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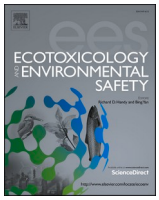

Fragrance materials (FMs) affect the larval development of the copepod *Acartia tonsa*: An emerging issue for marine ecosystems

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ABSTRACT

Fragrance materials (FMs) are used in a variety of detergents and cosmetics, including household and personal care products. Despite their widespread use and the growing evidence of their occurrence in surface waters worldwide, very little is known about their toxicity towards marine species, including a key component of the marine food webs such as copepods. Thus, we investigated the toxicity of six of the more long-lasting and stable commercial fragrances, including Amyl Salicylate (AMY), Oranger Crystals (ORA), Hexyl Salicylate (HEX), Ambrofix (AMB), Peonile (PEO), and Benzyl Salicylate (BZS), to assess their ability to impair the larval development of the calanoid copepod *Acartia tonsa*. FMs inhibited the development of *A. tonsa* significantly at concentrations by far lower than the effect-concentrations reported in the literature for aquatic species. The more toxic FMs were HEX ($EC_{50} = 57$ ng L^{-1}), AMY ($EC_{50} = 131$ ng L^{-1}) and ORA ($EC_{50} = 766$ ng L^{-1}), while the other three compounds exerted toxic effects at concentrations higher than 1000 ng L^{-1} (LOEC at 1000 ng L^{-1} for PEO and BZS, and at 10,000 ng L^{-1} for AMB). Early life-stage mortality was unaffected by FMs at all the tested concentrations. A comparison with water concentrations of FMs reported in the literature confirmed that FMs, especially HEX and AMY, may act as contaminants of potential concern in many aquatic habitats, including urban areas and remote and polar environments.

1. Introduction

Fragrance materials (FMs), also called fragrance mixtures or oils, are formulations used in the majority of consumer products, including both household and personal care products (Api et al., 2015). However, despite their widespread use and the growing evidence of their worldwide occurrence in surface waters, from urban areas to remote and polar environments (Vecchiato et al., 2020, 2018, 2017, 2016), very little is known about the environmental fate and toxicity of these compounds. Toxicity data are available only for a restricted number of compounds and, in most cases, they concern lethal effects on freshwater fishes and daphnids (Belsito et al., 2007; Natsch et al., 2018). Furthermore, effects on marine species were wholly overlooked, notwithstanding coastal areas and oceans are the final repository for most of the discharged FMs.

Marine copepods are a significant component of the marine ecosystem and play a key role in marine and coastal food webs (Turner, 2004). Several factors make them a useful bioindicator for assessing the adverse effects of chemicals, including worldwide distribution, easy culturing, short generation times and ecological relevance (Picone et al., 2018). Moreover, endocrine regulation of critical processes including moulting, sexual differentiation and growth, makes copepods particularly well suited for detecting the effects of chemicals that may affect or interfere with physiological regulation and neuroendocrine signalling (Andersen et al., 2001). The larval development of *Acartia tonsa* and *Nitocra spinipes,* in particular, is a very sensitive endpoint for assessing the short-term toxicity of pharmaceuticals, synthetic hormones, brominated flame retardants, and synthetic musk fragrances such as 1-(4-*tert*-butyl-2,6-dimethyl-3,5-dinitrophenyl)ethanone (musk ketone), 1-(3,5,5,6,8,8-hexamethyl-6,7-dihydronaphthalen-2-yl)ethanone (Tonalide™), 4,6,6,7,8,8-hexamethyl-1,3,4,7-tetrahydrocyclopenta[*g*]isochromene (Galaxolide™) and 1-(6-*tert*-butyl-1,1-dimethyl-2,3-dihydroinden-4-yl)ethanone (Celestolide™) (Andersen et al., 2001; Breitholtz et al., 2003; Wollenberger et al., 2003).

The present work aims at providing a first screening of the toxicity of commercially available FMs on the larval development of marine copepods. To this purpose, we selected six of the more long-lasting and

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stable commercial fragrances among those detected in the surface water worldwide. The IUPAC (and Givaudan trade names) of the commercial fragrances used for testing are: pentyl-2-hydroxybenzoate (Amyl Salicylate - AMY), 2-acetonaphtone (Oranger Crystals - ORA), hexyl-2-hydroxybenzoate (Hexyl Salicylate - HEX), dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-*b*]furan (Ambrofix - AMB), 2-cyclohexylidene-2-phenylacetonitrile (Peonile - PEO), and benzyl-2-hydroxybenzoate (Benzyl Salicylate - BZS). Then, we exposed fertilised eggs of *A. tonsa* to 5 nominal concentrations of each chemical, to cover the range from environmental detected concentration in the Venice Lagoon (Vecchiato et al., 2016) to acute-effect levels reported in the literature (Belsito et al., 2007; Natsch et al., 2018). At the end of the exposure, we generated point-estimate toxicity data, including effect concentrations for 20% and 50% inhibition of larval development (EC_{20} and EC_{50}, respectively). We also calculated hypothesis-based toxicity data, such as no observed effect concentrations (NOECs) and lowest observed effects concentrations (LOECs), although their use in ecotoxicology is criticised (Warne and Van Dam, 2008). Finally, we compared these screening toxicity data with the environmental concentrations reported in the literature to predict whether FMs in surface waters may be of concern for the larval development of crustaceans.

2. Materials and methods

2.1. Acartia tonsa culturing

The original strain of *A. tonsa* was purchased from Guernsey Sea Farms Ltd, Port Vale, Guernsey, United Kingdom. In our laboratory, we started new cultures by adding 600–800 freshly released eggs to 1.8-L of a 20‰ salinity culture medium prepared according to ISO 16778 (ISO, 2015), by diluting a 10% hypersaline water with Elendt M7 medium. Recipes for 10% hypersaline water and Elendt M7 medium are reported in Supplementary material.

The cultures were kept at 20 ± 1 °C, under continuous aeration using a 16-h light and 8-h dark photoperiod and fed ad libitum with a mixture of three marine flagellates, the chlorophyte *Tetraselmis suecica* and the haptophytes *Pavlova lutheri* and *Tisochrysis lutea*. The algae were added four times per day using a timer-controlled peristaltic pump; algal cultures were maintained in Guillard's F/2 medium, at the same condition of temperature and photoperiod as copepods' cultures.

Newly released eggs were removed daily from the culture by siphoning out the medium from the bottom of the culture flask and then filtering the decanted medium through two sieves with mesh sizes of approximately 170-μm and 50-μm, respectively. This procedure allows separating the eggs (passing through the 170-μm sieve but retained by the 50-μm sieve) from detritus, faecal pellets and copepods. Each culture was used for testing for a period of up to 6–7 weeks. Two different cultures were used during the testing period, for a total of five testing sessions; eggs from culture AT17/20 were used for testing PEO (09/06/2020), AMB and HEX (16/06/2020) and AMY (22/06/2020), while eggs from culture AT18/20 were used for testing ORA (29/06/2020) and BZS (13/07/2020).

2.2. Chemicals

Givaudan SA (Vernier, Switzerland) provided the fragrances used in this work. Commercial and IUPAC names, CAS number, molecular weight, vapour pressure (hPa) at 20 °C, water solubility (mg L^{-1}) and coefficient of partitioning between octanol and water (log K_{ow}) are summarised in Table 1.

The selected FMs have a molecular weight ranging between 152 and 288 and their persistence as fragrances (expressed as tenacity on blotter) ranges from a few weeks to months. Their chemical stability and persistence as fragrances are indications of their possible persistence in the environment.

AMY, HEX and BZS are fragrance ingredients used in many personal care products such as decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents (Lapczynski et al., 2007a, 2007b, 2007c). As concern the other FMs, ORA is widely used in perfume, soap and detergent compositions, PEO in laundry products and perfumes, while AMB is one of the most useful chemicals in modern perfumery, produced from natural ingredients (eindex.givaudan.com).

Stock solutions at 25 mg L^{-1} were prepared for each FM in 99% ethanol and then diluted to test concentrations with the 20‰ salinity medium used for culturing the copepods.

2.3. Toxicity testing

The larval development test with *A. tonsa* is a semi-static test with a renewal of test solution after 48-h (day-2).

The experiment started on day-0 by adding a known number of eggs (50−80) to each 100 mL glass beaker containing 30 mL of test solution. This was done by 1) collecting the eggs from the culture as reported above, 2) filtering aliquots of egg suspension (100–200 μL) through a cellulose filter, 3) counting under a dissecting microscope all the eggs recovered on the filter, 4) inoculating them into test beakers using a disposable sterile syringe and the appropriate FM solution, and 5) checking under a dissecting microscope for eggs remained on the filter. On day-2, an additional 30 mL of test solution was added to each beaker. Hatched larvae were fed on day-0 and day-2 with 100 μL of a concentrated mixture of *T. suecica, T. lutea* and *P. lutheri* obtained by centrifuging cultured algae per 8 min at 2000 g. The obtained algal density in the test beakers was higher than 6×10^4 cell mL^{-1}. Test vessels were maintained at the same conditions as the cultures (T = 20 ± 1 °C; 16-h light, 8-h dark photoperiod), but without aeration.

The test ended on day-5 when approximately 40% of the larvae in negative controls reached the copepodite-I stage. Under the testing conditions, the metamorphosis onset from the last naupliar stage (nauplius-VI) to the first copepodite stage (copepodite-I) is on day-5. This is a relatively easy endpoint to determine due to the two larval stages' different morphology. According to Andersen et al. (2001), to ensure that at least 40% of all test organisms had reached the copepodite stage, the ratio of nauplii to copepodites was determined in one control replicate after exactly 5-d. After a visual check of the developmental stage, the content of the beaker was fixed and stained by adding 0.5-mL of Lugol's solution to the medium. The development stage was examined

Table 1
Trade and IUPAC names, CAS number, molecular weight (MW), tenacity on blotter, octanol-water partition coefficient (logK_{OW}), water solubility (mg L^{-1}) and vapour pressure (VP, hPa) at 20 °C for the selected fragrance materials. Data were retrieved from eindex.givaudan.com and echa.europa.eu.

Trade name	Label	IUPAC name	CAS-number	MW	VP	Solubility	logKow
Amyl salicylate	AMY	Pentyl 2-hydroxybenzoate	2050–08–0	208	0.0040	5.5	4.4
		3-methylbutyl 2-hydroxybenzoate	87–20–7			6.3	
Oranger crystals	ORA	2-acetonaphtone	93–08–3	170	0.0013	133	2.8
Hexyl salicylate	HEX	Hexyl-2-hydroxybenzoate	6259–76–3	222	0.0013	2	4.9
Ambrofix	AMB	Dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-*b*]furan	6790–58–5	236	0.0013	1.88	>6.0
Peonile	PEO	2-cyclohexylidene-2-phenylacetonitrile	10,461–98–0	197	0.0004	7.5	4.0
Benzyl salicylate	BZS	Benzyl-2-hydroxybenzoate	118–58–1	228	<0.0013	8.8	4.0

by filtering the content of the beaker through a mixed cellulose ester filter with gridlines (Whatman ME25, diameter 47-mm, porosity 0.45-μm). If this control contained less than 40% copepodites, the test was allowed to run for 1 additional hour before another control was sacrificed. This procedure was repeated until 40% of the larvae reached the copepodite-I stage in the fixed control; however, the exposure was extended for no longer than 5-hours.

All unhatched eggs, nauplii and copepodites recovered on the filter were counted under a dissecting microscope to calculate the larval development ratio (LDR), namely the ratio of copepodites on total larvae (Eq. 1). Dissolved oxygen (DO) and pH were measured on day-0, before the inoculation of the eggs, and on day-5, before fixing with Lugol's solution. Five nominal concentrations, spaced by a factor 10, were tested for each fragrance to cover the range of environmental and acute-effect concentrations reported in the literature (Belsito et al., 2007; Natsch et al., 2018; Vecchiato et al., 2017, 2016). Six and twelve replicates per each fragrance concentration and the negative control were used, respectively. The negative control consisted of 20‰ salinity culture medium aliquots.

To check for possible solvent-induced effects, ethanol's toxicity diluted in 20‰ salinity medium was tested within the concentration range 0.0002–2%.

2.4. Chemical analysis

The actual concentrations of the FMs in the experiments were verified analyzing an aliquot of the test solutions at concentrations bracketing the lowest effect levels (10 and 100 ng L^{-1} for AMY, ORA and HEX; 100 and 1000 ng L^{-1} for PEO, BZS and AMB). Water samples (0.5 L) were spiked with phenanthrene ^{13}C as internal standard and extracted using Oasis HLB cartridges 6cc (0.2 g), Waters, Milford, MA, (USA), previously conditioned with 10 mL of *n*-hexane, 10 mL of dichloromethane and 10 mL of ultrapure water. Samples were eluted with 1 mL of toluene, 15 mL of dichloromethane, 10 mL of *n*-hexane and dried with Na_2SO_4. Eluates were reduced to 100 μL under a gentle nitrogen flow at 23 °C (Turbovap II®, Caliper Life Science, Hopkinton, MA, USA) and analyzed by GC-MS/MS (Trace 1310 - TSQ 9000 Thermo Fisher). GC operating conditions were: He at 1.2 mL min^{-1} with a 60-m HP-5MS column (0.25 mm I.D., 0.25 μm; Agilent Technologies, Avondale, USA); oven starting at 120 °C (1 min), increasing to 180 °C at 25 °C min^{-1}, to 250 °C at 10 °C min^{-1} and to 310 °C at 20 °C min^{-1} (1 min). Results are reported in the Supplementary Material Table 3S and showed a good correspondence between nominal and real concentrations for all the six FMs tested. For this reason, results and discussion were based on nominal concentrations.

2.5. Data analysis

The results of the test were expressed as Larval Development Ratio (LDR) ± standard error (SE). LDR is reported as the number of copepodite-I larvae divided by the total number of early stages (nauplii plus copepodite-I) recovered at the end of the test:

$$LDR = \frac{copepodites}{nauplii + copepodites} \quad (1)$$

LDR obtained for each test concentration was then normalised to the average control LDR in order to compare graphically results obtained in different tests.

Early Life Stage mortality (ELS-m) was calculated to estimate the quality of the eggs, as follows:

$$ELS_m = 100 \cdot \frac{initialeggs - (unhatchedeggs + nauplii + copepodites)}{initialeggs - unhatchedeggs} \quad (2)$$

One-way ANOVA with Dunnett's post hoc test was used to calculate No-observed effect concentrations (NOECs) and lowest-observed effect concentrations (LOECs), as the highest tested concentration not statistically different from control (NOEC) and lowest tested concentration statistically different from control (LOEC). Data normality and variance homoscedasticity were checked using Kolmogorov-Smirnov and Levene's test based on medians, respectively. All these statistical analyses were performed using the software package IBM SPSS Statistics v.25.

Effective concentrations 20% (EC_{20}s), and effective concentrations 50% (EC_{50}s) were computed using a statistical program for continuous response developed at the Technical University of Denmark (Christensen et al., 2009), by assuming a log-normal distribution of the observed effects at the tested concentrations.

2.6. Quality assurance/Quality control (QA/QC)

On day-5, control results were deemed as acceptable whether an average LDR of 0.5 ± 0.2 and average early-life stage mortality (ELS-m) less than 30% were observed. The quality of the eggs produced by each culture was verified by using 3,5-dichlorophenol (3,5-DCP) as a reference toxicant (positive control), according to Andersen et al. (2001). The acceptability interval for the EC_{50} of 3,5-DCP is 31–250 μg L^{-1} (Picone et al., 2018).

3. Results

3.1. QA/QC

Five testing sessions were performed, by using eggs collected from 2 different *A. tonsa* cultures (AT17/20 and AT18/20). Average LDR obtained in the five testing sessions after five days of exposure was 0.39 ± 0.02 (n = 5), with a minimum of 0.34 ± 0.02 (culture AT17/20, 09/06/2020) and a maximum of 0.45 ± 0.02 (culture AT18/20, 13/07/2020). Mean ELS-m was less than 30% in all tests, with a minimum value of 28 ± 2% (culture AT17/20, 22/06/2020) and a maximum of 29 ± 2% (culture AT18/20, 13/07/2020). Details on mean ELS-m and LDR obtained in the negative control for each testing session are reported in Supplementary material - Tables 1S and 2S.

In both positive control tests, the EC50 for 3,5-DCP fell within the acceptability interval of the control chart (49 μg L^{-1} and 44 μg L^{-1} for culture AT17/20 and AT18/20, respectively).

Ethanol did not affect significantly the larval development of *A. tonsa* at the tested concentrations (0.0002–2%) ($F_{5,23} = 1.837$; $p = 0.156$). At 0.2% and 2% we observed a minor reduction of LDR as compared with control (14% and 22% inhibition, respectively) but it was not significant (Dunnett *post-hoc* T-test: $p = 0.348$ and $p = 0.065$, respectively).

3.2. Toxicity testing

ELS-m was unaffected by FM's concentration for all the six compounds; results of the one-way ANOVA on ELS-m data are summarised in Supplementary Material Table 1S.

Conversely, significant effects were observed on LDR. Toxicity data obtained for LDR, including EC_{20}, EC_{50}, NOEC and LOEC are summarised in Table 2. According to the calculated EC_{50}s (Fig. 1), HEX and AMY were the more toxic chemicals, with HEX exerting a 50% inhibition of

Table 2
Summary of toxicity data obtained for the larval development ratio (LDR) endpoint. All data are reported as ng L^{-1}.

Fragrance	NOEC	LOEC	EC_{20}	EC_{50}
AMY	<10	10	1.3	131
ORA	10	100	40	766
HEX	<10	10	1.0	57
AMB	1000	10,000	727	26,276
PEO	100	1000	121	2034
BZS	100	1000	53	2795

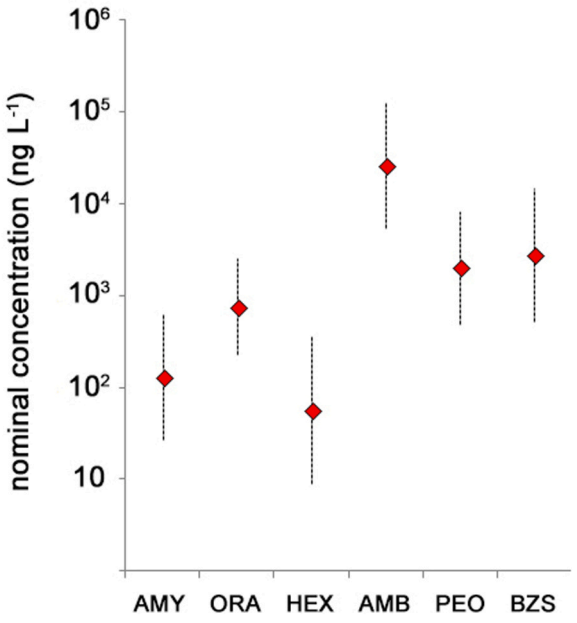

Fig. 1. EC_{50} and 95% confidence interval calculated for the FMs.

larval development at concentration as low as 57 ng L^{-1}. ORA was less toxic than HEX and AMY; however, it exerted a 50% effect at concentration as low as 766 ng L^{-1}. PEO and BZS generated a similar EC_{50} and were two order of magnitude less toxic than HEX. The less harmful FM for the larval development of the copepods was AMB, with an EC_{50} of one order of magnitude higher than PEO and BZS.

HEX and AMY inhibited the larval development of *A. tonsa* significantly at the lowest tested concentration (10 ng L^{-1}), impeding the calculation of NOEC and providing EC_{20}s of 1.0 and 1.3 ng L^{-1}, respectively. Concentration-effect curves of HEX and AMY had a similar log-linear trend up to a concentration of 1000 ng L^{-1}, with HEX affecting somewhat more severely the larval development of *A. tonsa* than AMY (Fig. 2). At concentrations higher than 1000 ng L^{-1}, AMY provided still a log-linear decrease of the larval development with increasing concentrations, while HEX exhibited a U-shaped curve. PEO, AMB, BZS and ORA did not affect larval development at 10 ng L^{-1}, while at 100 ng L^{-1} only ORA exerted significant effects (Dunnett *post-hoc* T-test: $p = 0.011$). PEO and BZS affected significantly the larval development at a concentration of 1000 ng L^{-1} (Dunnett *post-hoc* T-test: $p = 0.012$ and $p < 0.001$, respectively), while AMB toxicity was

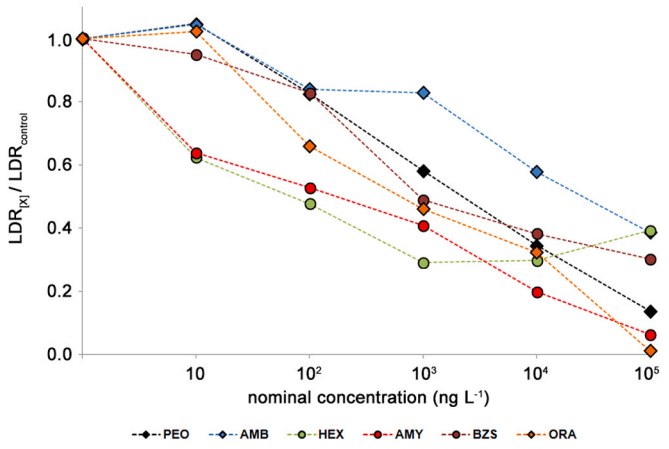

Fig. 2. Concentration-effect curve for the tested FMs. Larval development ratios (LDR_x) are reported as value normalized to control LDR.

significant at 10,000 ng L^{-1} (Dunnett *post-hoc* T-test: $p = 0.003$). At 100,000 ng L^{-1}, larval development was reduced by more than 85% as compared with control by AMY, ORA and PEO, while effects of HEX, BZS and AMB were less marked.

4. Discussion

4.1. Toxicity of FMs towards A. tonsa

The toxicity data obtained for the tested FMs evidenced that these compounds inhibit the larval development of *A. tonsa* significantly at concentrations by far lower than the effect-concentrations reported in scientific literature and databases for aquatic species. Focusing on PEO and the salicylates, acute effects on freshwater fishes, algae and *Daphnia magna* were observed at concentrations ranging from 0.7 to >10 mg L^{-1} (Belsito et al., 2007; Natsch et al., 2018), while EC_{20}s (1–121 ng L^{-1}) and EC_{50}s (57–2795 ng L^{-1}) estimated for the larval development of *A. tonsa* are at least three orders of magnitude lower. Similarly, lethal effects of ORA on mosquito instars occurred at concentrations higher than 1 mg L^{-1} (LC_{50}s in the interval 2.37–7.80 mg L^{-1}; Borovsky et al., 1987), while effects on *A. tonsa* were significant at 100 ng L^{-1} (LOEC) and the EC_{50} was calculated at 766 ng L^{-1}. To the best of our knowledge, acute toxicity data are not available for AMB.

Furthermore, our data underline that FMs are among the most effective inhibitors of *A. tonsa* larval development. In the literature, only the toxicity data reported for the antifouling active ingredient tributyl (chloro)stannane (TBT) are lower than those calculated for HEX, and AMY ($EC_{10} = 0.7$ ng L^{-1}, $EC_{50} = 3$ ng L^{-1}; Kusk and Petersen, 1997); other persistent, bioaccumulative and toxic chemical such as brominated flame retardants provided EC_{50}s similar than those calculated for ORA, BZS and PEO (BDE-99, BDE-100; Wollenberger et al., 2005). Endocrine-disrupting chemicals such as natural hormones (e.g. 17β-estradiol, estrone, progesterone, 20-hydroxyecdysone, insect juvenile hormone III), pharmaceuticals (e.g. 17α-ethinylestradiol, flutamide, tamoxifen), and industrial products (e.g. bisphenol A, 4-octylphenol) were by far less toxic than HEX, AMY, ORA, PEO and BZS, and provided EC_{50}s similar or 1–2 orders of magnitude higher than the EC_{50} calculated for AMB (Andersen et al., 2001). Similarly, also for other chemicals used in personal care products the EC_{50}s reported in the literature are similar (Tonalide, $EC_{50} = 26{,}000$ ng L^{-1}) or up to 1 order of magnitude higher ($EC_{50} = 59{,}000$, 66,000 and 160,000 ng L^{-1} for Galaxolide, Musk ketone and Celestolide, respectively) than the EC_{50} calculated for AMB in the present study (Wollenberger et al., 2003).

4.2. Environmental significance of FMs toxicity

The ability of FMs to act as potent inhibitors of larval development in copepods may be of ecological concern, due to the relevance of copepods in marine and coastal food webs. In particular, the analysis of literature data available for surface waters evidenced that AMY and HEX represent a contaminant of potential concern both in coastal, urban environments as well as in polar areas (Vecchiato et al., 2018, 2017, 2016).

The comparison between toxicity data and FMs concentrations measured in 41 surface water samples collected in the Venice Lagoon (Vecchiato et al., 2016), evidenced that AMY and HEX are the FMs of major concern in the urban canals of Venice and Burano islands (Fig. 3). HEX occurred at concentrations above LOEC in approximately 75% of analysed water samples and exceeded the estimated EC_{50} in approximately 50% of them. AMY showed a similar trend. Thus, for both HEX and AMY, major effects on larval development may be expected. PEO and BZS are of less concern since only the maximum measured concentrations approximated EC_{50}s. Nevertheless, although major effects due to PEO and BZS may be generally excluded, the occurrence of minor toxic effects is plausible, due to the number of samples with concentrations exceeding EC_{20}s. Minor toxicity due to ORA is likely only for a few samples (4 out of 41), while AMB is not of concern, since all surface

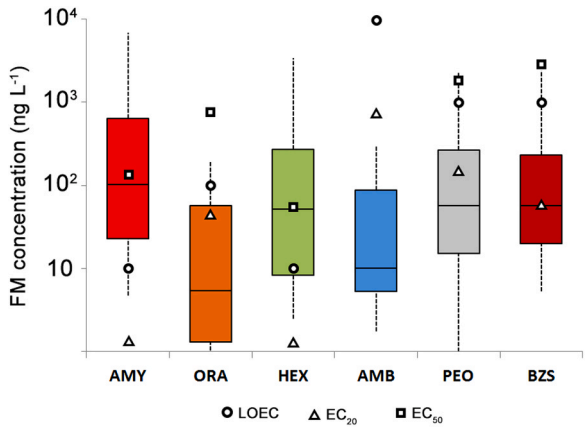

Fig. 3. Comparison between environmental concentrations of FMs (box plots) reported for urban canals of the Venice Lagoon and toxicity data calculated for FMs. Boxes identify interquartile ranges, while whiskers identify minimum and maximum concentrations.

water samples were far below LOEC and EC_{20}.

In the Sicily Channel, Central Mediterranean, FMs concentrations in surface water were by far lower than in the Venice Lagoon (Vecchiato et al., 2018). However, alongside coastal areas, where FMs concentrations reflect local land-based pollution, AMY (59 ng L^{-1}) and HEX (37 ng L^{-1}) occurred at concentrations above NOEC, EC_{20}s and LOEC and thus may affect the larval development of *A. tonsa*, although expected effects are minor. In contrast, PEO, AMB, BZS and ORA were below the NOECs.

Similarly, also in seawater samples collected in Terra Nova Bay, Antarctica, AMY (1.4–29 ng L^{-1}) and HEX (0.65–15 ng L^{-1}) occurred at concentrations above EC_{20}s and LOECs during the seasonal snow melt, probably reflecting the release of particle-bound contaminants and/or as a consequence of discharges of treated waste-waters from the Mario Zucchelli Research Station (Vecchiato et al., 2017). As concern the other compounds, ORA and PEO were not detected, while BZS and AMB were below the estimated NOECs.

4.3. Possible mechanisms of effect

Toxicological effects of FMs were studied mainly in humans, other mammals and fishes, both in vivo and in vitro (Patel, 2017; Patel et al., 2020), while molecular mechanisms of toxicity of FMs were poorly investigated in invertebrates; thus, the cause of their toxicity toward aquatic invertebrates is mostly unknown. Nevertheless, since many FMs are suspected of having xenoestrogenic activity, endocrine-mediated toxic effects should be taken into consideration.

Different studies investigated the potential endocrine-disrupting effects of salicylate esters, showing that many of them, including BZS, display intense xenoestrogenic, antiestrogenic and antiandrogenic activity in mammals, both in vitro and in vivo (Charles and Darbre, 2009; Kiyama and Wada-Kiyama, 2015; Kunz and Fent, 2006; Zhang et al., 2012). Since some insect uses BZS as a pheromone for triggering with conspecifics (Estrada et al., 2011; Farine et al., 1994), then it cannot be excluded that also in crustaceans BZS or other salicylates may be involved in endocrine regulation and signalling that may be disrupted by the occurrence of FMs in surface waters.

Endocrine-disrupting effects have also been suggested for ORA: it is suspected of causing a precocious metamorphosis in termites (*Coptotermes formosanus*) by antagonising the activity of juvenile hormones, following a mechanism already observed in other arthropods and induced by precocenes (Ibrahim et al., 2004). However, ORA is also recognised as a potent repellent and inhibitor of food consumption for insects (Ibrahim et al., 2004).

However, although endocrine disruption of FMs has been observed or hypothesised in mammals or arthropods, the effects we observed on copepod larval development cannot be unquestionably attributed to endocrine disruption mediated by FMs. A retard in larval development can be also determined by a reduced energy intake or an increased energy demand to cope with the presence of the chemical stressor, without any interference with the endocrine signalling (Barata et al., 2004). Further investigations are needed to clarify the possible role of FMs as inhibitors of enzymes involved in the molting process (Zou and Fingerman, 1999) and/or their ecdysteroid agonists/antagonists behaviour by using in vitro tests such as the *Drosophila melanogaster* B_{II} cell line assay (Dinan et al., 2001; Pounds et al., 2002).

5. Conclusion

This work is the first laboratory research intended to investigate the toxicity of FMs towards brackish and marine invertebrate species.

The data revealed that FMs, especially HEX and AMY, are potent inhibitors of the larval development, suggesting that FMs may act as contaminants of concern in many estuarine and coastal environments and that monitoring of FMs in surface waters should be implemented in monitoring programmes.

The comparison with environmental concentrations confirmed that HEX and AMY might exert major toxicological effects in highly urbanised estuarine areas such as the Venice Lagoon, but also minor effects in environments with a lower direct anthropogenic impact, as the offshore sea or the polar areas.

Future developments of this research should characterise the environmental effects and fate of fragrances, involving the assessment of the possible chronic effects on planktonic and benthic species, as well as bioaccumulation and biomagnification of the fragrances.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

CRediT authorship contribution statement

Marco Picone: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Gabriele Giuseppe Distefano:** Investigation, Resources. **Davide Marchetto:** Investigation, Resources. **Martina Russo:** Investigation, Resources. **Marco Vecchiato:** Investigation, Resources, Data curation, Writing - original draft. **Andrea Gambaro:** Supervision, Writing - review & editing. **Carlo Barbante:** Writing - review & editing. **Annamaria Volpi Ghirardini:** Supervision, Writing - review & editing. Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112146.

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