC, 4-methylbenzylidene camphor; OMC, octyl-methoxycinnamate; PP, propyl paraben; RT-PCR, reverse transcription and polymerase chain reaction; UV, ultraviolet

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1. Introduction

Organic chemicals that absorb ultraviolet radiation, which are called UV screens, are added to sunscreen products in concentrations of up to 10% for skin protection. Some of these compounds are also included in other cosmetics such as beauty creams, lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and bubble bath powders, for product stability and durability. The use of UV screens is increasing because of growing public concern about skin damage, due to sunlight. Thus far, in studies of humans, acute and subchronic systemic toxicity of these compounds has been considered to be rather low (Okereke et al., 1995; Hayden et al., 1997), although there have been some problems, such as photoallergic reactions (Schauder and Ippen, 1997). However, it has been reported recently that these UV screens, induce pS2 protein in MCF-7 breast cancer cells, and that 4-methyl-benzylidene camphor (4-MBC) and uterotropic assay showed dose-dependent increases in uterine weight due to 4-MBC and octyl-methoxycinnamate (OMC) (Schlumpf et al., 2001).

Parabens are the most commonly used preservatives in cosmetics, toiletries, pharmaceuticals, and foods because of their relatively low toxicity in humans and their effective antimicrobial activity (Elder, 1984). However, it has been reported that the estrogenic activity of parabens and their main metabolites may be demonstrated by in vitro recombinant yeast assay, human estrogen receptor (ER) assay, and E-screen (Lemini et al., 1997; Routledge et al., 1998; Blaier et al., 2000; Pedersen et al., 2000; Satoh et al., 2000; Okubo et al., 2001). In particular, propyl paraben (*n*-propyl-*p*-hydroxy-benzoate; PP) has the highest estrogenic activity among paraben esters (Routledge et al., 1998; Blaier et al., 2000; Nishihara et al., 2000; Satoh et al., 2000; Okubo et al., 2001). It has also been reported that PP directly affects the male reproductive system (Song et al., 1989; Song et al., 1991; Oishi, 2002).

These previous reports revealed that 4-MBC and OMC in UV screens, and PP in preservatives have estrogenic activity especially in in vitro system. Recently, international and national estrogenic compound screening programs have established methods for the screening and testing of environmental chemicals under the Organization for Economic Co-operation and Development (OECD) and the US Environmental Protection Agency (EPA). Examples are use of sensitive fish species (e.g. fathead minnow *Pimephales promelas*, zebrafish *Danio rerio*, rainbow trout *Oncorhynchus mykiss*, and medaka *Oryzias latipes*) for specific tests (Hutchinson and Pickfold, 2002). In particular, medaka fish have been used in Japan as a model in systems for monitoring chemicals in water. Vitellogenin (VTG) is well known as an estrogen-responsive phosphoprotein, and can also be used as a biomarker of contaminant exposure in fish such as medaka. VTG is a complex egg yolk precursor protein in oviparous vertebrates and is produced in the liver (Ng and Idler, 1983). Recently, it has become possible to conveniently measure medaka plasma VTG concentration, since enzyme-linked immunosorbent assay (ELISA) has been developed for measurement of medaka VTG (Nishi et al., 2002). Moreover, it has been reported that choriogenin (CHG), known to be a liver-derived precursor protein for the inner layer subunits of the fish egg envelopes (Murata et al., 1997), responds to estrogenic compounds (Lee et al., 2002). These reports show that two proteins, VTG and CHG, are key molecules that are present in medaka livers, and are estrogen-responsive proteins.

In the present study, we analyzed the VTG plasma concentration of medaka exposed by various concentrations of 4-MBC, OMC, and PP as compared to exposure to 17β-estradiol (E_2) using a VTG ELISA system (Nishi et al., 2002). We also measured mRNA expression levels of the VTG subtypes VTG-1 and VTG-2 (Shimizu et al., 2002), of the CHG subtypes CHG-L and CHG-H (Murata et al., 1997), and of medaka sex hormone receptors, such as ERα, ERβ, and androgen receptor (AR) in the liver. In this study, we found increased plasma VTG concentration and increased mRNA expression level of VTG-1, VTG-2, CHG-L, and CHG-H in medaka exposed to 4-MBC, OMC, and PP.

2. Materials and methods

2.1. Chemicals

OMC (Eusolex 2292) and 4-MBC (Eusolex 6300) were purchased from Sigma–Aldrich (Tokyo, Japan),

and PP and E_2 were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Fish and study design

Adult male Japanese medakas (*O. latipes*) (body length 2.5–3.5 cm) were purchased from a local commercial supplier. Experimental fish were maintained for 7 days in 2 l of pure water, and were fed daily with tropical fish flake food (TetraFin, Tetra GmH, Melle, Germany). The acclimated fish (each treatment group: $n = 5$) were exposed to 4-MBC (0.039, 0.39, and 3.9 mM), OMC (0.034, 0.34, 3.4, and 34 mM), PP (0.055, 0.55, 5.5, and 55 mM) or E_2 (3.7, 37, and 185 nM). Solutions including these chemicals were changed every other day for 1 week. Although we exposed male medakas to concentrations of estradiol similar to those of UV screens and PP, the fish perished. Moreover, since Schlumpf et al. (2001) have reported that similar effects of estradiol appear at about 1/1000, the concentration in UV screens, in MCF-7 cell proliferation assay, we used this as the fixed treatment concentration. Non-treated control fish were only exposed to solvent (ethanol final concentration: 0.1%). Blood was collected by cutting the isthmus of the fish, and then placing a disposable micropipet (Fisher Scientific, St. Louis, MO, USA) at the cut end of the ventral artery. For quantification of VTG, blood was placed in a microcentrifuge tube that contained assay buffer, in an ELISA system for VTG (EnBioTech Laboratories, Tokyo, Japan). In addition, the livers were removed from the fish and were frozen in liquid nitrogen for real-time reverse transcription, and polymerase chain reaction (real-time RT-PCR) analysis.

2.3. ELISA of plasma VTG concentration

ELISA of the plasma VTG concentration was performed using a system produced by EnBioTech Laboratories. Samples and standards were analyzed in triplicate. Data are shown as mean.

2.4. Analysis of mRNA expression by means of real-time RT-PCR

Real-time RT-PCR analysis was carried out to estimate the mRNA expression of VTG-1, VTG-2, CHG-L, CHG-H, ERα, ERβ, and AR in the livers. After the collection of blood, the livers ($n = 5$) were separated and frozen in liquid nitrogen. The cDNA templates for real-time RT-PCR were synthesized from poly(A)+ RNA of the fish livers using reverse-transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD). PCR was performed using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacture's instructions, but with one modification: reaction volume was adjusted to 25 μl. The primer pairs are shown

Table 1
Primer pairs using real-time RT-PCR

Gene name (accession no.)	Primer (upper: sense primer; lower: antisense primer)	PCR product (bp)
VTG-1 (AB064320)	5′-CTCCAGCTTTGAGGCCATTTAC-3′	81
	5′-ACAGCACGGACAGTGACAACA-3′	
VTG-2 (AB074891)	5′-CCAAGACCAAAGACCTGAACC-3′	167
	5′-TAAGATTAGGGAACCAGTAGT-3′	
CHG-L (AF500194)	5′-CAACATCTGCTGCTTATCCCC-3′	257
	5′-GACATCGCCTTCCCATTCCAG-3′	
CHG-H (AF500195)	5′-TACTTTCCCGTCACTTATTGC-3′	189
	5′-TTCCACGACCAGAGTTTCAAC-3′	
ERα (D28954)	5′-CCTCTCCCCACCTCGCCTCTC-3′	97
	5′-CAAAAAGTCCACAGCAGCCAC-3′	
ERβ (AB070901)	5′-CTGTTAGATGCCTCGGACCTT-3′	204
	5′-GATTGGCTGGCTGGTTTCGTG-3′	
AR (AF178118)	5′-CTCCTCACCAGCCTTAACGA-3′	142
	5′-AGACCATCACTCCCACCCAA-3′	
β-Actin (S74868)	5′-TCCACCTTCCAGCAGATGTG-3′	76
	5′-AGCATTTGCGGTGGACGAT-3′	

in Table 1. Statistical evaluations were performed by ANOVA test. Data are shown as mean ± S.E.M., and statistical significance is defined as $P < 0.01$ against non-treated control.

3. Results

3.1. Plasma VTG concentration in medaka

Plasma VTG levels increased dose-dependently after all chemical treatments. However, plasma VTG levels were only slightly higher than those of the non-treated control following treatment with large doses of OMC. Plasma VTG levels of medaka treated with 4-MBC and PP were similar to those obtained following treatment with 10^{-5} times the E_2 concentration (Fig. 1).

3.2. Estimation of mRNA expression of VTG, CHG, and sex hormone receptors using real-time RT-PCR analysis

To analyze the mRNA expression of egg proteins VTG and CHG, as well as sex hormone receptors ERα, ERβ, and AR in chemical-treated and non-treated medaka, we performed real-time RT-PCR. Each primer pair is summarized in Table 1.

The expression levels of VTG-1 and VTG-2 increased dose-dependently following all the chemical treatments (Fig. 2). Moreover, the expression levels of CHG-L and CHG-H also increased dose-dependently after all chemical treatments, and the expression level of CHG-L was more dose-dependent than that of CHG-H. In addition, increased CHG-L mRNA expression was noted in treatments with concentrations lower than that of CHG-H mRNA (Fig. 3).

The expression level of ERα in sex hormone receptors increased with high dose treatments, except in the case of 4-MBC. However, the expression levels of ERβ and AR were not significantly different from those of the non-treated control, except in the case of ERβ expression following high-dose exposure to PP and E_2 (Fig. 4).

4. Discussion

In the present study, we analyzed the VTG plasma concentration and liver mRNA expression level of VTG, CHG, and sex hormone receptors of medaka exposed by various concentrations of 4-MBC, OMC, and PP as compared to exposure to E_2.

We found for the first time that increase of plasma VTG concentration was induced in male medaka exposed to OMC and 4-MBC. In UV screens, plasma

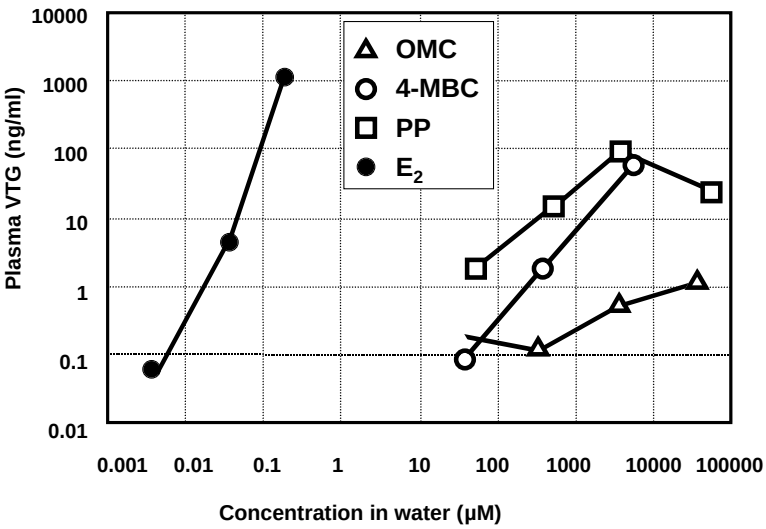

Fig. 1. Dose-response relationship of plasma VTG level to OMC, 4-MBC, PP, and E_2. Data are shown as mean ($n = 5$).

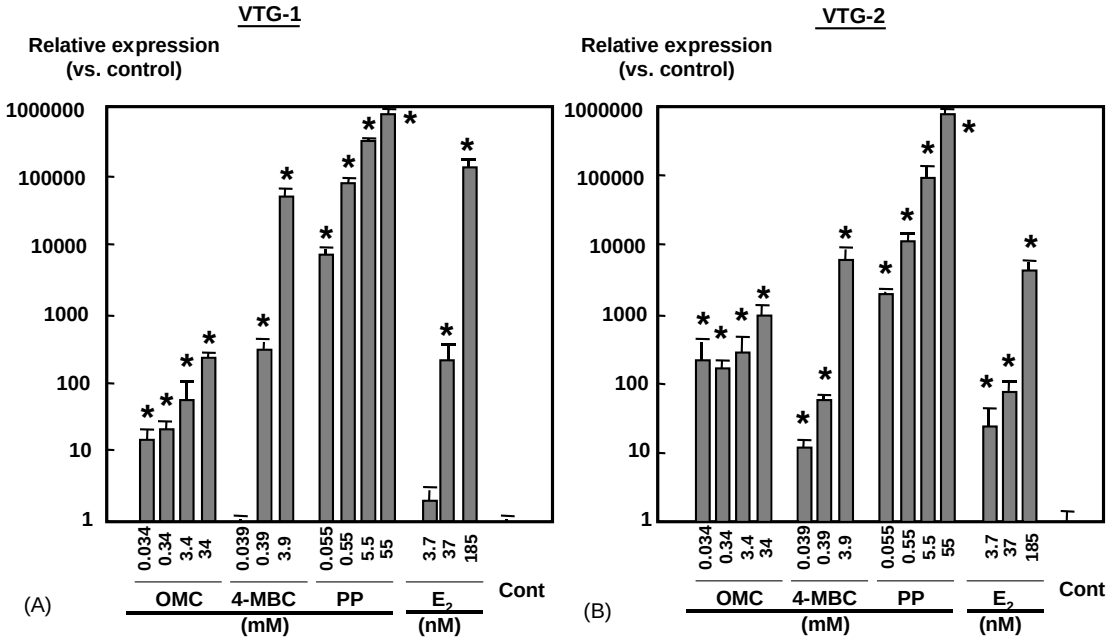

Fig. 2. Real-time RT-PCR analysis of (A) VTG-1 and (B) VTG-2 in medaka liver. Relative intensity of VTG-1 and VTG-2 mRNA expression was calculated against each β-actin mRNA expression, and expression in non-treated control was normalized as one. Data ($n = 5$, mean ± S.E.M.) are presented as relative values compared to non-treated control. $^{*}P < 0.01$ compared with non-treated control was considered significant.

VTG concentration of medaka exposed to 4-MBC was higher than that of medaka exposed to OMC (Fig. 1). It has been reported in regard to UV screens that induction of pS2 protein in MCF-7 is more sensitive following 4-MBC treatment than after OMC treatment, and that in uterotropic assay, the uterine weight is higher following 4-MBC treatment than after OMC treatment (Schlumpf et al., 2001). Therefore, our results are consistent with previous reports.

Moreover, we showed for the first time that mRNA expression levels of the VTG subtypes VTG-1 and VTG-2, and of the CHG subtypes CHG-L and CHG-H, increased in male medaka exposed to OMC and 4-MBC (Figs. 2 and 3). In particular, VTG-1 and VTG-2 mRNA expression levels were 10^6 and 10^5 times higher than the non-treated controls, respectively, following exposure to 4-MBC, and were similar to those following E_2 exposure (Fig. 3). These results suggest that plasma VTG concentration is closely related to VTG-1 and VTG-2 mRNA expression in the liver following exposure to 4-MBC and E_2. Moreover, since the mRNA expression levels of CHG-L and CHG-H were also higher than in the non-treated control, and were similar to those following E_2 exposure (Fig. 3), the mRNA expression levels of CHG-L and CHG-H may be closely related to plasma VTG concentration and liver VTG expression following exposure to 4-MBC and E_2. Particularly, our results as to CHG expression level are consistent with previous reports, since the mRNA expression level of CHG-L was higher than that of CHG-H (Lee et al., 2002).

However, despite the fact that little VTG was detected in plasma, exposure to OMC induced mRNA expression of VTG-1, VTG-2, CHG-L, and CHG-H in the liver (Figs. 1–3). We also found significant increases in the ERα mRNA expression level in the liver following exposure to OMC, as compared with the non-treated control (Fig. 4). It has been reported that ERα gene expression is positively autoregulated upon exposure to estrogenic compounds (Islinger et al., 2003). However, since the dose-dependent effect of

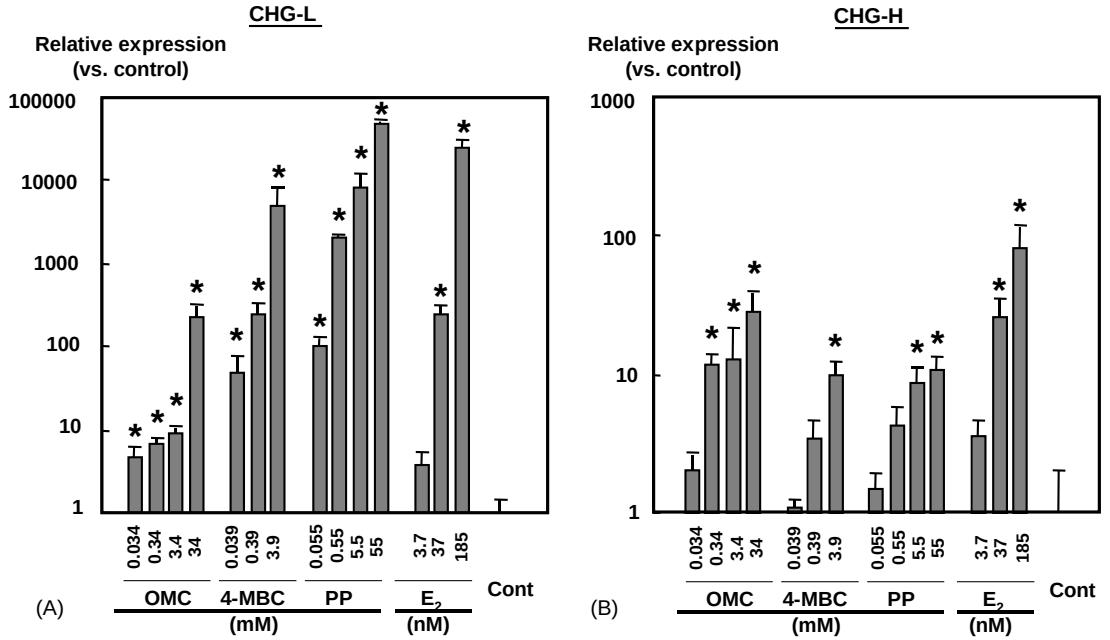

Fig. 3. Real-time RT-PCR analysis of (A) CHG-L and (B) CHG-H in medaka liver. Relative intensity of CHG-L and CHG-H mRNA expression was calculated against each β-actin mRNA expression, and the expression in non-treated control was normalized as one. Data ($n = 5$, mean ± S.E.M.) are presented as relative values compared to non-treated control. $^{*}P < 0.01$ compared with non-treated control was considered significant.

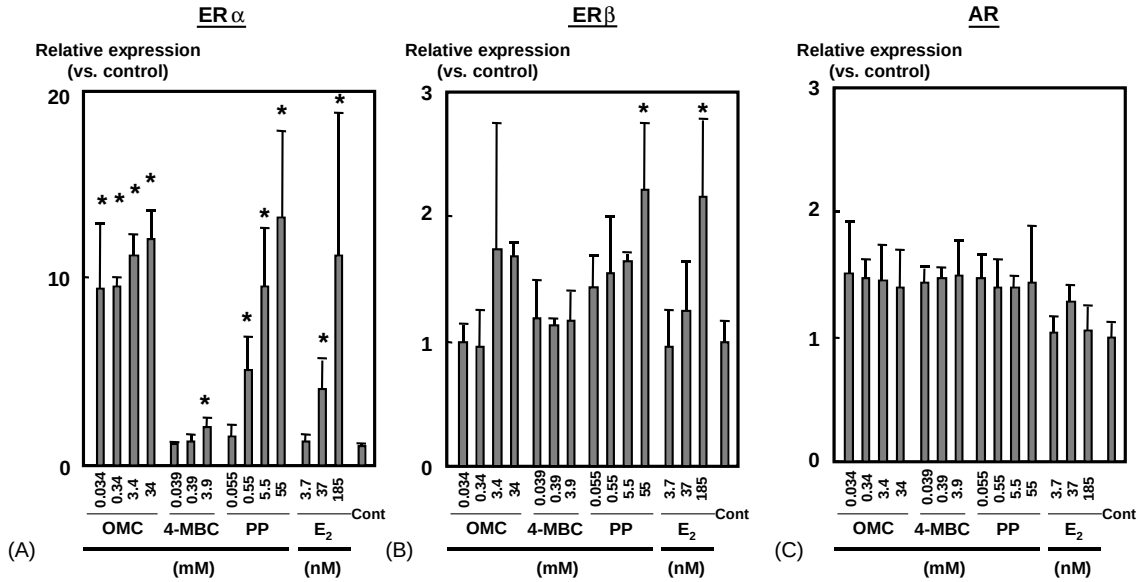

Fig. 4. Real-time RT-PCR analysis of (A) ERα, (B) ERβ, and (C) AR in medaka liver. Relative intensity of ERα, ERβ, and AR mRNA expression was calculated against each β-actin mRNA expression, and the expression in non-treated control was normalized as one. Data ($n = 5$, mean ± S.E.M.) are presented as relative values compared to non-treated control. $^{*}P < 0.01$ compared with non-treated control.

exposure to OMC on expression of VTG, CHG, and ERα was not significant, exposure to OMC might affect estrogenicity in fish. Our results assume that OMC is an estrogenic compound, and an integrative assay system for hepatic VTG, CHG, and ERα mRNA expression levels, including plasma VTG concentration, may be needed for the estimation of estrogenic activity. Moreover, future studies will have to focus on other effects of OMC.

Moreover, we also found an increase of plasma VTG concentration and mRNA expression of VTG-1, VTG-2, CHG-L, CHG-H, and ERα in the livers of male medaka exposed to the PP (Figs. 1–4). Our results are consistent with previous reports (Routledge et al., 1998; Blaier et al., 2000; Nishihara et al., 2000; Pedersen et al., 2000; Satoh et al., 2000; Okubo et al., 2001).

In conclusion, we found that exposure to UV screens OMC and 4-MBC, and PP preservative, induced increases in plasma VTG concentration, and in the expression of VTG-1, VTG-2, CHG-L, and CHG-H. OMC, 4-MBC, and PP have recently become to be widely used in cosmetics and pharmaceuticals. Our results suggest that such UV screens and preservatives affect not only mammals, including humans, but also fish. In future study, we need to reconsider the potential benefits of these chemicals from both medical and ecological perspectives.

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Effects of the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) on expression of genes involved in hormonal pathways in fathead minnows (*Pimephales promelas*) and link to vitellogenin induction and histology

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ABSTRACT

UV-filters are increasingly used in cosmetics and in the protection of materials against UV-irradiation, and ultimately they reach aquatic systems. The lipophilic UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) belongs to one of the most frequently used UV-filters and accumulates in aquatic animals. Despite its ubiquitous presence in water and biota, very little is known about its potential hormonal effects on aquatic organisms. In our study, we evaluated the effects of measured water concentration of 5.4, 37.5, 244.5 and 394 μg/L EHMC on the expression of genes involved in hormonal pathways in the liver, testis and brain of male and female fathead minnows (*Pimephales promelas*). We compare the transcription profile with the plasma vitellogenin (VTG) content, secondary sex characteristics, and gonad histology. Transcripts of the androgen receptor (*ar*) were significantly down-regulated in the liver of females at 37.5, 244.5 μg/L and 394 μg/L EHMC. Additionally, the 3β-hydroxysteroid dehydrogenase (*3β-HSD*) transcript was significantly decreased in the liver of males at 37.5, 244.5 and 394 μg/L EHMC, and at 244.5 and 394 μg/L EHMC in females. The expressional changes were tissue-specific in most cases, being most significant in the liver. Vitellogenin plasma concentration was significantly increased at 244.5 μg/L EHMC in males. EHMC induced significant histological changes in testes and ovaries at 394 μg/L. Testes displayed a decrease in spermatocytes, and ovaries a decrease in previtellogenic oocytes. The induction of VTG plasma concentration and the histological changes in gonads suggest an estrogenic and/or antiandrogenic activity of EHMC. On the other hand, the gene expression profile shows an antiestrogenic (e.g.: down-regulation of *esr1*) activity of EHMC. In conclusion, our data demonstrate that EHMC displays low but multiple hormonal activities in fish.

1. Introduction

UV-absorbing compounds (UV-filters) are increasingly used in personal care products in particular to protect the human skin from direct exposure to harmful UV-radiation from sunlight. There are two types of UV-filters, physical filters such as titanium dioxide and zinc oxide, which mainly scatter and reflect UV-light, or organic compounds absorbing UV-light. UV-filters are widely used in cosmetics (lipsticks, shampoos, creams, fragrances, skin lotions, hair sprays) and in the UV-protection of numerous materials and products (Balmer et al., 2005; Fent et al., 2010a). Currently, 28 UV-filters are registered in the European Union (Schlumpf et al., 2008). UV-filters enter directly into surface water via recreational activities or indirectly via wastewater, which was found to be the dominant source in rivers (Balmer et al., 2005; Fent et al., 2010). Many UV-filters used in sunscreens are lipophilic and can accumulate in biota, which has been demonstrated for 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC; formerly octylmethoxycinnamate, OMC) (Fent et al., 2010a).

Recent findings demonstrate that several UV-filters may have hormonal activity in mammals (Schlumpf et al., 2008) and in fish (Kunz et al., 2006a,b; Coronado et al., 2008; Weisbrod et al., 2007; Fent et al., 2008; Zucchi et al., 2011). They may negatively affect corals (Danovaro et al., 2008) or the reproduction of *Daphnia magna* (Fent et al., 2010b). Hormonal activity *in vitro* of benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-4 (BP-4), 3-benzylidene camphor (3-BC) and ethyl-4-aminobenzoate (Et-PABA) has also been shown. In addition to estrogenic activity (Schreurs et al., 2002; Inui et al., 2003; Kunz et al., 2006a; Coronado

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et al., 2008), 3-BC, BP-2 and BP-3 impair fertility and reproduction in fish (Kunz et al., 2006b; Weisbrod et al., 2007; Fent et al., 2008; Coronado et al., 2008). Despite these few studies, ecotoxicological consequences of UV-filters in personal care products are fairly unknown and need to be further investigated.

EHMC is one of the most widely used UV-filters in topically applied sunscreens. Environmental concentrations between 0.01 and 0.1 μg/L were reported in treated, and up to 19 μg/L in untreated municipal wastewater (Balmer et al., 2005). Lower concentrations occur in lakes, rivers (Straub, 2002; Balmer et al., 2005; Fent et al., 2010), and coastal seawater, where up to 390 ng/L was detected (Langford and Thomas, 2008). EHMC occurred even at very remote environments such as the Pacific Ocean (Polynesia) (Goksoyr et al., 2009). Furthermore, concentrations of 0.26–5.61 μg/L were reported in untreated drinking water (Loraine and Pettigrove, 2006). EHMC is very lipophilic ($\log K_{ow}$ of 6.1, Balmer et al., 2005) and accumulates in aquatic biota of different trophic levels with concentrations of up to 340 ng/g lipids in cormorants (Fent et al., 2010).

Despite its widespread presence in the environment and its accumulation tendency, little is known about environmental risks of EHMC. Multiple hormonal activities were shown *in vitro* including antiestrogenic, antiandrogenic and weak androgenic activity (Kunz and Fent, 2006). High concentrations of EHMC (9.87 mg/L) led to induction of vitellogenin (VTG) in male medaka (Inui et al., 2003), whereas no VTG induction was observed in fathead minnows at lower concentrations (Kunz et al., 2006a). Therefore, there is a need for a better understanding of potential environmental risks associated with EHMC contamination, also because the mode of action of EHMC is unknown.

The goals of this study are two-fold. First, molecular effects of EHMC on gene expression are evaluated by applying a targeted gene approach. We focus on seven transcripts involved in hormonal pathways and steroidogenesis in three different organs (liver, gonads and brain), both in male and female fish. Second, we determine whether gene expression alterations are linked to physiological effects. Thus, we compare plasma concentrations of VTG, secondary sex characteristics and the histology of gonads with gene expression changes.

2. Materials and methods

2.1. Chemicals

2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC, CAS No. 5466-77-3) and 3-(4-methylbenzylidene)-camphor (4MBC; CAS No. 36861-47-9) were purchased from Merck (Glattbrugg, Switzerland), ethanol (EtOH), methanol and dichloromethane in HPLC grade from J.T, Baker (Stehelin AG, Basel, Switzerland) and formic acid from Sigma Aldrich (Fluka AG, Buchs, Switzerland), Cremophor RH40 from BASF (BASF Chem Trade GmbH, Ludwigshafen, Germany), paraffin tissuewax, xylol, UltraClear and haematoxylin from Medite, Nunningen (Switzerland) and eosin from Carl Zeiss AG, Feldbach (Switzerland).

Stock solutions of EHMC were prepared by dissolving the compound in ethanol at a concentration of 20 g/L. Subsequently 1:1 dilutions of the stock solutions were prepared in 20% Cremophor RH40 (dissolved in nanopure water) to obtain a concentration 10 g/L EHMC. Working solutions were stored in the dark at 4 °C between uses.

2.2. Maintenance of fish

Sexually mature fathead minnows (*Pimephales promelas*) were obtained from Osage Catfisheries Inc., Osage Beach, MO/USA. Fish were acclimatized in 300 L culture tank for 1 month prior to the experiment. The fish were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 μS/cm. The water temperature was held constant at 25 ± 1 °C with the photoperiod was set at 16:8 h light/dark. Fish were fed twice daily with brine shrimp (*Artemia salina*) and white mosquito larvae.

2.3. Experimental design

The experimental design was similar as in previous studies (Kunz et al., 2006b; Weisbrod et al., 2007). The experimental setup consisted of four replicate tanks of each water controls, solvent controls (1:1 dilution of EtOH and 20% Cremophor RH40) and four nominal EHMC concentrations (30, 300, 1000 and 3000 μg/L). At the beginning of the experiment, four female and two male fish per group were randomly selected and assigned to well-aerated 20 L replicate stainless steel tanks at 25 ± 1 °C and a photoperiod at 16:8 h light/dark. A 21 d pre-exposure period was performed in aquaria containing a stainless steel funnel as a spawning substrate. This was done in order to establish the reproductive capacity of unexposed fish and to provide tank-specific baseline data for statistical comparison once the exposure with EHMC was initiated. After the 21 d pre-exposure, fish were exposed for 14 d to nominal values of 30, 300, 1000 and 3000 μg/L EHMC, respectively. During exposure survival, reproductive behavior, secondary sex characteristics and fecundity (in terms of cumulative number of spawned eggs per day) were determined. Eggs attached to the spawning substrate were removed and counted daily. Due to the very low fecundity, data on egg laying were not valid and therefore not included in our study.

A static-renewal procedure was used during the pre-exposure and exposure period. Thereby food remains and feces were removed after 48 h by siphoning two thirds of the water and replaced by new exposure water containing the appropriate EHMC or solvent concentrations. This water renewal procedure was chosen to minimize handling stress and disturbances for the fish. The quality of the exposure water was continuously monitored by determining oxygen concentration (>70%), the pH-value (6.7–7.2) and the temperature (25 ± 1 °C) (OECD 229).

Mortality and abnormal behavior were recorded daily. At the end of the exposure experiment (day 14), all fish were anaesthetized in clove oil solution (Fluka AG, Buchs, Switzerland). Individual length and weight of fish (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males) were measured in order to assess the condition factor (CF = weight (g)/length (mm) × 100). To determine plasma concentration of VTG, blood was collected from the caudal vein of male and female fish (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males) using a heparinised capillary tube (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) and transferred into a labeled Eppendorf® tube. Plasma was then separated from the blood by centrifugation (full-speed for 2 min) and the plasma was stored at −80 °C until analysis.

After sampling of the blood, gonads of female and male fish (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males) were removed and weighted in order to assess the gonadosomatic index (GSI = 100 × gonad weight/body weight). Gonads, liver and brain from female and male fish were immediately excised and stored in RNA*later* (Qiagen, Basel, Switzerland) at −80 °C for determining mRNA content of selected genes by quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR). For analysis of the secondary sex characteristics, nuptial tubercles are counted in male fish, and classified according to the OECD guideline 229.

Table 1
Primer sequences for quantitative real-time PCR analysis and sources: *18S rRNA* (18S ribosomal RNA), *ar* (androgen receptor), *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a), *cyp19a1b* (cytochrome P450, family 19, subfamily A, polypeptide 1b), *esr1* (estrogen receptor), and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*), *ptgds* (prostaglandin D2 synthase) and *vtg1* (vitellogenin 1).

Gene	Sequence accession #	dir	Sequence	Product size (bp)
18S rRNA[a]	Y855349.1	fw	aaa cgg cta cca cat cca ag	116
		rv	tta cag ggc ctc gaa aga ga	
ar[b]	AY727529	fw	gtg cca tgc gct tcc aa	150
		rv	ctg acc ttt gtg ggc aag ga	
cyp19a1a[b]	AF288755	fw	gga gag ctg agc gct gag a	58
		rv	gga gcc gcg atc aac atc t	
cyp19a1b[b]	AJ277866	fw	gga cgt ttc caa tag act ctt cct aa	72
		rv	ata gcg atg gat ctt tat cag caa	
esr1[b]	AY727528	fw	aac tca tct ttg ctc agg atc tca	64
		rv	agc cat ccc ctc gac aca t	
3β-HSD[c]	BC045457	fw	atg aga tgc cct acc caa aga c	76
		rv	ccc ttt acc ttt gtg cca ttg	
ptgds[b]	UniGene Ppr.12268	fw	ttg gac acc ggc atc ctt	80
		rv	ttt aag aga ccc tca ggc atc tg	
vtg1[b]	AF130354	fw	gct gca gag gcc att tct aag a	68
		rv	agc att gcc cag aac ttt cag	

[a] Data source: Wintz et al. (2006).
[b] Data source: Dorts et al. (2009).
[c] Data source: Garcia-Reyero et al. (2009).

2.4. qRT-PCR analysis

Total RNA was extracted from the brain, liver and gonads using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were determined spectrophotometrically using a NanoDrop ND-1000 UV-VIS Spectrophotometer at 260 nm.

1 μg of total RNA template was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma–Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C, 1 h at 37 °C, and the reaction was stopped by heating at 95 °C for 5 min.

cDNA was used as a template to perform qPCR using SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland).

We selected genes with focus on hormonal activity of UV-filters (Kunz and Fent, 2006; Kunz et al., 2006a; Weisbrod et al., 2007; Coronado et al., 2008; Zucchi et al., 2011) and on those altered by 17α-ethynylestradiol (EE2) (Hoffmann et al., 2006). Expressional changes on the estrogen receptor alpha (*esr1*), vitellogenin 1 (*vtg1*), and prostaglandin D2 synthase (*ptgds*) (Hoffmann et al., 2006; Dorts et al., 2009) were selected for estrogenic/antiestrogenic activity, mRNA changes on the androgen receptor (*ar*) for effect on the androgenic/antiandrogenic pathway. Effects on steroidogenesis were evaluated by determination of transcripts of P450 aromatases (*cyp19a1a* and *cyp19a1b*) and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*).

Gene-specific primers for 18S ribosomal RNA (*18S rRNA*), androgen receptor (*ar*), cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1b*), estrogen receptor *alpha* (*esr1*), and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*), *ptgds* (prostaglandin D2 synthase) and *vtg1* (vitellogenin 1) were obtained from fathead minnow published primers sequences (Table 1). The *18S rRNA* was selected in this study as reference gene for normalization, because their expression profile did not vary either under experimental conditions or in different analysed tissues.

Real time PCR amplification was performed on a Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 61 °C for 60 s followed by a melting curve analysis post run.

For calculation of mRNA expression levels, normalization was performed against the reference gene *18S rRNA* (*18S*). The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Transcriptional alterations of the different genes are expressed as fold change (log 2).

2.5. Vitellogenin analysis

For quantification of VTG a commercially available, homologous enzyme-linked immunosorbent assay (ELISA) for fathead minnow VTG from Biosense Laboratories (Biosense, Bergen, Norway) was used. The assay was performed according to the manufacture's protocol.

2.6. Histological analysis of testes and ovaries

Testes and ovaries of one male and one female fish per replicate tank were examined histologically to reveal changes induced by EHMC exposure. Whole animals were fixed in neutral buffered formalin (Roth, Arlesheim, Switzerland), dehydrated in graded ethanol and xylene, paraffinized in a tissue processor (Gewebeentwässerungsautomat TPC 15 Duo, Medite, Nunningen, Switzerland) and finally embedded in paraffin wax. Sections were taken along the long axis of the gonads at 5 μm intervals, in a serial step fashion. Two serial sections were collected from 3 steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of 6 tissue sections/sample.

Cross sections of 5 μm thickness were stained with haematoxylin and eosin. Obtained sections were microscopically examined to determine the reproductive condition of fish. Pictures of testes and ovary were made using Olympus BX41 microscope (Olympus Schweiz AG, Volketswil, Switzerland, 40× magnification).

The ovaries were evaluated based upon relative frequencies of primary oocytes, previtellogenic, vitellogenic oocytes and atretic follicles. Frequencies were evaluated by counting the different stages in three randomly selected fields of vision/female. For each male, pictures of three randomly selected fields of vision were taken and the areas of the different stages (spermatogonia, spermato-

Table 2
Nominal and measured concentrations of EHMC in exposure waters at the day of the substance addition (day 1, 3, 7, 10), 24 h after the addition (day 4, 8, 11) and 48 h after the addition (day 5, 9, 12) during the 14 d exposure. Arithmetic means ± standard deviations of 3 replicates.

Nominal Concentration	Exposure waters						
	Measured concentration (μg/L)						
	0 h	% of nominal	24 h	% of nominal	48 h	% of nominal	Arithmetic Mean
30 μg/L	14.6 ± 0.7	48.7	1.55 ± 1.6	5.1	0.19 ± 0.2	0.6	5.4
300 μg/L	97.97 ± 80	32.7	12.85 ± 12.6	4.3	1.64 ± 1.9	0.5	37.5
1000 μg/L	675.91 ± 116.8	67.6	55.85 ± 54	5.6	1.77 ± 1.3	0.2	244.5
3000 μg/L	1165.17 ± 1.3	38.8	14.95 ± 18.6	0.5	2.42 ± 3.7	0.1	394.2

cytes and spramatides) were measured using the software ImageJ (Abramoff et al., 2004).

2.7. Chemical analysis

To determine actual EHMC concentrations during exposure different amounts of aquaria water were taken during the experiment prior to water renewal (0 h) and after 48 h exposure from randomly selected replicate tanks. Aliquots of 250 mL were taken at the lower EHMC concentrations (30 and 300 μg/L) and the controls, and 25 mL for the higher EHMC concentrations (1000 and 3000 μg/L) for EHMC analysis. Water sampling took place on days 1–3, 7–9 and 10–12, respectively. The water samples were stored in the dark at −20 °C until analysis by HPLC. Extraction of water samples and chemical analysis was performed according to Kunz et al. (2006b). To control for the influence of Cremophor RH40 on the EHMC stability, a control experiment including three replicate tanks without fish was performed with 1000 μg/L EHMC over 48 h.

2.8. Data analysis and statistics

The data were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov–Smirnov test and variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p > 0.05$) to compare treatment means with respective controls. Results are given as means ± standard error of means. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. EHMC concentration and gross effects

To determine actual EHMC concentrations in exposure water, samples were analysed by HPLC-DAD at 0 h, 24 h and 48 h. The EHMC concentrations were much lower than nominal at the beginning (0 h), and they decreased further at 24 h and 48 h (Table 2). The arithmetic means at these time points are taken as exposure concentrations, as we assume that arithmetic means are best estimates.

To evaluate the role of Cremophor RH40 as a solubilizing agent, a control experiment was performed in tanks lacking fish with nominal concentration of 1000 μg/L EHMC using EtOH as a solvent, and a 1:1 dilution of EHMC in 20% Cremophor RH40. The data show that Cremophor RH40 was not responsible for the drop of the EHMC concentration in the tank as it was even more pronounced with EtOH, nor is this drop due to metabolism by fish. The median of EHMC concentration using Cremophor RH40 as solvent were 280 and 420 μg/L, respectively, in the two replicates, immediately after giving EHMC into tank water.

No significant differences in survival, body length and in the gonadosomatic index (Table 3; Figs. 1 and 2) occurred in any of the EHMC-exposed fish compared to control fish.

Data on expressional changes, VTG and histology from control groups and from the four different EHMC exposure treatments are depicted in the graphs (Fig. 1–6 and in supplementary material, Figs. S1) as water (water control), solvent control (Crem/EtOH), environmentally realistic concentration, 5.4 μg/L (*E*), low concentration, 37.5 μg/L (*L*), medium concentration, 244.5 μg/L (*M*) and high concentration, 394 μg/L (*H*).

3.2. Alterations in gene expression

There were no significant differences between the solvent control and the water control in any of the transcripts. Therefore, only the qRT-PCR data relative to the solvent control are shown in Fig. 3(A–D).

The expression pattern in males showed an overall tendency (albeit not significant) for an up-regulation of the *esr1* transcript mostly in the brain (Fig. 3A). However, in the liver of females *esr1* expression was down-regulated in a concentration-dependent manner, being significant at 394 μg/L EHMC. As observed in males, *esr1* mRNA showed a slight increase (albeit not significant) in the brain of females (Fig. 3A). In the liver of both males and females *vtg1* mRNA showed an overall up-regulation trend (although not significant).

In female fish, the expression of the androgen receptor (*ar*) was significantly down-regulated in the liver by 37.5, 244.5 and 394 μg/L EHMC, as well as in the ovary, although not significantly (Fig. 3C). In the liver of males the transcription of *ar* tended to be down-regulated in a concentration-dependent manner (Fig. 3C). A significant inhibition of *3β-HSD* transcript was noted in the liver of

Table 3
Body weight, length and survival of exposed fish after 14 d of exposure ($n = 24$, 16 females and 8 males per treatment).

	Exposure (μg/L)	Survival (%)	Male		Female	
			Body weight (g)	Body length (mm)	Body weight (g)	Body length (mm)
Controls	Water	100	3.09 ± 1.2	67.6 ± 6	1.47 ± 0.3	53.3 ± 2.9
	Solvent	100	3.57 ± 0.5	72.5 ± 3.9	1.70 ± 0.3	55.1 ± 3.6
EHMC	5.4	100	2.88 ± 0.4	66.3 ± 4.2	1.62 ± 0.2	53.9 ± 2.3
	37.5	96	2.99 ± 0.1	67.9 ± 2.1	1.54 ± 0.3	53.5 ± 4.1
	244.5	96	2.86 ± 0.6	65.5 ± 4.5	1.64 ± 0.3	55.9 ± 3.5
	394.2	100	2.72 ± 0.4	65.0 ± 3.0	1.51 ± 0.3	54.3 ± 3.0

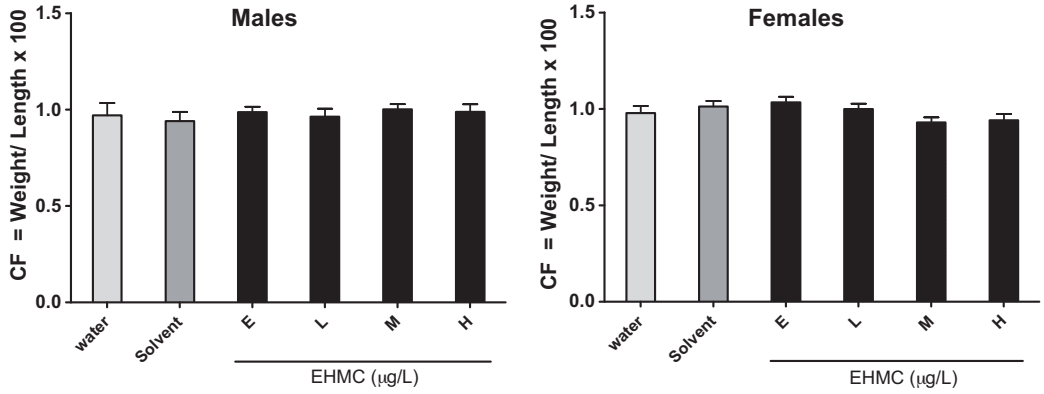

Fig. 1. Condition factor (CF) of female and male fathead minnows. Controls (grey bars) and fish exposed to increasing EHMC concentrations (black bars). Values are means ± SEM (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males). No significant differences occurred.

male fish at 37.5, 244.5 and 394 µg/L EHMC, and at 244.5 µg/L and 394 µg/L EHMC in female fish (Fig. 3D). Furthermore, a tendency of *3β-HSD* mRNA down-regulation was noted at all concentrations in the ovary (Fig. 3D).

No significant mRNA alterations in any of the tissues of male and female fish were noted for *cyp19a1a*, *cyp19a1b* and *ptgds* transcripts, thus they were in the range of inter-individual variability (supplementary material, Fig. S1).

In summary, even if some transcripts altered by EHMC displayed a tendency, the observed alterations lacked statistical significance in most cases. Nevertheless, significant down-regulation occurred in the liver for transcripts involved in hormonal pathway such as *esr1*, *ar*, and *3β-HSD*. Some gene expression profiles varied among tissues, and in some cases, between male and female fish.

3.3. Plasma vitellogenin and secondary sex characteristics

The plasma VTG concentration in male was significantly increased by 224.5 µg/L EHMC (mean concentration: 13.06 µg/mL) as compared to water (mean concentration: 0.37 µg/ml) and solvent control (mean concentration: 0.22 µg/mL). The observed increase in plasma VTG in the 394 µg/L EHMC dose group (mean concentration: 4.95 µg/mL, Fig. 4A) lacked statistical significance. VTG plasma concentrations in female fish did not significantly change (supplementary material, Fig. S2).

No significant alterations in the number and score of nuptial tubercles of EHMC-exposed males as compared to control fish were noted (supplementary material, Fig. S3).

3.4. Histology of testis and ovary

We analysed the histology of testis and ovary of fish from the water and solvent controls, and in all EHMC groups. In the testis of males exposed to the highest EHMC concentration, alterations in the frequency of cells at different stages of spermatogenesis (spermatocytes and spermatides) occurred (Fig. 5A). These testes were characterized by a significant decrease in spermatocytes compared to solvent control fish and an increase in mature spermatides. The EHMC-induced changes on the histology of testis are shown in Fig. 6.

In females exposed to EHMC, there was also a visible alteration in the frequency of cells at the different stages of oogenesis (primary oocytes, previtellogenic, vitellogenic oocytes and atretic follicles) (Fig. 5B). A reduced number of primary oocytes occurred in the ovary of females exposed to the highest EHMC concentration. On the other hand, a significant increase in vitellogenic oocytes was noted compared to the solvent control. The changes induced by EHMC in the ovary are depicted in Fig. 7.

4. Discussion

EHMC is one of the most frequently used UVB-filter in sunscreen formulations. Previously, we showed that this UV-filter has multiple hormonal activities *in vitro* (Kunz and Fent, 2006). Estrogenic activity has been reported at high concentrations in male medaka EHMC (Inui et al., 2003), while no significant VTG induction was observed in juvenile fathead minnows exposed to low aqueous concentrations (Kunz and Fent, 2006).

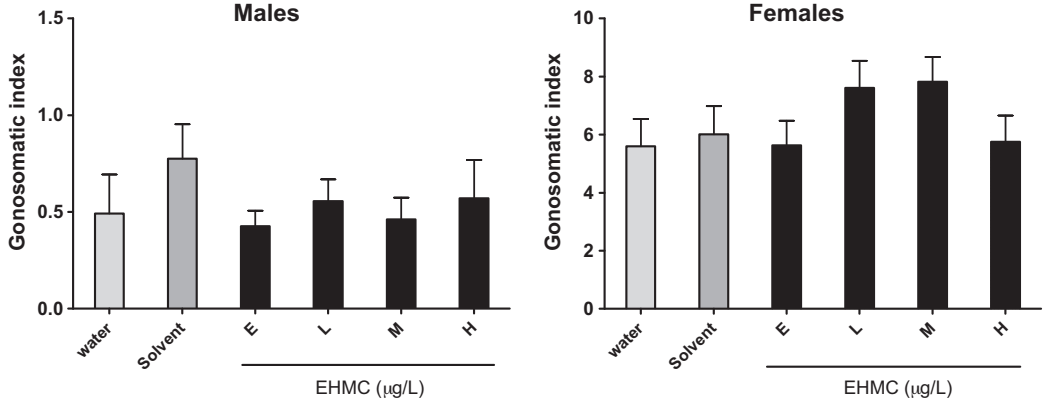

Fig. 2. Gonosomatic index (GSI) of male and female fathead minnows. Controls (grey bars) and fish exposed to increasing EHMC concentrations (black bars). Values are means ± SEM (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males). No significant differences occurred.

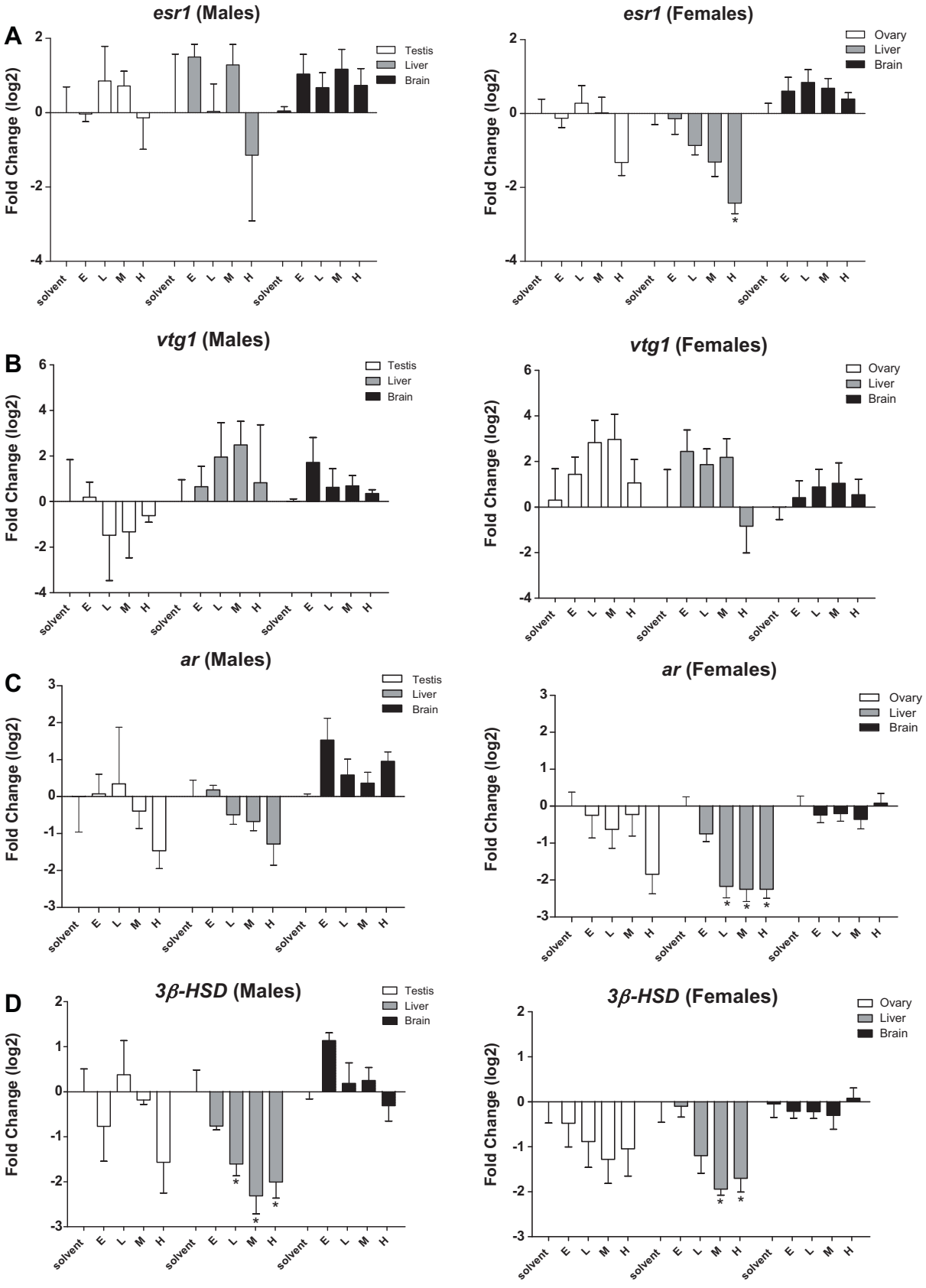

Fig. 3. Relative gene expression of *esr1* (A), *vtg1* (B) *ar* (C) and *3β-HSD* (D) in liver, brain and gonads of male and female fathead minnows after EHMC exposure. Data in the graphs are represented as follows: solvent (Crem/EtOH), *E* (environmental concentration: 5.4 μg/L), *L* (low concentration: 37.5 μg/L), *M* (medium concentration: 244.5 μg/L) and *H* (high concentration: 394 μg/L). Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log 2) in mRNA abundance as compared to solvent controls (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males) were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± SEM. Asterisks indicate significantly altered expression compared to controls ($^*p < 0.05$).

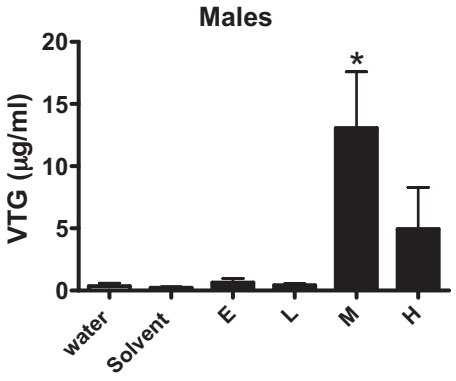

Fig. 4. Concentration of plasma vitellogenin in male fathead minnows of water and solvent control, and fish exposed to EHMC concentrations (black bars). Data in the graph are represented as follows: water (water control), solvent control (Crem/EtOH), *E* (environmental concentration: 5.4 µg/L), *L* (low concentration: 37.5 µg/L), *M* (medium concentration: 244.5 µg/L) and *H* (high concentration: 394 µg/L). Results are given as mean value ± SEM (solvent control: 6 males; water control and EHMC treatments: 8 males). Asterisk denotes significant induction of vitellogenin in males exposed to 244.5 µg/L as compared to control groups ($^*p < 0.05$).

In our present study we demonstrate that EHMC displays endocrine activity in adult fathead minnows (*Pimephales promelas*) based on data on alterations in gene expression, VTG plasma concentrations and gonad histology. The activity of EHMC seems not high, and the toxicological profile seems complex due to the multiple hormonal activities exhibiting different net effects in expressional profiles and associated physiological effects.

4.1. Water analysis

The measured EHMC concentrations were significantly lower than the nominal concentration and decreased further during exposure. The decrease was due to several factors including adsorption of highly lipophilic EHMC ($\log K_{ow}$ 6.1, Balmer et al., 2005) to surfaces such as tank walls, spawning substrate etc., as well as uptake into fish and eggs. The low concentration of EHMC (as compared to nominal values) at the beginning and the further decrease during exposure is partly due to the use of a solubilizing agent (in the present study Cremophor RH40), and to a minor extent, to dimerization of EHMC (Broadbent et al., 1996). The control experiment (data not shown) demonstrated that Cremophor RH40 itself does not led to the EHMC decrease observed during the exposure time (0–48 h); an immediate drop in EHMC concentration occurred with both solvents, Cremophor RH40 and EtOH. Therefore, our exposure was rather pulsative than constant, and consequently, exposure

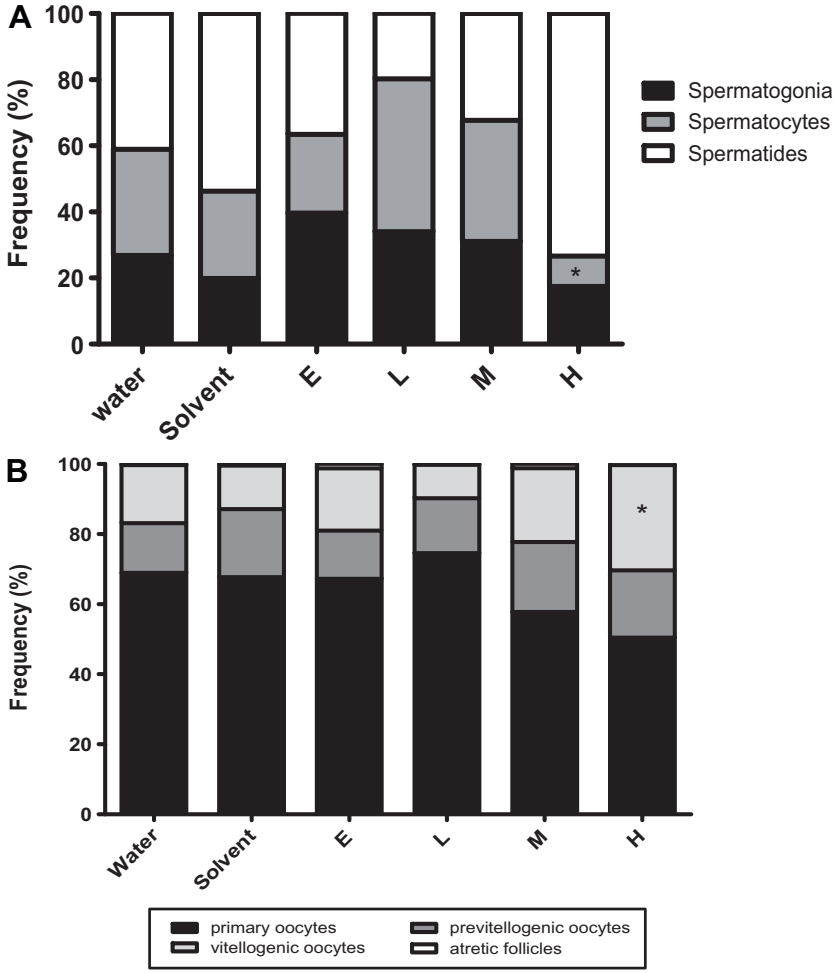

Fig. 5. Relative percentage of cells at the different stages of (A) spermatogenesis in testis of male, and (B) oogenesis in ovary of female fathead minnow after EHMC exposure determined in three randomly selected areas per fish. Data in the graphs are represented as follows: water (water control), solvent (Crem/EtOH), *E* (environmental concentration: 5.4 µg/L), *L* (low concentration: 37.5 µg/L), *M* (medium concentration: 244.5 µg/L) and *H* (high concentration: 394 µg/L). Results are given as means ± SEM (solvent control: 3 females and 3 males; water control and EHMC treatments: 4 females and 4 males). Asterisks denote significant difference from solvent control at $p \leq 0.05$.

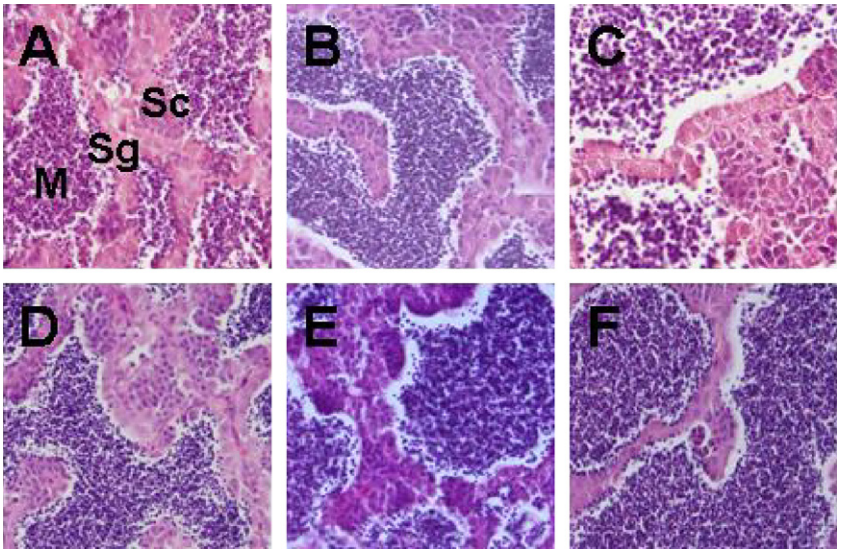

Fig. 6. Histological sections of typical seminiferous tubules in testis of males ($n = 3$ in solvent control, $n = 4$ in water control and EHMC treatments). (A) Water control, (B) solvent control, (C) fish exposed to 5.4 μg/L EHMC, (D) fish exposed to 37.5 μg/L EHMC, (E) fish exposed to 244.5 μg/L EHMC and (F) fish exposed to 394 μg/L EHMC. Visible are the increase in spermatides and the decrease in spermatocytes. Spermatogonia (Sg), spermatocytes (Sc) and spermatides (mature sperms M) are marked.

concentrations were much lower than under a constant EHMC exposure. This control experiment also shows that metabolism is not the reason for the drop in EHMC.

The measured EHMC concentrations of 5.4 and 37.5 μg/L are in the range of levels found in wastewater, but are higher than those found in coastal waters (390 ng/L) (Langford and Thomas, 2008). Maximal wastewater concentrations were 19 μg/L in Switzerland (Balmer et al., 2005), and 1.7 μg/L in Spain (Rodil et al., 2009). In a lake with swimming activity EHMC levels reached up to 3.0 μg/L (Rodil et al., 2009).

4.2. Gene expression analysis

Exposure of female fathead minnows to 394 μg/L EHMC led to a significant decrease in the expression of *esr1* in the liver. The modulation of this sex-steroid receptor is in line with previous UV-filter studies (Schlumpf et al., 2008), where often an inhibition of ERα was observed, in particular by 4-MBC. The down-regulation of *esr1* in the liver suggests an antiestrogenic activity of EHMC, which is in accordance to previous *in vitro* data (Kunz and Fent, 2006). In contrast to our findings, Inui et al. (2003) reported increases of mRNA expression of *esr1*, in male medaka (*Oryzias latipes*) after exposure to very high EHMC levels (9.87 mg/L). Additionally, *vtg* mRNA was up-regulated (although not significantly) in male liver at 244.5 μg/L EHMC. The lack of statistical significance in the induction of *vtg* in male liver may partly be due to different time-course of induction and stability of mRNA as compared to the VTG protein. The transient EHMC concentration may have resulted in a transient *vtg* mRNA induction.

In our study EHMC significantly inhibited the expression of *ar* in female liver at 37.5 μg/L, which is in line with data obtained *in vitro* in recombinant yeast (Kunz and Fent, 2006). In male medaka, antiandrogenic activity was not observed (Inui et al., 2003), which is apparently consistent with our observation in males. In com-

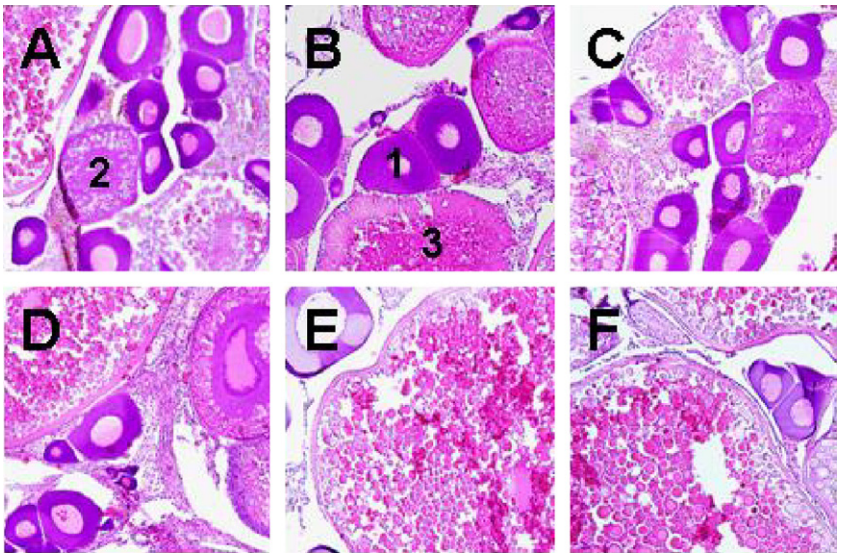

Fig. 7. Histological section of a typical ovary ($n = 3$ in solvent control, $n = 4$ in water control and EHMC treatments). (A) Water control, (B) solvent control, (C) fish exposed to 5.4 μg/L EHMC, (D) fish exposed to 37.5 μg/L EHMC, (E) fish exposed to 244.5 μg/L EHMC and (F) fish exposed to 394 μg/L EHMC. The decrease in primary oocytes and the increase in vitellogenic oocytes is visible. (1) primary oocytes, (2) previtellogenic oocytes and (3) vitellogenic oocytes.

parison, the estrogenic EE2 results in down-regulation of the *ar* transcript (Filby et al., 2007), whereas the antiandrogenic flutamide results in a strong down-regulation in hepatic *ar* mRNA. Based on these data an antiandrogenic activity of EHMC cannot be ruled out in the liver of females, but seems not to occur in males.

EHMC exposure also affected the mRNA expression of an important enzyme involved in steroidogenesis. 3β-Hydroxysteroid-dehydrogenase (3β-HSD) is involved in the conversion of pregnenolone to progesterone (Arukwe et al., 2008). In our study, a significant down-regulation of *3β-HSD* transcripts was observed in the liver (Fig. 4D). In the ovaries, there was also a tendency for down-regulation. The inhibition of *3β-HSD* may result in imbalance of steroid hormones, and in turn, reproductive dysfunction in fish (Villeneuve et al., 2008).

Potential changes in hormones may also in part be a reason for the observed histological changes in testes and ovaries of EHMC-exposed fish (Figs. 5–7). The fact that exposure of fish to E2 or EE2 (Govoroun et al., 2001; Baron et al., 2005) led to inhibition of *3β-HSD* mRNA suggests that EHMC exhibits an estrogenic activity in the liver, and to a lesser extent in ovaries.

In summary, our data indicate that EHMC displays multiple hormonal activities, including antiestrogenic activity (down-regulation of *esr1*), estrogenic activity (down-regulation of 3*β*-HSD), and antiandrogenic activity (down-regulation of *ar*). However, significant alterations were observed in the liver of male and female fish only.

4.3. Plasma vitellogenin

EHMC led to VTG induction in male fathead minnows, which was significant at 244.5 μg/L EHMC. Additionally, *vtg* mRNA is induced at this concentration (although not statistically significantly). The lack of statistical significance in the induction of *vtg* in male liver may partly be due to different time-course of induction and stability of mRNA versus protein. The transient EHMC exposure (due to immediate decrease of EHMC after water-renewal) may have affected the transcriptional level, but less the VTG protein, which is more stable. Statistical significance may also be lacking, because only 4 males per group were analysed for *vtg* mRNA induction, which is in contrast to 8 males for VTG plasma protein determination. Induction of plasma VTG as a biomarker for estrogenic compounds (Sumpter and Jobling, 1995) was demonstrated for many environmental chemicals (e.g. Jobling et al., 1998; Sohoni et al., 2001). VTG induction is associated with adverse effects on fertility and reproduction (Sumpter and Johnson, 2005; Tyler et al., 1998). We previously showed that estrogenic UV-filters including benzophenone-1, benzophenone-2 and 3-benzylidene camphor led to VTG induction (Kunz et al., 2006b; Kunz and Fent, 2009), which was paralleled by adverse effects on fertility and reproduction (Kunz et al., 2006b; Weisbrod et al., 2007). VTG induction and associated reduced fertility was also shown for benzophenone-3 in medaka (Coronado et al., 2008). Very high EHMC concentrations of 9.87 mg/L resulted in *vtg* mRNA induction in medaka (Inui et al., 2003). Data of our present study also suggest an estrogenic activity of EHMC in fathead minnows, but it remains open, whether this is based on direct interaction of the compound with estrogen receptors, and/or indirectly by the action on enzymes involved in steroidogenesis.

4.4. Histology

EHMC induced effects on gonad histology of male and female fish. Males exposed to 394 μg/L EHMC displayed significant alterations in the frequencies of different spermatogenic stages in testes, as compared to control males. Spermatogenesis appeared to be inhibited, as testes were characterized by enlarged areas of mature sperms and a reduced presence of spermatocytes. Spermatogonia apparently did not undergo any further differentiation into spermatocytes. Similarly, in fish exposed to other UV-filters, namely 3-BC (Kunz et al., 2006b) and BP-2 (Weisbrod et al., 2007), an inhibition of testicular development was also shown. Similar to EHMC, an increase in the frequency of spermatides and a decreased frequency of spermatocytes were observed. The histological effects in the testes are also analogous to E2 and EE2, showing inhibition of testicular development, depending on the dose (Gimeno et al., 1998; Miles-Richardson et al., 1999a; Pawlowski et al., 2004). Analogously, an inhibition of testicular development was reported for fish exposed to the weak estrogen receptor agonist 4-nonylphenol, which led to a significant reduction of fecundity (Harries et al., 2000; Miles-Richardson et al., 1999b) and a significant necrosis of sperm cells and spermatozoa (Miles-Richardson et al., 1999b). These data lead to the conclusion that the histological effects of EHMC in the testis indicate an overall estrogenic or antiandrogenic effect.

EHMC exposure also affected ovaries in female fathead minnows, where a significant increase in vitellogenic oocytes was observed at 394 μg/L EHMC. Different effects were found after exposure of fathead minnow to 3-BC (Kunz et al., 2006b), BP-2 (Weisbrod et al., 2007) and additional compounds (e.g. Leino et al., 2005). 3-BC and BP-2 and high concentrations of weak estrogen receptor agonists such as methoxychlor also resulted in increased follicular atresia (Ankley et al., 2001). Therefore, EHMC is concluded to exhibit a different activity on the ovary than the previously analysed UV-filters 3-BC and BP-2.

In summary, the data on gonad histology suggest an estrogenic or antiandrogenic effect of EHMC in testis. This may be related to the effect of EHMC on either steroid hormone receptors or steroidogenesis, or a combination of both (Jensen et al., 2001).

5. Conclusions

Induction of VTG and alterations in the histology of testes indicate an estrogenic and/or antiandrogenic activity of EHMC. The observed changes in gonad histology suggest a negative interference with maturation of sperms (significantly more mature spermatides and significant less immature spermatocytes in seminiferous tubules) and oocytes (significantly more vitellogenic and less primary oocytes). The targeted gene analysis showed that EHMC displays multiple hormonal activities including estrogenic (down-regulation of 3*β-HSD*), antiestrogenic (down-regulation of *esr1*), and antiandrogenic activity (down-regulation of *ar* in the liver of females). These expressional changes are supported by our previous *in vitro* study showing multiple hormonal activities of EHMC (Kunz and Fent, 2006). Additionally, the data demonstrate that the toxicological profile of EHMC seems complex due to the multiple hormonal activities. Most sensitive expressional changes of EHMC were observed at concentrations in the range of those found in municipal wastewater (Balmer et al., 2005), or in most contaminated surface waters (Rodil et al., 2009; Straub, 2002; Balmer et al., 2005; Fent et al., 2010; Langford and Thomas, 2008). Furthermore, EHMC accumulates in all trophic levels (Fent et al., 2010), and may contribute to the additive action of UV-filters (Kunz and Fent, 2009). Forthcoming studies are needed to investigate the effects of EHMC on fertility and reproduction of fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.01.013.

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