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Effects of ultra-violet filters oxybenzone (benzophenone-3) and 4-methylbenzylidene camphor on different life-history traits of the copepod *Acartia tonsa* Dana

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ABSTRACT

The UV filters 2-hydroxy-4-methoxybenzophenone, or benzophenone-3 (BP3), and 4-methylbenzylidene camphor, or enzacamene (4-MBC), are recognised as toxicants that might impair different life-history traits in marine invertebrates and threaten the resilience of critical ecosystems such as coral reefs. Their effects on different life-traits of the calanoid copepod *Acartia tonsa* were tested, including (i) hatching, early-life stages mortality and larval development from the egg to the copepodite-I stage, (ii) the attainment of sexual maturity and reproduction in young adult copepods, (iii) the reproduction of mature copepods (F_0 generation) and hatching, larval mortality and larval development in their offspring (F_1 generation). The chemicals exerted effects on different life traits of the copepods, with BP3 displaying clearer and more consistent impacts than 4-MBC. BP3 did not affect larval survival and development of the parental generation and reproduction and feeding up to 500–600 μg L^{-1}. At the same time, it severely delays the larval development of the offspring at a concentration as low as 7.4 μg L^{-1}, showing a transgenerational effect at concentrations frequently measured in surface waters. Conversely, 4-MBC severely affected larval survival and development at a concentration of 46 μg L^{-1}, caused mortality in the parent generation at 9.3 μg L^{-1}, but did not inhibit larval survival and development of the offspring up to a concentration of 2.1 μg L^{-1}. At the same time, it exerts a significant stimulatory effect on egg production by generation F_0 and larval development of generation F_1 compared to the control, suggesting a probable toxicant-mediated endocrine disruption.

Keywords:UV-filters Sunscreens Zooplankton,Transgenerational effect Enzacamene Benzophenone-3

1. Introduction

In the last decades, organic ultraviolet (UV) filters have been increasingly used in personal care and sunscreen products to protect humans from UVA and UVB solar radiation, thereby preventing photoaging, sunburns, and the risk of skin cancer (Sander et al., 2020). Furthermore, several organic UV filters are used in other consumer products to prevent polymer and pigment photodegradation, including food packaging, plastics, paints, textiles, detergents, and disinfectants (Cadena-Aizaga et al., 2022a). Consequently, these compounds have been extensively released into the environment through boating, residential, and municipal wastewaters, as well as through washing off the skin during aquatic recreational activities at the sea or other water reservoirs (Langford and Thomas, 2008; O'Malley et al., 2020; Ramos et al., 2016). In particular, leaching from the skin during recreational activities such as swimming, sailing, and bathing results in summer peaks of UV filter concentrations in surface waters and sediments of beaches and coastal areas highly frequented by tourists (Astel et al., 2020; Cadena-Aizaga et al., 2022a; Langford and Thomas, 2008), possibly posing a threat for wildlife and fisheries (Downs et al., 2022a).

Two of the organic UV filters approved for sunscreen formulations and frequently detected in surface waters worldwide are 4-methylbenzylidene camphor (4-MBC) and 2-hydroxy-4-methoxybenzophenone (BP3), also known as benzophenone-3 (Mitchelmore et al., 2021; Tashiro and Kameda, 2013). For both 4-MBC and BP3, there is relevant literature highlighting their adverse effects in vitro and in vivo on several biological models, including microalgae (Paredes et al., 2014; Vieira Sanches et al., 2021), marine and freshwater invertebrates (Corinaldesi et al., 2017; Cuccaro et al., 2023; Lambert et al., 2021), and vertebrates, including humans (Ghazipura et al., 2017; Mustieles et al., 2023; Portrais et al., 2019). In addition, both 4-MBC and BP3 have been proven to bioaccumulate in marine biota (Cuccaro et al., 2022; Scheele et al., 2023; Vidal-Liñán et al., 2018). Toxicological effects of 4-MBC and BP3 in marine species were often associated with their ability to generate oxidative stress and act as endocrine disruptors at different levels (i.e., by inhibiting spermatogenesis, altering the HPG-axis, affecting the thyroid and estrogenic systems), leading to detrimental effects on the reproduction (Ghazipura et al., 2017; Lax et al., 2023). Furthermore, both 4-MBC and BP3 showed the ability to cause bleaching in corals by favouring viral infections (Danovaro et al., 2008), and BP3 has also been proven to generate genotoxicity and larval deformation in corals (Downs et al., 2015). Consequently, this evidence of adverse effects on different animal models and critical ecosystems, such as coral reefs, led to the introduction of regulatory measures and local bans on the use of 4-MBC and BP3 in sunscreen products (Lax et al., 2023; Miller et al., 2021). In the European Union, 4-MBC is approved at a maximum concentration of 4 % in sunscreen products intended for body application (Regulation 1229/2009/EC), while such filter is not approved as a sunscreen active ingredient in Japan and the United States (Downs et al., 2022b; Lax et al., 2023). As concern BP3, it is allowed in the European Union up to a maximum concentration of 6 % in sunscreens and up to a maximum of 0.5 % in other cosmetic products (Regulation 238/2017/EC), and similar limits were set by Canada, the United States, South Korea, and Japan (Mitchelmore et al., 2021; Wnuk et al., 2022). Furthermore, regulatory measures intended to ban the use of BP3 in sunscreen products to protect coral reefs were adopted by Hawaii, the Republic of Palau, the U.S. Virgin Islands, the Marshall Islands, Aruba, Mexico, Thailand and Bonaire, while the Republic of Palau and Thailand are the only countries to have approved a ban for 4-MBC, to date (Khanna and Singh, 2023; Miller et al., 2021; Narla and Lim, 2020; Ouchene et al., 2019). Moreover, BP3 was recently detected in the snow deposition from glaciers of the Svalbard Islands, accounting for processes of long-range atmospheric transport of this contaminant towards remote polar environments (D'Amico et al., 2024).

Besides coral reefs, coastal plankton is the biological compartment that is expected to be more exposed to UV filters and their adverse effects. Organic and inorganic UV filters, indeed, accumulate in surface waters along the coastlines (Labille et al., 2020), which receive the products directly washed off from skin and clothing during recreational activities and released in wastewater by urban and industrial sources (Cadena-Aizaga et al., 2022a). Copepods dominate the zooplankton in estuaries and coastal areas, and here they occupy a critical trophic position in the food web since they graze on the phytoplankton, consume detritus, and serve as a food reservoir for consumers of higher trophic levels, including fish and larvae of fish and invertebrates (Turner, 2004). Copepods graze primarily in surface waters, and then they are vulnerable to contaminants discharged in marine and coastal areas, including UV filters, which might both critically affect copepod's physiological and life history traits or accumulate in their tissues, making them a vector for the transfer of pollutants to their consumer. Consequently, the effects on copepods might have significant consequences at the ecosystem scale, especially in areas where the ecosystem's functioning is strictly regulated by the lower trophic levels (Irigoien et al., 2004).

We performed a suite of bioassays aiming at identifying potential impairments caused by 4-MBC and BP3 on different life-history traits of the calanoid copepods *Acartia tonsa*, including: (i) the larval development from the egg stage to the copepodite-I stage, (ii) the attainment of sexual maturity and the onset of the reproduction, (iii) the egg hatching ratio, and (iv) the effects on F_1 generation larval survival and development. We chose to focus on effects at the individual level to capture integrated organismal responses and explore the toxic effects of environmental pollutants at a high level of biological organisation. Indeed, each level of biological organisation (from molecular to individual, population, and biosphere) provides valuable insights and contributes in different ways to understanding the impact of toxicants on biota (Newman, 2010). The test species, *A. tonsa*, was chosen based on its well-known ecology and wide diffusion (Camatti et al., 2019; Drillet et al., 2008; Peck et al., 2015), easy maintaining and handling for toxicity testing (Kusk and Wollenberger, 1999), suitability for the measure of different sensitive endpoints within the same experiment (Andersen et al., 1999; Picone et al., 2022), and because it previously demonstrated sensitivity to different classes of chemicals (Kusk et al., 2011; Picone et al., 2021; Wollenberger et al., 2003, 2005a). The observed effects were discussed considering the known effects of UV filters on arthropods under the perspective of the Dynamic Energy Budget (DEB) theory, which provides a rational and consistent framework to evaluate ecotoxicological test results (Kooijman, 2009). Although it is not regularly applied in ecotoxicology, the DEB approach is practical and allows interpreting effects on survival, growth, and reproduction as changes in the energy flow with the individuals (Klok et al., 2012). This approach was successfully used to assess the effects of PAHs and open loop exhaust gas cleaning systems discharges on copepods (Klok et al., 2012; Picone et al., 2023).

2. Materials and methods

2.1. Chemicals

Native UV filters 2-hydroxy-4-methoxybenzophenone (BP3) and 4-methylbenzylidene camphor (4-MBC) were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Stock solutions of OXY and 4-MBC were prepared in acetone (Merck KGaA, Darmstadt, Germany), as both substances are poorly soluble in water. To exclude possible effects of acetone on the observed results, we analysed the concentrations at which acetone may affect *A. tonsa*. Literature data indicate that acetone does not exert detrimental effects on larval development up to a concentration of 157 mg L^{-1} (Andersen et al., 1999), while preliminary laboratory tests showed no effects on egg production or pellet production up to a concentration of 7.3 mg L^{-1} (Picone, unpublished data). The acetone concentrations used in our tests were well below these thresholds ($<$74 mg L^{-1} for the larval development test and $<$ 7 mg L^{-1} for the egg production and long-term tests), confirming that acetone was unlikely to influence the observed results.

2.2. Copepod culturing

The copepods used for the toxicity tests were reared in an in-house laboratory culture at the Ecotoxicology Laboratory of Ca' Foscari University (Venice, Italy), according to the procedure outlined in (Picone et al., 2021, 2022). Accordingly, approximately 600/800 individuals were maintained in 2-L glass bottles filled with a 20 ‰ salinity medium, prepared according to ISO 16778 standard method (ISO, 2015), and fed four times per day with an algal mixture (*Tetraselmis suecica*, *Tisochrysis lutea*, and *Isochrysis galbana*) dispensed through a peristaltic pump, to keep an algal concentration in the culture of about 10^3 cell mL^{-1}. The cultures were cleaned thrice per week to remove the excess food, the faecal pellets, and the eggs released by the copepods to avoid overcrowding. A new culture was started every four to five weeks by adding 600/800 freshly collected eggs to a bottle filled with 20 ‰ salinity medium. Besides being provided for by standard methods and published protocols (ISO, 2015; Picone et al., 2021, 2022), the salinity of 20 ‰ was chosen because *A. tonsa* is the dominant species in the North Adriatic Sea estuaries at salinities around 20–25 ‰, whereas it is replaced by other species (e.g., *A. clausii*) at higher salinities (Bianchi et al., 2003; Camatti et al., 2019).

The algal clones used as food were individually cultured using Guillard's F/2 as the culture medium (Guillard and Ryther, 1962). The cultures were maintained at 20 °C in a climatic room under a 16:8 light: dark photoperiod. The 20 ‰ salinity medium used for culturing the copepods was also used as the dilution water for preparing solutions and as the negative control in toxicity tests after being laced with the algal mixture to obtain a concentration of approximately 10^3 cells mL^{-1}.

2.3. Toxicity tests

Three different testing procedures were used: 1) the larval-development test to assess effects on the early-larval stages, from the egg to the copepodite-I (C–I) stage; 2) the egg production test to assess effects on the attainment of sexual maturity, and reproduction in young adult copepods; 3) the long-term exposure test to assess effects on reproduction of mature copepods (F_0 generation) and their offspring (F_1 generation). In the larval development test, a concentration range (i.e., 0.04–657 μg L^{-1}) bracketing environmental concentrations and effective concentration towards different coral species was tested (Mitchelmore et al., 2021), while for the eggs production and the long-term exposure tests, the range was narrowed in consideration of the longer exposure time, the higher sensitivity of the measured endpoints, and the results obtained with the larval development test.

The **larval development test** was performed according to the ISO 16778 standard method (ISO, 2015), modified as reportedin Picone et al. (2021). Freshly released eggs collected from the in-house laboratory culture were exposed to different UV-filter concentrations (range: 0.04–46 μg L^{-1} and 1–657 μg L^{-1} for 4-MBC and BP3, respectively) and a negative control in 100 mL glass beakers filled with 25 mL of testing solution. The experiment started by adding a known number of eggs between 50 and 80 to each beaker at $t = 0$, according to the procedure detailed in Picone et al. (2021). The concentrations set for each UV-filter were selected after a preliminary test performed using a limited number of replicates ($n = 3$). The test solution was then refreshed on day 2 by adding 25 mL of solution, including food, to each test beaker, without removing the nauplii, to minimise stress on the larvae (ISO, 2015). The exposure ended on day 5, when approximately 40 % of the control larvae reached the C–I stage by staining the beaker's content with Lugol's solution and filtering it through a cellulose nitrate filter with gridlines. All the recovered larvae (i.e., nauplii and copepodites) and unhatched eggs were then counted under a dissecting microscope to calculate hatching rate (H), early-life stage mortality (ELS_m), and larval development ratio (LDR), according to the following equations:

$$H = \frac{eggs_{t_0} - eggs_{t_5}}{eggs_{t_0}}$$

$$ELS_m = 1 - \frac{nauplii - copepodites}{eggs_{t_0} - eggs_{t_5}}$$

solution at the appropriate UV-filter concentration and laced with food (to obtain a food concentration $> 6 \times 10^4$ cells mL^{-1}), and finally reintroducing the copepods retained by the filter into the testing culture. Viability and motility of copepods were visually checked under a stereomicroscope before being reintroduced into the testing culture. The filtered test solution was recovered into 50 mL polypropylene vials for measuring dissolved oxygen and pH and into 250 mL amber glass bottles for measuring the actual concentration of BP3 and 4-MBC. On day 10, the exposure was ended by filtering the whole content of the testing culture through a 150 μm mesh size sieve. The filtered medium was collected in 50 mL polypropylene vials to measure pH and dissolved oxygen and in 250 mL amber glass bottles to verify actual concentrations. At the same time, copepods were poured into crystallising dishes and checked under a dissecting stereoscope for their motility, developmental stage, and sex. Then, ten mature females per treatment were selected and individually placed into 30 mL glass vials filled with 25 mL of the test solution at the same concentration as their testing culture. The content of each vial was monitored daily from day 11 to day 14 to count the produced eggs. Since egg production in copepods can be influenced by limited feeding (Jensen et al., 2008; Klok et al., 2012), we also counted faecal pellets and used this count as a proxy for feeding. This operation was done by transferring the female to another vial filled with the test solution, filtering the vial's content through a cellulose-nitrate filter with gridelines, and counting all the recovered eggs and faecal pellets under a dissecting stereoscope. The data were reported as egg production per female per day (egg female^{-1} d^{-1}) and pellet production per female per day (pellet female^{-1} d^{-1}), for each treatment. Only the data obtained from females that survived until day 14 were used for the statistical analyses.

The **long-term exposure** was performed according to the method reported by Picone et al. (2022) with a few modifications. Testing cultures were prepared by adding 400/600 freshly released eggs to 2-L glass bottles filled with test solutions and the negative control. In this case, treatments were selected based on the response of the egg and faecal pellets production test and analytically verified (initial concentrations: 0.15–0.41–4.3 μg L^{-1} for 4-MBC, and 6.4–63 - 590 μg L^{-1} for BP3). One culture per treatment was used, including the negative control (Kusk and Wollenberger, 2007; Picone et al., 2022). The testing cultures were then tapped, sealed with Parafilm®, and placed on a vertical rotatory mixer under the same conditions reported for the eggs and faecal pellets production test. The test solution was renewed thrice per week up to day 21. It was done by siphoning off 50 % of the testing culture, filtering it through 60 μm and 20 μm mesh size sieves to recover the copepods (60 μm), nauplii, and eggs (20 μm), and adding freshly prepared test solution at the appropriate UV-filter concentration and laced with food. Co-

$$LDR = \frac{copepodites}{nauplii + copepodites}$$

Where $eggs_{t0}$ and $eggs_{t5}$ are the number of eggs introduced in the test beakers at the beginning of the test and the unhatched eggs recovered on the filter on day 5, respectively. The test was run in 6 replicates. Physicochemical parameters (dissolved oxygen and pH) were measured in a dedicated replicate at the beginning and end of the test.

The egg production test was performed according to an internal procedure. Briefly, 200/300 eggs freshly released by the in-house culture were added to 500 mL glass bottles filled with testing solutions, and the negative control. Test concentrations were selected based on the larval development test outcome and analytically verified (initial concentrations: 0.04–0.11–1.4–9.4 μg L^{-1} for 4-MBC, and 3.7–18–288–657 μg L^{-1} for BP3). Then, the bottles (hereafter, testing culture) were tapped, sealed with Parafilm®, placed on a rotatory mixer, and wheeled at 1 rpm for ten days, under natural photoperiod (16:8 light:dark) and at $T = 20 \pm 2$ °C. One culture per treatment was used (including the negative control). Test solution was renewed on day 2, day 5, and day 7, by siphoning off 50 % of the testing culture, filtering it through a 60 μm mesh size sieve to recover the copepods, adding freshly prepared test pepods retained by the 60 μm mesh size filter were checked for their viability and motility under a dissecting microscope and then reintroduced into the testing culture, while eggs and nauplii were stored separately in glass vials and at 4 °C. Under test conditions ($T = 20 \pm 2$ °C, 16:8 light:dark, algal density $> 6 \times 10^4$ cells mL^{-1}), the eggs hatch within 24 h, the larvae reach the C–I stage after 5/6 days, and the adult stage is reached after 10/11 days. On day 19, ten females were collected from their treatment and checked under a dissecting microscope to confirm sex and maturity. Then, they were individually placed into 30- mL glass vials filled with 25 mL filled with 25 mL of the test solution at the same concentration as their testing culture and 100 μL of the algal mix at 10^4 cells mL^{-1}, as previously described for the egg production test. The exposure in the vials lasted 72-h and the egg production was verified at 24-h intervals by transferring the females to a new set of vials filled with saline medium and food and then filtering the content through a cellulose nitrate filter (Ø 47 mm, porosity 0.45 μm) with gridlines. From day 19 to day 21, eggs produced by copepods exposed to UV treatments and the control were collected from their respective culture, stored at 4 °C and then used to start a larval development test on the F_1 generation, according to the abovementioned procedure. In the larval development of F_1 generation, the eggs were exposed only to the 20 ‰ salinity medium to disentangling effects on the reproduction due to the exposure of parents (F_0) from effects due to F_1 exposure to UV filters. Aliquots for chemical analyses were preserved in amber glass bottles and stored at —4 °C on day 0 and after test solution renewals on days 5, 9, 14, 19, and 21.

The acceptability criteria for test results are reported in Supplementary Material, together with the parameters measured during the testing with BP3 and 4-MBC (Supplementary Material – Table S2, Table S5, and S6).

2.4. Chemical analyses

The UV-filter concentration in the water used for the solution renewals was tested analysing 200 mL water samples through solid phase extraction (SPE, Waters Oasis® HLB cartridges, 200 mg, 6 cm^3, Waters Corp., Milford, MA USA). The SPE cartridges were previously conditioned with 10 mL of *n*-hexane, 10 mL of dichloromethane, followed by 10 mL of ultrapure water (ELGA, High Wycombe, UK), and later eluted with 1 mL of toluene, 15 mL of dichloromethane, and 10 mL of *n*-hexane. Pesticide-grade solvents (Romil Ltd., Cambridge, UK) were used. Samples were dried with an aliquot of Na_2SO_4 and concentrated to 250 μL under nitrogen flow at 23 °C (Turbovap II®, Caliper Life Science, Hopkinton, MA, USA). Phenanthrene ^{13}C (Cambridge Isotope Laboratories, Andover, MA USA) was used as Internal Standard, spiking 50 μL at the concentration of 1 ng/ μL to samples prior to extraction, according to previously developed methods (D'Amico et al., 2022; Vecchiato et al., 2021).

Instrumental analyses were conducted by GC–MS/MS (Trace 1310 - TSQ 9000 Thermo Fisher onto a 60-m HP-5MS (0.25 mm I.D., 0.25 μm; Agilent Technologies, Avondale, USA). Oven conditions were: 120 °C for 1.0 min, increased to 180 °C at 25 °C min^{-1}, then to 250 °C at 10 °C min^{-1}, and finally to 310 °C at 20 °C min^{-1}. The SRM transitions for quantification (Q) and confirmation (q) were respectively 227.1 → 184 (CE 20; Q) and 151 → 95 (CE 10; q) for BP3; 254.1 → 239.1 (CE 10; Q) and 254.1 → 211.1 (CE 10; q) for 4-MBC; 184 → 158 (CE 22; Q) and 184 → 157 (CE 28; q) for Phenanthrene ^{13}C.

The Method Detection Limits (MDLs) were calculated as three times the standard deviations of the mean blanks ($n = 3$), which resulted respectively 1.4 ± 0.5 ng L^{-1} for BP3 (MDL = 1.6 ng L^{-1}), and 1.9 ± 1.6 ng L^{-1} for 4-MBC (MDL = 4.9 ng L^{-1}). Samples were not blank corrected.

2.5. Data handling and analysis

In the larval development and long-term tests, one-way analysis of variance (ANOVA) and Tukey's HSD *post-hoc* test were used to assess the effect of UV filter concentrations at the end of the exposure on hatching, early-life stage mortality, and larval development ratio compared to the control. Before ANOVA, variance homogeneity was tested using Levene's test, while residual normality was verified using the Shapiro-Wilk normality test. When the residual normality condition was not satisfied, Kruskal-Wallis and Wilcoxon rank-sum tests were used to check for treatment differences.

To investigate the effects of UV filter concentrations on the reproduction of *A. tonsa* over the duration of the experiment, we used Generalized linear mixed models (GLMM). GLMMs were fitted with egg production (log-transformed) as the dependent variable, with either BP3 or 4-MBC concentration, day of observation, and pellet production (used as a proxy for feeding; log-transformed) as fixed predictors, and individuals as a random factor. (Jensen et al., 2008; Klok et al., 2012). The interactions between either BP3 or 4-MBC concentration and day of observation were also tested (BP3:day and 4-MBC:day). Models fitting was performed using the "*lme4*" R-package (Bates et al., 2015), while type III ANOVA using Wald χ^2 test and *post-hoc* pairwise comparisons were performed using the "*car*" and "*emmeans*" R-package, respectively (Fox and Weisberg, 2018; Lenth, 2023). All analyses were performed using R Statistical Software (R Core Team, 2022). The package "*ggplot2*" was used to prepare the graphs (Wickham, 2016).

3. Results

3.1. Chemical analysis

In the egg production test, the concentrations of 4-MBC were relatively constant over the test duration (Table 1). Similarly, BP3 test concentrations also remained relatively constant, even if they tended to decrease over the test duration (Table 1). In the long-term exposure test, the 4-MBC treatment with the lowest initial concentration (0.15 μg L^{-1}) was relatively stable over time. In contrast, the other treatments (initial concentrations 0.41 and 4.34 μg L^{-1}) showed a decreasing trend with increasing exposure time (Table 1). Conversely, BP3 concentrations showed different trends in the long-term exposure, with the treatment at the lowest concentration showing a tendency to increase over time, and the treatments at the higher concentration showing a decreasing trend, particularly irregular for the treatment with the highest initial concentration (590 μg L^{-1}).

Considering the variability observed in some treatment during testing, the exposure concentrations used for data analysis were the averages of the actual concentrations measured on days 0, 3, 5, 7 and 10 for the egg production test, and on days 0, 5, 9, 14, 19, and 21 for the chronic test: 0.05, 0.1, 1.3 and 9.3 μg L^{-1} for the egg production test with 4-MBC; 2.6, 16, 195, and 576 μg L^{-1} for the egg production test with BP3; 0.13, 0.25, and 2.1 μg L^{-1} for the chronic test with 4-MBC; 7.4, 41, and 468 μg L^{-1} for the chronic test with BP3.

3.2. *Effects of BP3 on A. tonsa*

Table 1

Summary of the chemical analyses performed on the aliquots of test medium retrieved from the treatments at the beginning of the test and before water renewals on days 3, 5, 7, and 10, for the egg and pellet production test, and on days 5, 9, 14, 19, and 21 for the long-term exposure test. In bold are reported the concentration at day 0 (second column) and the the average of the concentrations measured during the experiment (last column).

Egg and pellet production test							
	day 0	day 3	day 5	day 7	day 10	mean	
4-MBC concentrations (μg L^{-1})							
Treatment 1	**0.04**	0.04	0.07	0.06	0.04	**0.05**	
Treatment 2	**0.11**	0.08	0.07	0.12	0.13	**0.10**	
Treatment 3	**1.42**	0.97	1.67	1.37	0.98	**1.26**	
Treatment 4	**9.40**	8.30	13.3	17.40	8.20	**9.32**	
BP3 concentrations (μg L^{-1})							
Treatment 1	**3.7**	3.5	2.6	2.1	1.4	**2.6**	
Treatment 2	**18**	12	15	16	18	**16**	
Treatment 3	**288**	134	151	175	225	**195**	
Treatment 4	**657**	512	484	561	665	**576**	
Long-term exposure test							
	day 0	day 5	day 9	day 14	day 19	day 21	mean
4-MBC concentrations (μg L^{-1})							
Treatment 1	**0.15**	0.15	0.15	0.11	0.10	0.11	**0.13**
Treatment 2	**0.41**	0.32	0.25	0.20	0.18	0.17	**0.25**
Treatment 3	**4.33**	1.90	1.87	1.60	1.61	1.48	**2.13**
BP3 concentrations (μg L^{-1})							
Treatment 1	**6.4**	6.7	5.6	8.0	7.6	9.8	**7.4**
Treatment 2	**63**	41	31	37	40	33	**41**
Treatment 3	**590**	196	338	616	462	607	**468**

In the **larval development test** with BP3, no significant effects were observed on hatching (one-way ANOVA: F = 1.53, p = 0.224) and larval development ratio (one-way ANOVA: F = 1.12, p = 0.371), while a significant among-treatment difference was observed for early-life stage survival (one-way ANOVA: F = 3.19, p = 0.030). The larval mortality was significantly higher than in the control in the treatment at 18 μg L^{-1} BP3 (Tukey's HSD test: p = 0.039), where 23 % of the hatched larvae died (19 % increase in mortality compared with the negative control). Inhibition of larval development compared to the control was maximum at 657 μg L^{-1}, where it approximated 15 % (Supplementary Material – Figure S1). Dissolved oxygen and pH remained within the acceptable range for the larval development test, i.e., oxygen concentration > 70 % of the air saturation value and pH variation within one unit from the initial pH measure (Supplementary Material – Table S2).

In the **egg production test**, copepods recovered on day 10 had reached the adult stage in all tested concentrations ("treatments") and showed no sign of narcotisation (i.e., lack of movement or reaction to stimuli). Female mortality during the observation period (day 11- day 14) was lower than 16 % in all treatments and averaged 3 %. The onset of egg production was observed on day 11 in all the tested treatments, with a percentage of egg-lying females in the 33–60 % range. The percentage of females producing eggs increased in the following days and bracketed the ranges 40–80 % on day 12, 56–100 % on day 13, and 80–100 % on day 14 (Supplementary Material – Table S3). Accordingly, the daily egg production showed minimum values on day 11 and peaked on day 14 (Fig. 1, upper panel). In control, daily egg production ranged from 0.8 ± 0.4 egg female^{-1} d^{-1} (day 11) to 17.0 ± 1.9 egg female^{-1} d^{-1} (day 14), while in the BP3 treatments, the minimum and maximum productions were 1.0 ± 0.6 egg female^{-1} d^{-1} (day 11 at 2.6 μg L^{-1} BP3) and 18.6 ± 3.4 egg female^{-1} d^{-1} (day 14 at 16 μg L^{-1} BP3), respectively. Similarly, daily pellet production showed minimum values on day 11 and maximum values on day 14 (Fig. 1, lower panel). The average egg and pellet production per female per day over the 96 h of observation is reported in Table 2. The linear model evidenced a significant effect of pellet production (GLMM: $p < 0.001$), day of observation (GLMM: $p < 0.001$), and interaction BP3:day (GLMM: $p = 0.043$) on the egg production by the exposed copepod females (Supplementary Material – Table S4). Conversely, the effects of the different treatments were not significant (Supplementary Material – Table S4).

In the **long-term exposure test**, egg production differed significantly among treatments, although the observed significance was borderline (one-way ANOVA: F = 3.25, p = 0.037). However, none of the BP3 treatments differed significantly from the control, and a significant comparison was observed only between 7.4 and 41 μg L^{-1} BP3 (Tukey's HSD test: p = 0.042). The mean egg production observed in each treatment is reported in Table 3. Effects on generation F$_1$ are summarised in Fig. 2. The hatching was not affected by exposure to BP3 (one-way ANOVA: F = 0.04, p = 0.989), while early-life stage survival showed borderline statistical significance among the BP3 treatments (one-way ANOVA: F = 3.26, p = 0.043; Tukey's HSD test: p = 0.032 for the pairwise comparison 7.4 vs 41 μg L^{-1} BP3) but no significant differences with the negative control. Conversely, the larval development was significantly retarded in all the BP3 treatments, which exhibited a ratio of C–I larvae <30 % than in the control.

3.3. *Effects of 4-MBC on A. tonsa*

4-MBC did not affect hatching ratio in *A. tonsa* at concentrations tested in the larval development test (one-way ANOVA: F = 0.63, p = 0.679), while significant effects were observed for early-life stage survivals (one-way ANOVA: F = 68.7, $p < 0.001$) and larval development ratio (Kruskal-Wallis rank test: χ^2 = 17.02, p < 0.001), in both cases due to the relevant effects observed at the 46 μg L^{-1} concentration, in which approximately 90 % of the larvae died before the end of the exposure and none of the survived larvae reached the C–I stage (Supplementary Material – Figure S2). Dissolved oxygen and pH measured at the beginning and end of the test showed a variation within the acceptable intervals (oxygen >70 % air saturation; pH variation within one unit from initial pH) (Supplementary Material – Table S2).

In the **egg production test**, copepods recovered on day 10 had reached the adult stage in all tested concentrations ("treatments"), but at 9.3 μg L^{-1}, most of the individuals were dead or showed clear signs of narcotisation and died on day 11. In the other treatments, female mortality during the observation period (day 11- day 14) was lower than 20 % in all treatments and averaged 6 %. As in the test with BP3, the egg production began on day 11 in all the tested treatments, with a percentage of egg-lying females in the 50–90 % range, with the minimum observed in the control and the maximum at 0.1 μg L^{-1} 4-MBC. On day 12, the percentage of egg-lying females reached 100 % in all treatments, including the control (Supplementary Material – Table S3). The daily egg production showed minimum values on day 11 and peaked on day 13 in all treatments (Fig. 3, upper panel). In the control, the daily egg production ranged from 2.1 ± 0.8 egg female^{-1} d^{-1} (day 11) to 16.7 ± 2.7 egg female^{-1} d^{-1} (day 13), while in the 4-MBC treatments, the minimum and maximum productions were 3.7 ± 1.5 egg female^{-1} d^{-1} (day 11 at 0.05 μg L^{-1} 4-MBC) and 23.1 ± 2.1 egg female^{-1} d^{-1} (day 13 at 0.04 μg L^{-1} 4-MBC), respectively. As a general trend, egg production in the 4-MBC treatments was higher than in the control on days 11, 12, and 13, while on day 14 the egg production was relatively even among the treatments. The average egg production per female per day over the 96 h confirmed a slightly higher egg production in the 4-MBC treatments than in the control (Table 2). The trend observed for pellet production was very similar to that observed for egg production (Fig. 3, lower panel). Regression analysis (GLMM) evidenced a significant influence

(GLMM: $p < 0.001$) of pellet production (feeding) and day of observation on the egg production by female *A. tonsa*, with egg production on day 11 being significantly lower than on days 12, 13 and 14 (GLMM: $p < 0.001$). No effect of 4-MBC concentration on egg production was observed. Conversely, the interaction 4-MBC:day was significant (Supplementary Material, Table S4).

In the **long-term exposure test**, the mean egg production did not differ among treatments (one-way ANOVA: $F = 0.29$, $p = 0.832$), while endpoints measured on F_1 generation evidenced significant effects of 4-MBC compared to the control, but the lack of a clear effect-concentration relationship (Fig. 4). The hatching ratio was lower in the treatment at 0.25 μg L^{-1} 4-MBC than in the control and the other treatments, and the differences were statistically significant (Kruskal-Wallis rank test: $\chi^2 = 17.07$, $p < 0.001$; Wilcoxon rank sum test: $p < 0.013$). The larval survival, in contrast, was significantly higher at 2.1 μg L^{-1} 4-MBC than in the control and the lower 4-MBC treatments (Kruskal-Wallis rank test: $\chi^2 = 9.11$, $p = 0.028$; Wilcoxon rank sum test: $p < 0.041$). Finally, the larval development was lower in the control and at 0.13 μg L^{-1} 4-MBC than in the treatments at higher concentrations (Kruskal-Wallis rank test: $\chi^2 = 9.99$, $p = 0.019$). However, the differences were significant only between control and 0.13 μg L^{-1} 4-MBC (Wilcoxon rank sum test: $p = 0.034$), control and 2.1 μg L^{-1}4-MBC (Wilcoxon rank sum test: $p = 0.041$), and 0.13 μg L^{-1} 4-MBC and 2.1 μg L^{-1} 4-MBC (Wilcoxon rank sum test: $p = 0.008$).

4. Discussion

Our data on the calanoid copepod *A. tonsa* confirmed that impairments on life-history traits of marine invertebrates may occur at a wide range of BP3 and 4-MBC concentrations, depending on the tested toxicant and endpoint considered (Chen et al., 2018; Miller et al., 2021). For BP3, hatching, larval survival, and larval development of F_0 generation were not significantly affected up to 657 μg L^{-1} after 5 days of exposure. Similarly, survival was not affected after 11 days of exposure and egg production by F_0 showed no significant changes up to 576 μg L^{-1}. However, significant and pronounced effects on the F_1 generation larval development were already evident at 7.4 μg L^{-1}, although no significant effects were observed for F_1 hatching and larval survival up to 468 μg L^{-1}. Conversely, exposure to 4-MBC led to significant mortality and retarded larval development at 46 μg L^{-1}, after 5 days of exposure, and

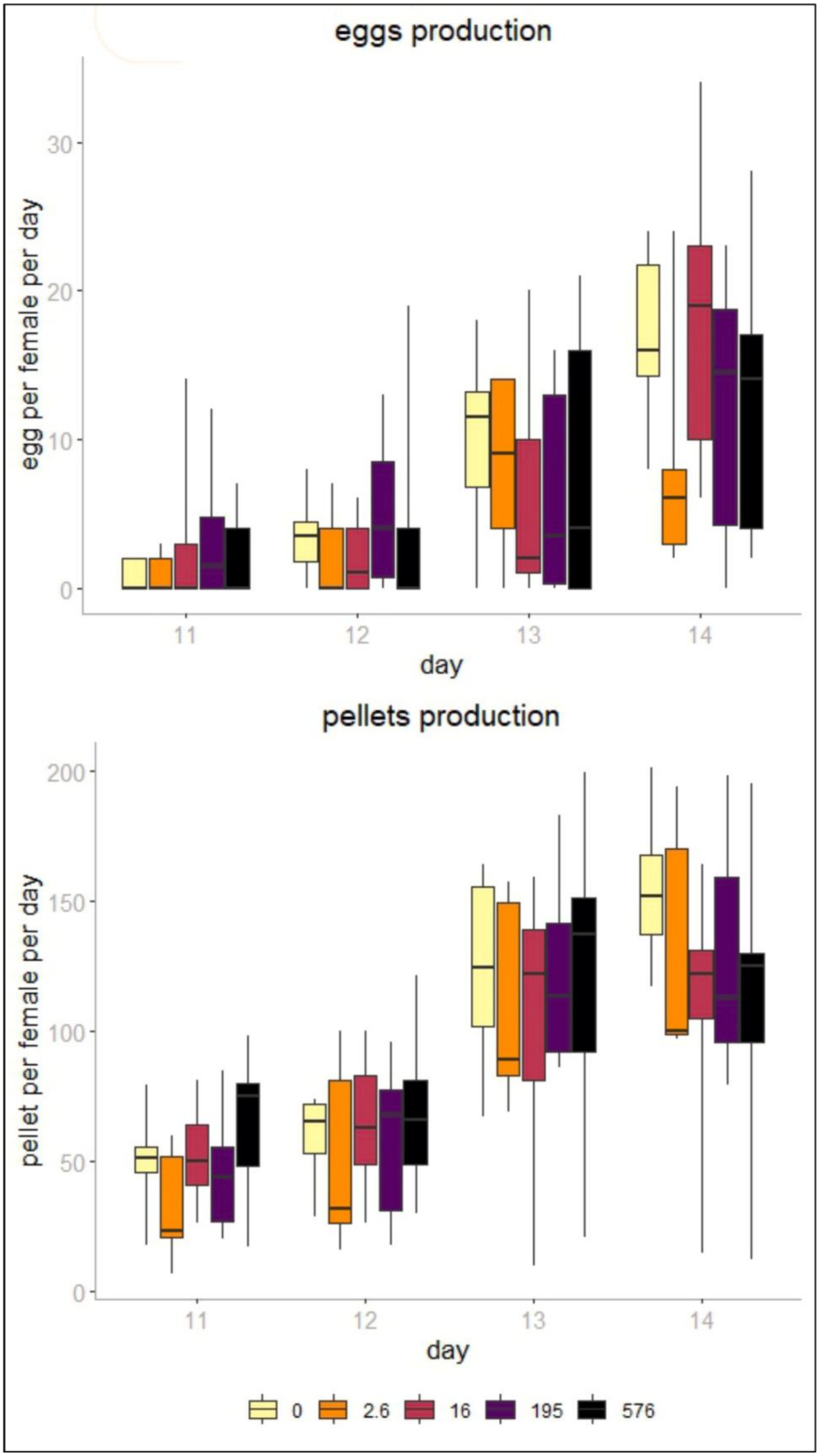

Fig. 1. Results of the egg and pellet production test performed on BP3. Concentrations are expressed as μg L^{-1}.

Table 2

Average egg and pellet production per female over the 96-h of observation. Data are reported as mean ± standard error (s.e.). The averages of the actual concentrations measured at days 0, 3, 5, 7 and 10 are reported in brackets.

	eggs female^{-1} d^{-1}	pellets female^{-1} day^{-1}
BP3 (μg L^{-1})		
Control	7.8 ± 1.2	97.8 ± 6.3
3.7 (2.6)	5.0 ± 1.7	81.3 ± 12.2
18 (16)	7.5 ± 1.9	83.1 ± 10.3
288 (195)	6.9 ± 1.5	88.2 ± 5.7
657 (576)	6.3 ± 2.0	91.8 ± 11.9
4-MBC (μg L^{-1})		
Control	11.6 ± 1.2	103.2 ± 5.7
0.04 (0.05)	14.8 ± 1.5	97.8 ± 4.7
0.11 (0.10)	14.2 ± 1.5	104.2 ± 4.7
1.4 (1.3)	13.1 ± 2.5	134.4 ± 11.4
9.4 (9.3)	–	–

Table 3

Average egg production per female in the long-term exposure test. Data are reported as mean ± standard error (s.e.). The averages of the actual concentrations measured at days 0, 5, 9, 14, 19 and 21 are reported in brackets.

	egg female^{-1} d^{-1}
BP3 (μg L^{-1})	
Control	8.9 ± 0.9
6.4 (7.4)	10.4 ± 0.8
63 (41)	5.9 ± 1.7
590 (468)	6.7 ± 1.4
4-MBC (μg L^{-1})	
Control	7.4 ± 1.1
0.15 (0.13)	7.6 ± 1.6
0.41 (0.25)	8.4 ± 1.3
4.34 (2.13)	8.9 ± 1.3

massive adult mortality at 9.3 μg L^{-1}, after 11 days. On the other hand, in the F_1 generation, 4-MBC caused only minor effects on hatching (at 0.25 μg L^{-1}) and larval development (at 0.25 μg L^{-1}) but significantly promoted larval development at 2.1 μg L^{-1}. In addition, 4-MBC promoted egg and pellet production compared to the control after 10 days of exposure, even if the results were not statistically significant.

In the case of 4-MBC, it is difficult to make predictions on its impact on copepods due to the generally lower environmental concentrations reported in the literature for this filter compared to BP3 and other compounds (Cadena-Aizaga et al., 2022a; Mitchelmore et al., 2021), and the alternation of inhibitory and stimulatory effects this contaminant exerted in *A. tonsa*. Conversely, for BP3, the data obtained for the F_1 generation evidenced a possible risk for adverse effects towards marine copepods. Indeed, this organic filter has often been detected in the surface water near coastal cities (Tsui et al., 2014), highly frequented beaches (Cadena-Aizaga et al., 2022a; Román et al., 2011; Sánchez Rodríguez et al., 2015), and near-reef areas (Bargar et al., 2015; Downs et al., 2015) at concentrations matching, or even exceeding, the threshold (7.4 μg L^{-1}) identified in our experiments as causing significant impairments in larval development in the F_1 generation.

Some authors have criticised the concern about the environmental impact of organic UV filters, arguing that acute toxicity tests with freshwater species reported BP3 as a low-risk chemical to aquatic ecosystems and UV filter concentrations in the sea are often much lower than those found to be effective in coral toxicity tests (Narla and Lim, 2020). Others suggest that climate change, water turbidity, eutrophication, and other contaminants are more likely responsible for coral reef decline and impacts to other aquatic ecosystems than UV filters (Wheate, 2022). Our results for BP3 support the possibility of a risk to copepod populations and highlight the potential environmental risks associated with BP3 contamination, which might affect copepod assemblages in the receiving waters and could have broader consequences at the higher organisation levels (i.e., community and ecosystem), given the crucial role played by copepods in the marine food webs.

The variability in some BP3 and 4-MBC treatments during the egg production and long-term exposure tests are difficult to interpret and can be attributed to different causes. Both compounds are known to be highly hydrophobic and resistant to photolysis, which might favour their enhancement in the short time through adsorption to particulate matter (Cooper et al., 2024). However, recent studies have identified that UV filters, including BP3 and 4-MBC, are susceptible to repartition in the atmosphere, where these compounds were detected mainly in the gaseous phase (Pegoraro et al., 2020). This suggests that evaporation may represent a possible removal mechanism for BP3 and 4-MBC, lowering their dissolved concentration in the solutions used for the tests. Further studies are needed to elucidate these compounds' possible metabolisation and accumulation in copepods throughout a toxicity test in a complex system containing copepods, algae, and bacteria (deriving from gut microbiota and faeces).

4.1. *BP3 affects the offspring of A. tonsa but not the parental generation*

Our results with *A. tonsa* indicated that BP3 caused a transgenerational effect on the offspring due to parental exposure since the generation F_1 was tested in a non-contaminated medium. Furthermore, since larval mortality was not observed in the offspring, the extent of the disturbance was low enough not to impair survival but high enough to delay their development to the C–I stage. A similar effect was observed in *A. tonsa* exposed to exhaust gas cleaning system discharges (Picone et al., 2023).

However, identifying the toxicity pathway behind the observed effects of BP3 on *A. tonsa* offspring is complex due to various biochemical and physiological traits that organic UV filters may alter in invertebrates. For example, BP3 is recognised to act as an endocrine disruptor (Carve et al., 2021). In particular, in dipterans, BP3 acts as an ecdysone-mimic toxicant, affecting larval development in the parental generation and impairing hatching and survival of offspring (Campos et al., 2019; Ozáez et al., 2016). Nevertheless, larval development of F_0 and hatching and survival of F_1 were not affected in *A. tonsa* at the tested BP3 concentrations (Supplementary Material – Figure S1 and Fig. 3). Consequently, the endocrine disruption is not probable as a potential pathway, even if only specific assays for potential ecdysteroid and juvenile hormone agonistic/antagonistic effects would clarify this aspect (Wollenberger et al., 2005b). Furthermore, BP3-induced damage to the gonads and alteration of mating can be excluded as potential mechanisms of action since parents exposed to BP3 produced viable eggs, and the hatched larvae survived up to the end of the exposure (Figure S3).

The causes of the retarded larval development in generation F_1 are probably to be identified in the lying of poor-quality eggs by parents exposed to BP3. Adult *A. tonsa* do not accumulate reserve in their body but invest their lipid metabolic production in egg lying (Jónasdóttir, 1994). Then, exposed parents might have deposited fewer lipids in their eggs due to increased energy costs for BP3 detoxification and elimination. As a result, their offspring could have limited energy reserve for growth, as the earlier naupliar stages do not feed and depend almost entirely on the lipid reserves provided by the mother (Landry, 1983). This hypothesis is consistent with the knowledge on BP3 metabolism in animals: it may undergo phase I and phase II biotransformation before elimination and is known to generate oxidative stress (Chaves Lopes et al., 2020; Kim and Choi, 2014). Consequently, exposure to BP3 might increase the costs for somatic maintenance (i.e., antioxidant defence) and detoxification (i.e., induction of phase I and phase II enzymes) for the exposed individuals. In copepods, it might result in less energy allocable to fuel the early nauplii (Klok et al., 2012). Future research

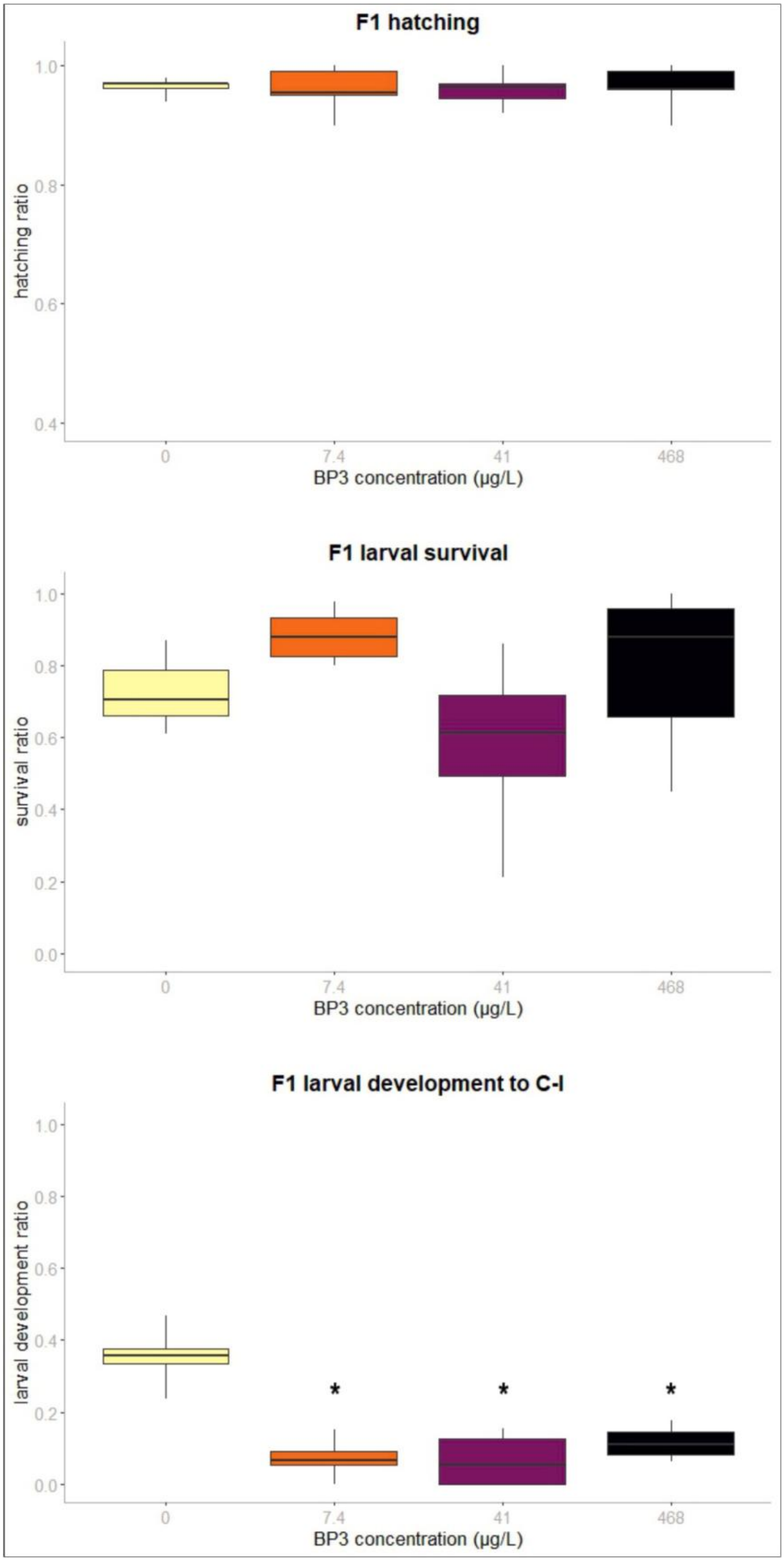

Fig. 2. Results of the larval development test on generation F_1 performed on BP3 after the long-term exposure of generation F_0. Asterisks designate BP3 treatments statistically different from control after one-way ANOVA or Kruskal-Wallis non-parametric test.

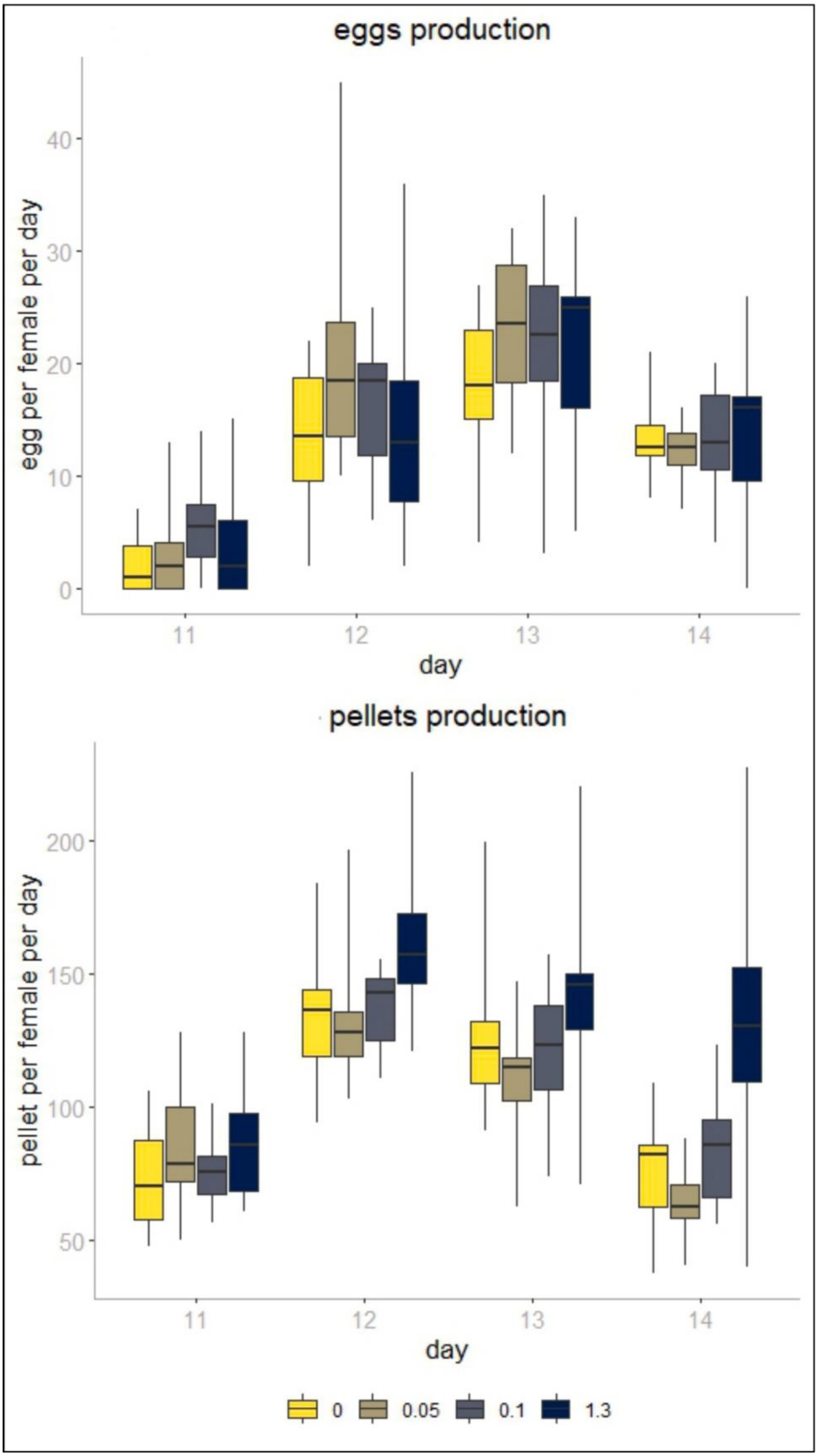

Fig. 3. Results of the egg and pellet production test performed on 4-MBC. Concentrations are expressed as μg L^{-1}.

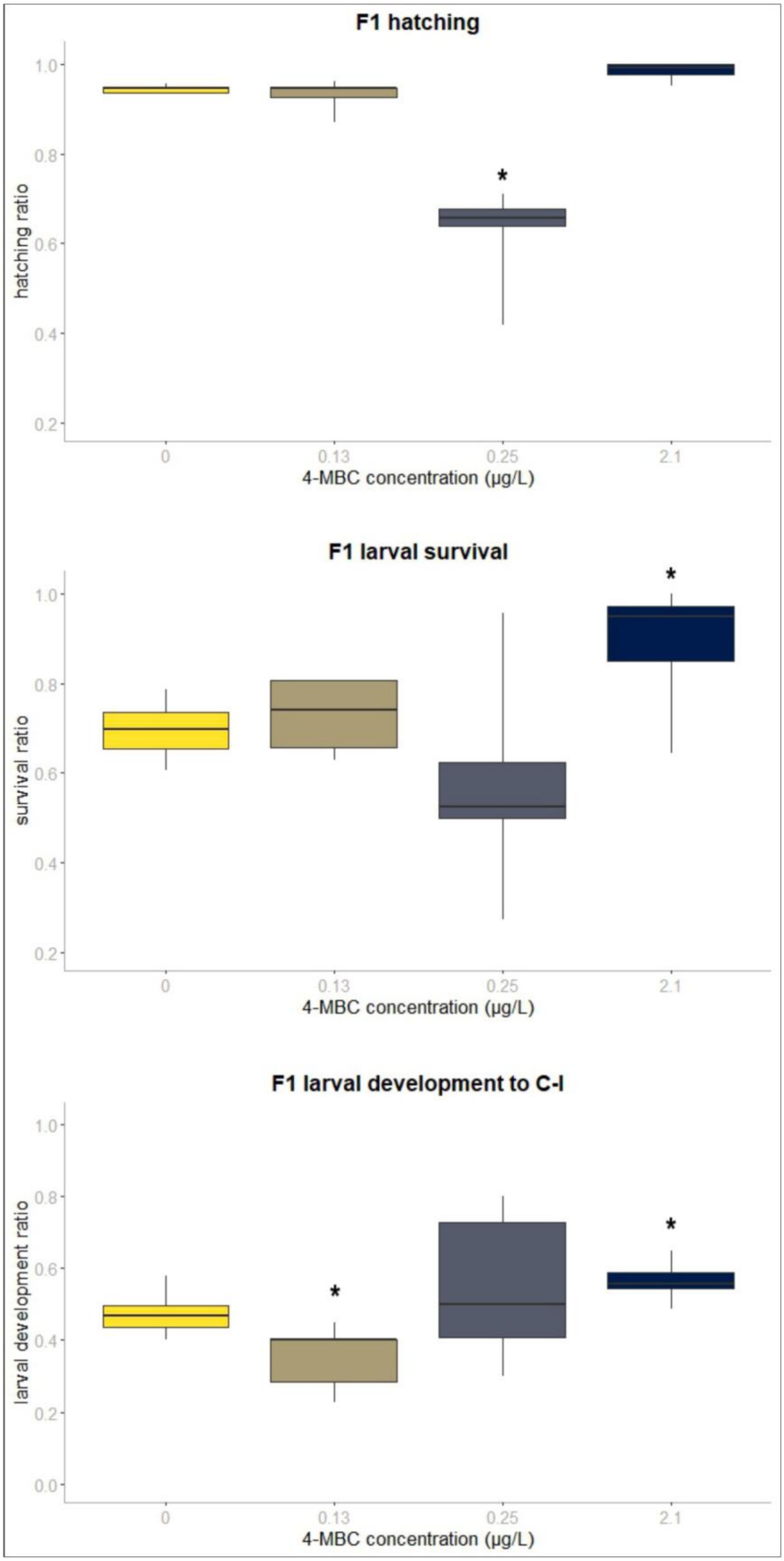

Fig. 4. Results of the larval development test on generation F_1 performed on 4-MBC after the long-term exposure of generation F_0. Asterisks designate 4-MBC treatments statistically different from control after one-way ANOVA or Kruskal-Wallis non-parametric test.

using biochemical oxidative markers may confirm this hypothesis.

A further possible cause of the retarded development in F$_1$ is the maternal transfer of lipid-associated BP3 or its phase I and phase II metabolites into the eggs during vitellogenesis (Niehoff, 2007). In this case, the contaminants associated with the lipids might be remobilised during the fuelling of the larvae, becoming available to the developing nauplii and affecting their development by impairing their energy allocation for growth or direct toxic effects on their tissues and organs.

4.2. 4-MBC is more toxic than BP3 for F$_0$, but has no adverse effects on F$_1$ at environmental concentrations

The results obtained with 4-MBC are more complicated to analyse since this compound caused both inhibitory and stimulatory effects in *A. tonsa. In the larval development and egg production tests,* 4-MBC exerted severe lethal effects on F$_0$ larval stages and adults at concentrations higher than 9.4 µg L^{-1}, resulting in more acutely toxic to copepods than BP3. However, these lethal effects were observed at concentrations higher than those currently measured in the surface waters, spanning from 0.0001 µg L^{-1} to 1 µg L^{-1} (Cadena-Aizaga et al., 2022b; Mitchelmore et al., 2021). Consequently, 4-MBC-mediated lethal effects are currently unlikely in aquatic ecosystems. On the other hand, the stimulatory effects observed in the larval development of the F$_1$ generation suggest that 4-MBC might act as an endocrine interferent in the moult and reproduction of *A. tonsa.* These effects should be carefully considered since they might occur at concentrations matching those reported in treated and untreated wastewater discharges (Balmer et al., 2005; Emnet et al., 2015) and in recreational basins (O'Malley et al., 2021).

A 4-MBC-mediated disruption of the endocrine regulation of moult and reproduction in copepods was already observed in the harpacticoid *Tigriopus japonicus* (Chen et al., 2018). In particular, a multigenerational exposure of *T. japonicus* to 4-MBC in the range 0.5 to 10 µg L^{-1} resulted in decreased times to the metamorphosis to copepodite and to maturity for generation F$_0$ to F$_3$, as well as in the up-regulation of the *EcR* gene, involved in the moulting regulation in arthropods (Chen et al., 2018). However, we have not observed an increased ratio copepodites:nauplii in the larval development test with F$_0$, which suggests that a decreased time to maturity is not a factor for the F$_0$ exposure. Nevertheless, a significantly higher C−I ratio was observed in F$_1$ at 2.1 µg L^{-1} 4-MBC compared to control and treatment at 0.13 µg L^{-1} 4-MBC. These results suggest a possible toxicant-induced reduced time to metamorphosis for the offspring (Fig. 4) not observed in the parental generation, thus a potential transgenerational 4-MBC-mediated endocrine disruption of the growth in *A. tonsa*. Conversely, in *A. tonsa* we did not observe any effect on F$_1$ hatching, as reported for *T. japonicus*, underlining different sensitivity to 4-MBC despite a possibly shared toxicity pathway in the two species.

5. Conclusions

The UV filters BP3 and 4-MBC can disrupt critical life-history traits in the calanoid copepod *A. tonsa*. We found that the two chemicals exert different effects on the test species. Our results suggest that categorizing the environmental hazard of 4-MBC is more challenging, and that further studies, possibly involving biochemical markers and endocrine activity assays, are needed to identify the toxicity pathways underneath the observed stimulation and the potential effects at the population level. On the contrary, the observed adverse effects of BP3 at low concentrations provide further support for the decisions by some national administration to ban or restrict its use. Overall, our research provided useful insights into the ecological risks posed by these UV filters, highlighting the need for more comprehensive studies to fully understand their environmental impact and guide future regulatory decisions.

CRediT authorship contribution statement

Marco Picone: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Del Vecchio:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Maria Pirvu:** Investigation, Formal analysis, Data curation. **Marco Vecchiato:** Writing – original draft, Investigation, Formal analysis, Data curation. **Annamaria Volpi Ghirardini:** Writing – review & editing, Supervision.

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.

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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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.117952.

Data availability

Data will be made available on request.

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