Aquatic Toxicology 194 (2018) 94–102

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Research Paper

Multigenerational effects of 4-methylbenzylidene camphor (4-MBC) on the survival, development and reproduction of the marine copepod *Tigriopus japonicus*

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ARTICLE INFO

Keywords:
4-Methylbenzylidene camphor
Aquatic toxicity
Multigenerational toxicity

ABSTRACT

One of the most widely used organic UV filters, 4-methylbenzylidene camphor (4-MBC), is present at high concentrations in offshore waters. The marine copepod *Tigriopus japonicus* was exposed to different concentrations of 4-MBC (i.e., 0, 0.5, 1, 5 and 10 μg L^{-1}) for 4 consecutive generations (F0-F3) to evaluate the impact of 4-MBC on marine ecosystems. The results showed that in the F0 generation, 4-MBC caused significant lethal toxicity in *T. japonicas* at concentrations of 5 and 10 μg L^{-1} and the nauplii were more sensitive to 4-MBC toxicity than the adults. However in the F1-F3 generations, 4-MBC exposure did not affect the survival rate. The hatching rate and the developmental duration from the nauplii to the copepodite (N-C) and from the nauplii to adult (N-A) decreased significantly in the F1-F2 generations and in the F2-F3 generations, respectively, even at the lowest exposure concentration (0.5 μg L^{-1}). In the subsequent two generations (i.e., the F4-F5 generations) of recovery exposure in clean seawater, the growth rates of the original 4-MBC exposure groups were still faster than the control in both the N-C and N-A stages, suggesting possible transgenerational genetic and/or epigenetic changes upon chronic 4-MBC exposure. The expression of the ecdysone receptor gene was up-regulated by 4-MBC, which was consistent with the decrease of the N-C/N-A duration. In addition, 4-MBC may induce oxidative stress and trigger apoptosis in *T. japonicas*, resulting in developmental, reproductive and even lethal toxicity. A preliminary risk assessment suggested that under environmentally realistic concentrations, 4-MBC had significant potential to pose a threat to marine crustaceans and marine ecosystems.

1. Introduction

Organic ultraviolet (UV) filters are substances designed to absorb harmful UV light and are widely used in personal care products (PCPs; e.g., sunscreens, shampoos, fragrances, cosmetics, soap, etc.) to protect the skin from UV irradiation. Because these filters are photostable and difficult to be oxidized, they are also added as stabilizers to food packaging materials, paints, textiles and other materials that must be protected from sunlight (Broniowska et al., 2016). Recently, the production volume of organic UV filters has increased rapidly (Ozáez et al., 2013) due to their extensive use in PCPs and many other commercial products. Personal hygiene practices (e.g., washing, showering and laundering) and sewage treatment plant effluents have led to the great entry of these substances into aquatic environments (Schmitt et al., 2008; Sieratowicz et al., 2011). As a result, these compounds are frequently detected in aquatic systems. Recent studies indicate that UV filters are considered to be ubiquitous environmental contaminants of increasing concern, due to their bioaccumulation potential and the suspicion that they may act as endocrine disruptors (Ozáez et al., 2013; Schmitt et al., 2008).

One of the most widely and frequently used organic UV filters is 4-methylbenzylidene camphor (4-MBC) (Wahie et al., 2007). The usual concentrations of 4-MBC in the cosmetic products are in the ranges of 0.5–4% (Orsi et al., 2006). It is difficult to be removed completely by sewage treatment processes and the removal efficiency is only in a range of 38%–77% (Tsui et al., 2014a). In addition to sewage treatment plant effluents, human hygienic practices are an important route for 4-MBC to directly enter the surface waters. Various environmental samples in many countries have been frequently found to contain 4-MBC, including the surface waters, wastewaters, sediments and aquatic organisms (Buser et al., 2006; Fent et al., 2010; Liu et al., 2011; Tsui et al., 2014b). For example, concentrations of 4-MBC were detected in a range

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https://doi.org/10.1016/j.aquatox.2017.11.008
Received 26 September 2017; Received in revised form 13 November 2017; Accepted 15 November 2017
Available online 16 November 2017
0166-445X/

of 173–379 ng L^{-1} and 346–1043 ng L^{-1} in the surface seawater at the recreational beaches in Hong Kong and Gran Canaria Island, respectively (Rodríguez et al., 2015; Tsui et al., 2014b). In the Turia River in Spain and Lake Cospuden in Germany, which are both recreation resorts, the concentrations of 4-MBC can reach 246 ng L^{-1} and 2592 ng L^{-1}, respectively (Moeder et al., 2010; Román et al., 2011). The compound 4-MBC is lipophilic with an octanol-water partition coefficient (log K_{ow}) of 4.95 (Gago-Ferrero et al., 2011), and therefore tends to accumulate in the sediment (Pintado-Herrera et al., 2017; Ramos et al., 2015) as well as in the biota (Liu et al., 2011, 2012). Concentration as high as 1800 ng g^{-1} lipid weight was found in fish collected from the Langete River in Switzerland (Buser et al., 2006).

Previous toxicological studies on 4-MBC have mainly focused on its endocrine disrupting effects in mammals, e.g., humans and rats. Only recently was the toxicity of 4-MBC evaluated in aquatic species. For example, 4-MBC exposure of freshwater zebrafish (*Danio Rerio*) and medaka (*Oryzias latipes*) reduced the hatching rate and caused estrogenic effects at concentrations above 1 mg L^{-1} (Inui, 2003; Torres et al., 2016). However, in aquatic invertebrates such as the freshwater midge *Chironomus riparius*, 4-MBC did not induce the expression of estrogen receptor genes, while the ecdysone receptor (EcR) gene was significantly up-regulated (Ozáez et al., 2013). In addition, 4-MBC exposure affected the development and reproduction of aquatic invertebrates. For instance, reduced body length of freshwater *Daphnia magna* and decreased reproduction of oligochaete *Lumbriculus variegatus* have been observed at the lowest observable effect concentrations (LOEC) of 200 μg L^{-1} and 6.18 mg kg^{-1} sediment (dry weight), respectively (Schmitt et al., 2008; Sieratowicz et al., 2011).

Although the toxicity of 4-MBC has been tested in several above-mentioned freshwater species, its toxicity in marine organisms is largely unknown. Short term exposure (48–96 h) to 4-MBC inhibited the growth of the microalgae *Isochrysis galbana*, induced mortality in mysid *Siriella armata* and caused malformations in mussel *Mytilus galloprovincialis* at LOECs of 75, 74 and 600 μg L^{-1}, respectively (Paredes et al., 2014). The toxicity of 4-MBC was also evaluated in sea urchin embryos (*Paracentrotus lividus*) in two different laboratories. Their results showed that after 48-h exposure, 4-MBC inhibited the growth and reduced the body length of *P. lividus* at 600 and 2 μg L^{-1}, respectively (Paredes et al., 2014; Torres et al., 2016). Other than these studies, very few others have evaluated toxicity, particularly the chronic toxicity of 4-MBC in marine species, although relatively high concentrations of 4-MBC have been frequently detected in seawaters (Román et al., 2011; Tsui et al., 2014b).

The marine copepod *Tigriopus japonicas* is widely distributed in the coastal areas of western Pacific regions (Guo et al., 2012). This copepod species has several advantages as a good model species for estuarine and marine toxicological studies: the different developmental stages and genders have distinctive characteristics; it has high fecundity, and a high tolerance to a wide range of temperature and salinity (Lee et al., 2008). Moreover, *T. japonicas* is easily maintained in the laboratory and has a relatively short life-cycle, so multi-generational experiments are feasible (Lee et al., 2008; Li et al., 2015; Hong et al., 2017).

In the present study, we investigated the multigenerational effects of 4-MBC on the survival, development and reproduction of *T. japonicas*. In addition, the transcription of genes related to oxidative stress, apoptosis and ecdysis was investigated to shed light on the potential toxic mechanisms of 4-MBC in marine copepods.

2. Materials and methods

2.1. Chemicals and reagents

The compound 4-MBC was purchased from the Tokyo Chemical Industry Co. (Tokyo, Japan); and the organic solvents (methyl alcohol, dichloromethane, acetonitrile) used for sample extraction and LC–MS/MS analysis were from Tedia Inc. (Fairfield, Ohio, USA). All other reagents were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA) except where indicated.

2.2. Copepod maintenance

T. japonicus was originally collected from Xiamen Bay (Guo et al., 2012), and a stock culture was maintained in 0.22 μm-filtered artificial seawater (salinity 30‰) in the laboratory (21 ± 1 °C, 14 h:10 h light-dark cycle) for more than five years before the experiments (Hong et al., 2017; Shi et al., 2017). An equal mixture of four algal species, *Platymonas subcordiformis*, *Chlorella pyrenoidosa*, *Dicrateria zhanjiangensis* and *Phaeodactylum tricornutum* was provided as daily food. The algae were cultured at 21 ± 1 °C, under a 14 h:10 h light:dark cycle in the f/2 medium using filtered (0.22 μm) and sterilized seawater. The conditions for copepod maintenance in the toxicity tests were the same as described above except where indicated.

2.3. 72 h acute immobilization toxicity test

T. japonicus adults were used in a standard static 72 h immobilization toxicity test. The copepods were exposed to nominal concentrations of 10, 50, 150, 1000, and 5000 μg L^{-1} (0.039, 0.20, 0.59, 3.9, 20 μM) 4-MBC in seawater. The solvent control group received the same concentration of DMSO as the treatment groups (0.04%, v/v). For each treatment group, 12 copepods were transferred by pipette to each well of a 12-well culture plate containing 3 mL of exposure media. The test solutions (> 80% of the working volume) were renewed every 48 h, and the immobilization of copepods was checked every day.

2.4. Multigenerational toxicity test

The nominal concentrations in the multigenerational toxicity test were 0, 0.5, 1, 5 and 10 μg L^{-1} (0, 2.0, 3.9, 20, 39 nM), all of which were below the LOEC in the 72 h acute toxicity test. The solvent control group received the same concentration of DMSO as the treatment groups (0.01%, v/v).

To start the first-generation life-cycle test (F0), 20 newly hatched nauplii (< 24h after hatching) per replicate were transferred to 5 mL glass beakers containing 3 mL of exposure media, and five replicates were included in each exposure group. Test solutions were freshly prepared and renewed daily (> 80% of the working volume) to maintain consistent concentrations of 4-MBC. *P. subcordiformis* was centrifuged to remove the culture medium and added at a density of 2×10^4 cells L^{-1} for the nauplii and at 2×10^5 cells L^{-1} for the copepodites. The nauplii were cultured under the previously mentioned conditions until all nauplii developed to the copepodite stage. Copepodites were transferred to a 50 mL glass beaker containing 20 mL test solution.

In total, five life history traits were recorded in the present study, i.e., the survival rate, the time necessary for nauplii to develop to copepodites (N-C), the time necessary for nauplii to develop to adults with egg sacs (N-A), the number of eggs produced in 10 days per female copepod and the hatching rate (i.e., the number of nauplii hatched out/total number of eggs produced per female copepod x 100%) (Fig. 1). The developmental stages and mortality were checked daily under a stereomicroscope and the N-C and N-A times were calculated. The survival rate was calculated after the maturation of all copepods. To measure the number of eggs produced in 10 days per female copepod and the hatching rate, ten females bearing an egg sac from each exposure group were individually transferred to 12-well culture plates containing 3 mL media per well. These females were cultured under the previously mentioned conditions for 10 days. The test solutions were renewed and *P. subcordiformis* was provided at a density of 2×10^4 cells L^{-1} everyday. The resulting nauplii and unhatched clutches were counted daily under the stereomicroscope.

For a life-cycle toxicity test of subsequent generations (i.e., F1, F2,

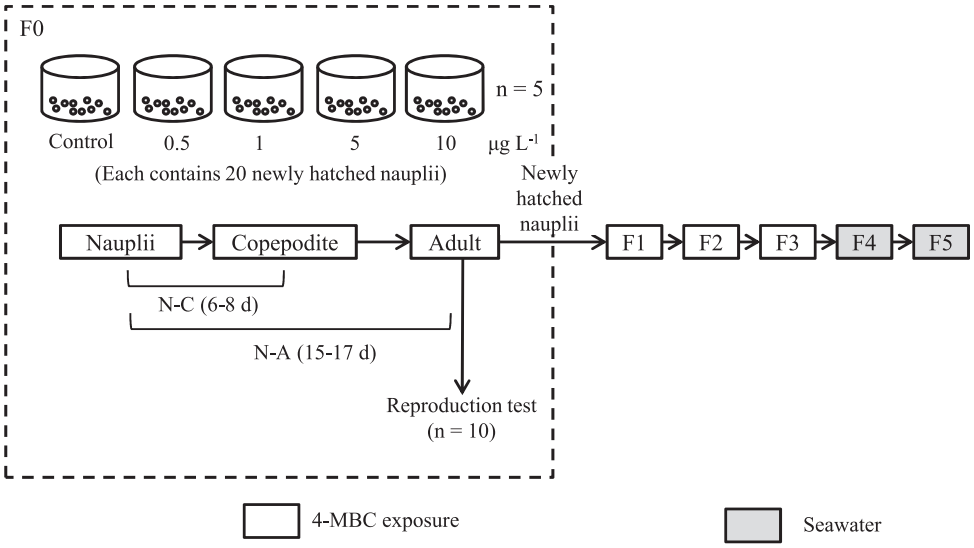

Fig. 1. Experimental design for multigenerational exposure. The F0 generation experiment started with exposure using newly hatched *T. japonicus* nauplii (< 24 h) collected from the stock culture maintained in the laboratory. *T. japonicus* were exposed to different 4-MBC concentrations (0, 0.5, 1, 5 and 10 μg L^{-1}) for 4 generations (F0-F3) and then transferred to clean seawater and the exposure was continued for another 2 generations (F4-F5).

F3), 20 nauplii hatched out in the first brood by the female copepods in the parent generation were transferred to a new glass beaker as one replicate. The experimental and exposure conditions were the same as those used in the F0 generation test.

2.5. Two generations of recovery test

To test whether the effects of 4-MBC on the life history traits of *T. japonicus* are lost when a population exposed to 4-MBC for multiple generations is recovered in a clean environment, we cultured the nauplii hatched out by the F3-generation copepods in clean seawater for two consecutive generations (i.e., the F4 and F5 generations) (Fig. 1). For each F4 and F5 generation, 20 nauplii from the parent generation (< 24 h) per replicate were transferred to 5 mL glass beakers containing clean seawater and each treatment had five replicates. The same experimental procedures were followed as in the previously mentioned multigenerational toxicity tests, and the same life history traits were observed and recorded.

2.6. Measurement of 4-MBC concentrations in the exposure media of multigenerational toxicity test

To determine the actual concentrations of 4-MBC in the exposure media in the multigenerational toxicity test, freshly prepared exposure media (T0) and media after 24 h exposure (T24) were collected for analysis. An aliquot of 10-mL of each medium sample was loaded onto an Oasis® HLB SPE column (Water Corporation, Milford, Massachusetts USA), which was preconditioned with methanol, dichloromethane and water to retain the organic compounds. After desalting the column with 6 mL H_2O, the organic fractions were eluted with 9 mL of a dichloromethane: methanol mixture (1:1, v/v), and the eluate was concentrated to 0.5 mL under a steady N_2 flow.

The quantification of 4-MBC was carried out on an Agilent 1290–6490 UPLC-triple quadruple mass spectrometry system (Agilent Technologies, Palo Alto, California, USA). The samples were loaded onto a Hypersil GOLD HPLC column (2.1 mm × 100 mm, particle size 1.9 μm, Thermo Scientific). The mobile phases consisted of two solvents: mobile phase A (0.1% formic acid in water, v/v) and mobile phase B (acetonitrile). The linear gradient program used for HPLC separation was as followed: isocratic at 5% B for 2 min (0–2 min), 5% to 100% B (2–10 min), isocratic at 100% B (10–12 min), and re-equilibration at 5% B for 3 min. The flow rate was 0.3 mL min^{-1}, and the LC eluate was directed to a triple quadruple mass spectrometer equipped with an electrospray ionization probe operated in a positive ion-mode. The compound 4-MBC was monitored in the multiple reaction monitoring mode with the transition event at m/z 255.0 > 105.0.

A 5-point calibration (i.e., 10, 100, 500, 1000, 1500 ng mL^{-1} in methanol) was performed to confirm the linearity of the mass spectrometer response, and a fine calibration curve was obtained with R^2 > 0.99. The procedural blanks that were included in each experimental run to confirm that no contamination was introduced via glassware or reagents contained no detectable 4-MBC. The recovery of 4-MBC was 88% in the spiked seawater blanks. The relative standard deviation was less than 20% in 3 sample replicates.

2.7. Real-time quantitative PCR (qPCR)

To investigate its toxic mechanism, a separate exposure was conducted using adult *T. japonicus* which were exposed to 0, 1 and 10 μg L^{-1} of 4-MBC. Each exposure replicate was cultured in a glass tank containing 600 mL of exposure media and four replicates were included in each group. The solvent control received the same concentration of DMSO as the treatment groups (0.01%, v/v). The exposure media were freshly prepared and renewed every 24 h. After 7 and 14 days exposure, 200 copepods per sample were collected, frozen immediately in liquid nitrogen and stored at −80 °C until further analysis.

Total RNA was extracted and an equal amount of RNA was reverse-transcribed using mixtures of oligo (dT) primer (5′-TTTTTTTTTTTTTTTTTTTT-3′) and random primers using M-MLV reverse transcriptase (BGI, Shenzhen, China) to generate cDNA. A CFX96™ Real-Time System (Bio-Rad Laboratories) using SYBR Green I was employed to carry out qPCR. The sequence of the primers for the qPCR of the superoxide dismutase (SOD), glutathione S-transferase (GST), P53, 8-oxoguanine DNA glycosylase (OGG1), ecdysone receptor (EcR), vitellogenin (VTG) and actin genes were based on published papers (Hwang et al., 2010; Kim et al., 2012; Rhee et al., 2013). The thermal cycling program consisted of an initial denaturation step at 95 °C for 3 min, followed by 50 cycles of 95 °C for 10 s and 65 °C for 35 s. A dissociation curve analysis was performed to confirm that only the targeted PCR product was amplified and detected. Two replicates of qPCR were performed for each sample as technical replicates. The transcription levels of the tested genes were analyzed using the 2- DΔCt method and then normalized to the levels of actin mRNA.

2.8. Data analysis

A statistical analysis of the toxicity was conducted using Sigma Plot

v.10.0 (Systat Software, Inc.) and SPSS Statistics 21.0 (IBM Software, Columbus, Ohio, USA). The significance of the differences was analyzed by a one-way analysis of variance (ANOVA) combined with an LSD test. Significant differences from the control are shown in tables and figures using asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The LC_{50} were calculated using the Probit analysis method conducted by SPSS Statistics 21.0.

3. Results

3.1. 72 h acute toxicity test

The survival rate of *T. japonicus* in the control group was > 90% and the mortality rate increased in a dose-dependent manner. At the two highest concentrations, i.e., 1000 and 5000 μg L^{-1}, 4-MBC caused 100% mortality. The LC_{50}, LOEC and NOEC (no observed effect concentration) of 4-MBC induced lethal toxicity in the 72 h acute toxicity test were 92.9, 10 and < 10 μg L^{-1}, respectively.

3.2. Multigenerational toxicity test (F0-F3 generations)

The multigenerational test was conducted at nominal 4-MBC concentrations of 0, 0.5, 1, 5 and 10 μg L^{-1}. The actual concentrations of 4-MBC in the control group media were below the detection limit. The measured concentrations were comparable to the nominal concentrations for all the 4-MBC treatment groups (Table 1). In addition, the measured concentrations of 4-MBC in the freshly prepared exposure media (T0) and in the media after 24 h exposure (T24) were very similar (Table 1), suggested that 4-MBC was not significantly degraded during the 24 h exposure period, which met the requirements of OECD Guidelines for Testing of Chemicals (OECD 211).

The survival rate of *T. japonicus* decreased with an increase in concentration in the first generation (F0 generation, Fig. 2A). Exposure to 1, 5 and 10 μg L^{-1} of 4-MBC gave rise to a cumulative mortality of 40%, 43% and 52%, respectively, by the end of the F0 generation (Fig. 2B). The LC_{50}, LOEC and NOEC of lethal toxicity in the F0 generation test were 8.5, 5 and 1 μg L^{-1}, respectively. In addition, mortality of the F0 generation frequently occurred in the first five days which were during the nauplii stage, and no obvious mortality was observed during the copepodite and adult stages, indicating that the nauplii were more sensitive to 4-MBC than the copepodites and adults (Fig. 2B). In the subsequent generations, i.e., F1-F3, the survival rates of *T. japonicus* in all the treatment groups were above 90% and did not show significant differences among groups (Fig. 2A).

In the control groups, the average time for nauplii to develop to copepodites (N-C) and for nauplii to develop to adults (N-A) in *T. japonicus* was 7.7 and 16.1 days, respectively. In the F0 and F1 generations, 4-MBC slightly decreased the N-C and N-A duration for approximately 1 day (Fig. 3), but only a few treatments showed statistically significant difference from the control. Stimulation of the growth rate by 4-MBC was much more obvious in the F2 and F3 generations, in which both the N-C and N-A development times in all of the 4-MBC treatment groups significantly decreased by approximately 2 days compared to the control, and the LOECs was 0.5 μg L^{-1} (Fig. 3, $p < 0.001$). Greater and more highly significant stimulation of the growth rate in the F2-3 generations than in the F0-1 generations (Fig. 3) suggested that the growth rate of *T. japonicas* was more sensitive to 4-MBC after multigenerational exposures.

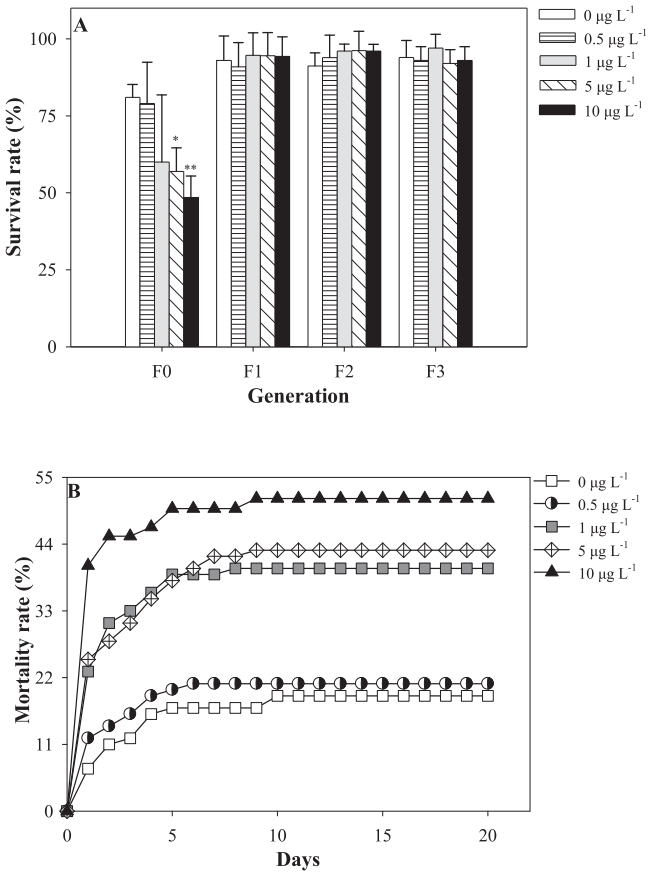

Fig. 2. Survival rates of *T. japonicus* in the F0-F3 generations (A) and the cumulative mortality rate in the F0 generation (B). *T. japonicus* was exposed to different 4-MBC concentrations (0, 0.5, 1, 5 and 10 μg L^{-1}) for four consecutive generations. Values that are significantly different from the control are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 5).

The number of eggs produced in 10-days per female in the control group was greater than 90 and the hatching rate was over 90% in the F0-F3 generations (Fig. 4). Exposure to 4-MBC significantly decreased the number of eggs produced in the F0 generation in the 10 μg L^{-1} treatment group, in which the number of eggs produced was reduced to 65 ± 16 (Fig. 4A, $p < 0.01$). However, in the F1-F3 generations, 4-MBC did not reduce the number of eggs produced as obviously as it did in the F0 generation (Fig. 4A). 4-MBC hardly affected the hatching rate in the F0 generation, though it had an apparent negative effect on the hatching rate in the F1 and F2 generations (Fig. 4B). At the two highest doses, i.e., 5 and 10 μg L^{-1}, the hatching rate in these two generations was reduced to approximately 70%. The hatching rate decreased significantly (Fig. 4B, $p < 0.01$) even at the lowest exposure concentration (0.5 μg L^{-1}). However, the negative effect on the hatching rate was alleviated in the F3 generation.

Table 1
Measured concentrations of 4-MBC in freshly prepared exposure media (T0) and in the media after 24 h exposure (T24) in the multigenerational toxicity test.

Nominal concentration (μg L^{-1})	**0.5**	**1**	**5**	**10**
T0 (μg L^{-1})	0.34 ± 0.07	0.70 ± 0.13	3.72 ± 0.27	9.56 ± 0.73
T24 (μg L^{-1})	0.38 ± 0.10	0.67 ± 0.03	3.77 ± 0.46	7.74 ± 0.88

3.3. Gene transcription induced by 4-MBC

The transcription of the oxidative stress biomarker genes SOD, GST and OGGT in *T. japonicus* generally increased in a dose dependent manner after exposure of *T. japonicus* to 4-MBC for 7 and 14 days (Fig. 5A–C). The transcription of OGG1, a gene responsible for the repair of oxidative DNA damage, was up-regulated by more than two-fold at the highest dose (10 μg L^{-1}) after 7-day exposure. Consistent with the up-regulation of oxidative stress responsive genes, the transcription

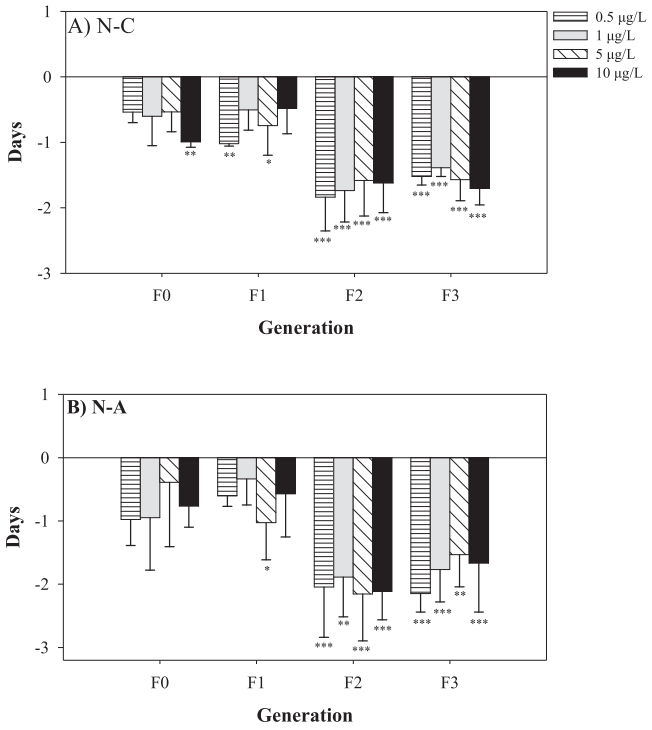

Fig. 3. Effects of 4-MBC exposure on the developmental duration of the nauplius phase (A) (from newly hatched nauplii to the copepodite, N−C) and maturation period (B) (from newly hatched nauplii to adults bearing egg sacs, N-A) in *T. japonicas* in the F0-F3 generations. *T. japonicus* was exposed to different 4-MBC concentrations (0.5, 1, 5 and 10 μg L^{-1}). The reduced developmental time compared to the control are shown and the data are expressed as the means ± SD (n = 5). Values that are significantly different from the control are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

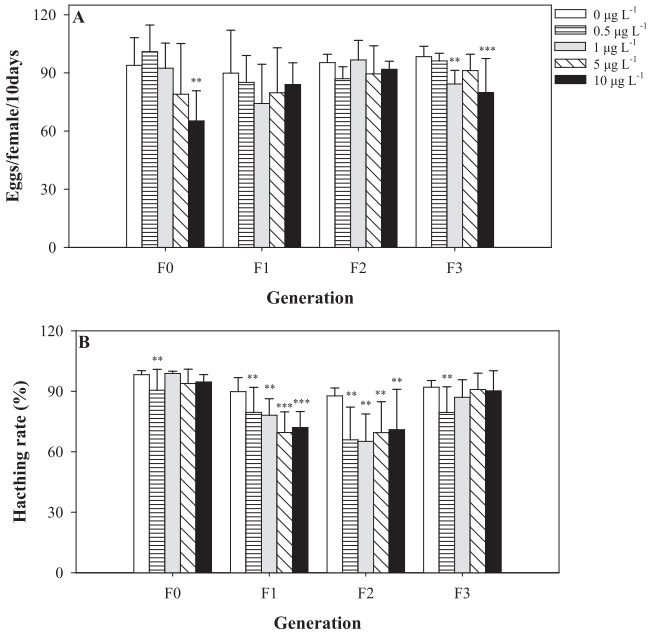

Fig. 4. Effects of 4-MBC exposure on the number of eggs produced per female in 10 days (A) and hatching rate (B) in *T. japonicas*. *T. japonicus* were exposed to different 4-MBC concentrations (0, 0.5, 1, 5 and 10 μg L^{-1}) for 4 generations (F0-F3). Data are expressed as the means ± SD (n = 10). Values that are significantly different from the control are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

of P53, a gene that plays an important role in DNA repair and apoptosis, was also significantly induced by exposure to 4-MBC (Fig. 5D). In addition, 4-MBC also significantly induced the transcription of the ecdysone receptor gene (EcR), but the transcription of VTG was not affected (Fig. 5E and F).

3.4. Two-generation recovery test (F4-F5)

After four consecutive generations of exposure to 4-MBC (F0-F3), we evaluated the survival, development and reproduction in the F4 and F5 generations, in which *T. japonicus* were exposed to clean seawater. The results indicated that the survival and reproduction (i.e., the numbers of eggs/female in 10 days and the hatching rate) in the F4 and F5 generations showed no significant differences among treatments (Table 2). Significant stimulation of the growth rate in both the N-C and N-A stages were still evident at the lower exposure concentrations, i.e., 0.5, 1 and 5 μg L^{-1} (Fig. 6). However, the developmental duration in the high dose group (10 μg L^{-1}) returned to normal and was not significantly different from the control in both the F4 and F5 generations (Fig. 6).

4. Discussion

In the present study, we evaluated the lethal, developmental and reproductive toxicities of 4-MBC in *T. japonicas* by acute and multigenerational exposure (Table 3). In the 72-h acute immobilization test, the LC_{50} of 4-MBC was 92.9 μg L^{-1} in *T. japonicas*, which was 6–8 folds lower than the values in the freshwater crustacean *Daphnia magna* (i.e., 560–800 μg L^{-1}) (Fent et al., 2010; Sieratowicz et al., 2011), suggesting that the marine crustacean species *T. japonicas* was much more sensitive to 4-MBC induced acute lethal toxicity than the freshwater species.

In the F0 generation life-cycle test, the most sensitive toxic endpoint in *T. japonicas* is mortality, as evidenced by the 43% of mortality at the LOEC of 5 μg L^{-1}, particularly during the nauplii stage (Table 3, Fig. 2). In comparison, in a one-generation toxicity test in *D. magna*, 4-MBC reduced reproduction and body length but not survival at the LOEC of 50 μg L^{-1} (Fent et al., 2010). These results also suggested that the marine crustacean *T. japonicas* was more sensitive to 4-MBC than the freshwater species *D. magna*. After exposure for more than one generation, the LOEC of a shorter developmental time (i.e., a decrease of the N-C and N-A duration) and reduced hatching rate was only 0.5 μg L^{-1}, which was much lower than the values for the F0 generation (Table 3 and Figs. 3 and 4), suggesting that the development and reproduction of *T. japonicas* became much more sensitive to 4-MBC after multigenerational exposure. It should be noted that an increasing development rate is not necessarily beneficial to the organism, the population, or the ecosystem. In the present study, we showed that the increase of the developmental rate indicated by the decrease of the N-C and N-A duration in the F0-F3 generations was accompanied by the reduction of the hatching rate, which resulted in lower reproduction (Figs. 3 and 4). It is possible that the compound 4-MBC disrupts the endocrine system in *T. japonicas*, thus affecting both development and reproduction.

To determine whether 4-MBC poses a potential threat to marine ecosystems, we performed a tentative environmental risk assessment based on the measured environmental concentration (MEC) of the Hong Kong seawater (0.379 μg L^{-1}, Tsui et al., 2014b) and the LOEC values from the multigenerational exposure in the present study (0.5 μg L^{-1}, Table 4). We derived a predicted no effect concentration (PNEC) according to European Chemicals Bureau (ECB, 2003) using the long term LOEC values and an assessment factor (AF) of 50 (PNEC = LOEC/AF). The risk classification was based on the following criteria: if MEC/PNEC < 1 no significant risk would be expected; if 1 < MEC/PNEC < 10, a small potential would exist for adverse effects; if 10 < MEC/PNEC < 100, a significant potential would exist for adverse effects; and if MEC/PNEC > 100, adverse effects would be expected (Rodríguez et al., 2015). We showed here that the calculated MEC/PNEC for 4-MBC is 37.9 (Table 4), suggesting that at

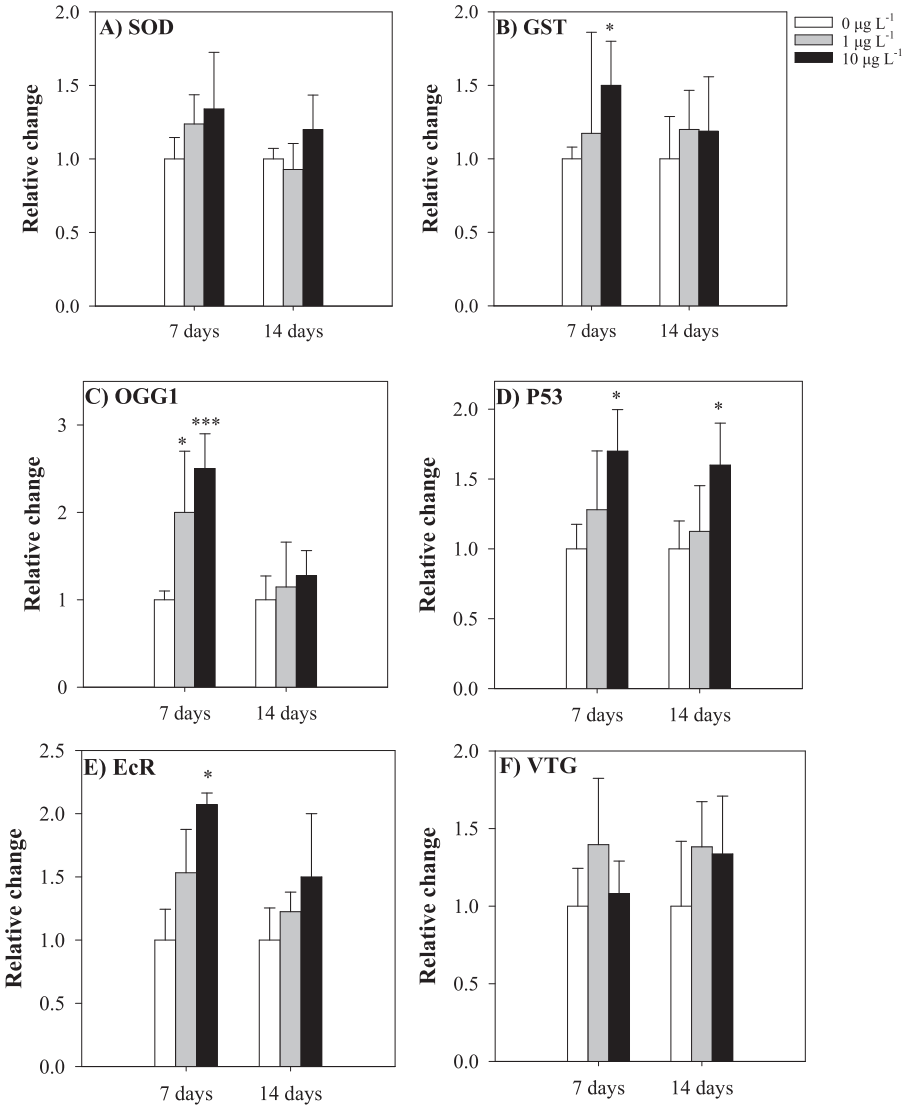

Fig. 5. Induction of the gene transcription of SOD (A), GST (B), OGG1 (C), P53 (D), EcR (E) and VTG (F) in *T. japonicus* after exposure to 0, 1 and 10 μg L^{-1} of 4-MBC for 7 and 14 days. The exposure was conducted separately from those for evaluating multigenerational toxicity. Data are expressed as the means ± SD (n = 4). Values that are significantly different from the control are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 2
Survival and reproduction of *T. japonicus* in the F4-F5 generation recovery test in which *T. japonicus* were exposed to clean seawater after four consecutive generations of exposure to different concentrations of 4-MBC (0, 0.5, 1, 5 and 10 μg L^{-1}). Data are expressed as the means ± SD (n = 5).

4-MBC exposure group in the F0-F3 generations (μg L^{-1})	Survival rate (%)		Eggs/Female/10 days		Hatching rate (%)	
	F4	F5	F4	F5	F4	F5
0	100 ± 0	96 ± 5	63 ± 10	80 ± 16	94 ± 7	99 ± 1
0.5	89 ± 10	91 ± 9	66 ± 11	72 ± 15	88 ± 11	98 ± 2
1	95 ± 7	90 ± 7	57 ± 13	70 ± 11	89 ± 10	99 ± 1
5	98 ± 3	90 ± 6	72 ± 10	65 ± 11	95 ± 6	96 ± 9
10	92 ± 8	98 ± 3	64 ± 14	74 ± 7	94 ± 12	98 ± 2

There are no significant difference among treatments.

environmentally realistic concentrations, 4-MBC has a significant potential to pose a threat to marine crustaceans and marine ecosystems.

The concentrations of UV filters in the water samples collected from some recreational beaches have seasonal variation. High concentrations were detected in the summer and usually lasted for at least three months (Bratkovics et al., 2015), which is in the same time scale as our multigenerational exposure. Moreover, in some beaches, e.g., Canary Island, the bathing season lasts nearly all year and the temporal fluctuation of UV filter concentrations is less pronounced (Rodríguez et al., 2015). As the developmental and reproductive toxicity of 4-MBC is more significant after multigenerational exposure, it is important to apply chronic toxicity data in the risk assessment of 4-MBC.

Recent studies on the estrogenicity of 4-MBC in vertebrates have raised concerns about the endocrine disruption potential of 4-MBC. For example, 4-MBC could bind to the estrogen receptor β and increase the uterine weight in rats (Schlumpf et al., 2004). Moreover, 4-MBC induced the expression of VTG in male *O. latipes*, which suggested its estrogenic activity (Inui, 2003). However, the expression of estrogen receptor or VTG was not up-regulated by 4-MBC exposure in the midge *C. riparius* (Ozáez et al., 2013), the frog *Pelophylax perizi* (Martins et al., 2017) or the copepod *T. japonicas* (the present study). Instead, 4-MBC significantly induced the expression of the EcR gene in *T. japonicus*

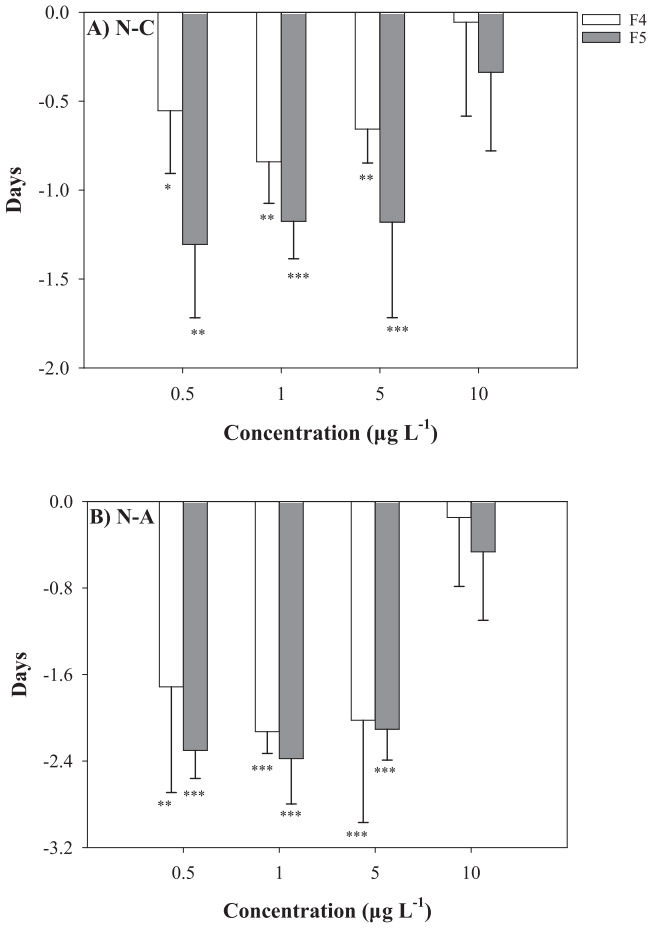

Fig. 6. The changes of the developmental duration of the nauplius phase (A) (from newly hatched nauplii to the copepodite, N-C) and maturation period (B) (from newly hatched nauplii to adult bearing egg sacs, N-A) in *T. japonicas* in the F4-5 generations, in which *T. japonicus* were exposed to clean seawater after four consecutive generations of exposure to different concentrations of 4-MBC (0, 0.5, 1, 5 and 10 μg L^{-1}). The reduced developmental time compared to the control are shown and the data are expressed as the means ± SD (n = 5). Values that are significantly different from the control are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(Fig. 5E). The molting of *T. japonicas* is an important process for its growth and development that is controlled by a cascade of hormone-receptor interactions, including EcR. In this context, the up-regulation of EcR transcription is consistent with the decrease of the N-C and N-A duration. In addition, 4-MBC exposure also stimulated the transcription of EcR in the midge *C. riparius* (Ozáez et al., 2013), further supporting that 4-MBC could up-regulate EcR in arthropods, disrupt their endocrine system and affect their molting process and development.

Furthermore, 4-MBC induced the expression of oxidative stress responsive genes (e.g., SOD and OGG1) and stress responsive genes (e.g., GST and P53) in *T. japonicas* (Fig. 5). Living organisms generate excessive reactive oxygen species (ROS) that damage important cellular components when they are exposed to certain environmental contaminants. The antioxidant enzymes are the first line of defense against ROS. SOD is an abundant superoxide radical scavenging enzyme in organisms that can transform harmful superoxide radicals into hydrogen peroxide (H_2O_2) (Fridovich, 1995). OGG1 encodes the DNA base-excision repair protein, 8-oxoguanine DNA glycosylase, which is the key enzyme in repairing oxidative DNA damage by removing 8-oxoguanine. 8-oxoguanine is one of the most abundant DNA oxidative damage products, and is generated by the attack of ROS on guanine, resulting in G to T transversion, if not properly repaired in a timely fashion (Ravanat et al., 2002; Sova et al., 2010). The induction of OGG1 expression suggested that the cellular DNA was undergoing ROS attack due to 4-MBC exposure, which was consistent with the increased transcription of the SOD gene. GST catalyzes the biotransformation of exogenous substances, which is an important enzyme for the detoxification of a large number of substances and could also response to oxidative stress (Sheehan et al., 2001). P53 is an important anticancer gene, which plays an important role in cell cycle checkpoints and triggers mitochondria-mediated apoptosis when cells are damaged by a variety of stresses, including oxidative stress (Harris and Levine, 2005). Previous studies have also consistently reported that 4-MBC induces oxidative stress responsive genes in aquatic organisms, e.g., in the frog *Pelophylax perizi*, and the protozoan *Tetrahymena thermophila* (Gao et al., 2013; Inui, 2003; Martins et al., 2017; Ozáez et al., 2016). In addition, the number of apoptotic neuroblastoma cells increased upon 4-MBC exposure (Broniowska et al., 2016). Taken together, it is possible that 4-MBC may induce oxidative stress and trigger apoptosis, which affects development and reproduction, and even results in a high mortality rate in *T. japonicas*.

In the F0 generation, 4-MBC caused significant mortality, while nearly no effects were observed on development and reproduction. However, in the F1 generation, 4-MBC did not cause remarkable negative effects, which may due to selection in the F0 generation that only robust individuals survived after one generation of 4-MBC exposure. In the F2-F3 generations, 4-MBC exposure still did not affect survival, but it reduced the hatching rate and the N-C/N-A durations significantly, suggesting that developmental and reproductive toxicity of 4-MBC is more pronounced after multigenerational exposure. It should be noted that in some cases, 4-MBC toxicity did not show a classical dose-response relationship. For example, the reduction of the hatching rate and N-C/N-A duration in the F2 and F3 generations did not follow an obvious dose-dependent manner (Figs. 3 and 4), possibly due to the selection of reproductive activity in the earlier generations, e.g., the F0 and F1 generations, in which exposure to higher doses resulted in a lower hatching rate and fewer number of eggs produced, and thus higher selection pressure.

After four generations of exposure to 4-MBC, the nauplii from the F3 generation were cultured in clean seawater for two more generations (i.e., the F4 and F5 generations). In the recovery generations, the survival and reproduction of the original 4-MBC exposed groups was not different from the control, suggesting that even after selection during the F0-F3 generations, the survival and reproduction of the population were insensitive to the changes from polluted seawater to a clean environment. A similar phenomenon has been noted in multigenerational toxicity studies of mercury (Li et al., 2015). In contrast, stimulation of the developmental rate in both the N-C and N-A stages was still maintained during exposure to clean seawater (Fig. 6). This finding is consistent with other studies on the copepod *Tigriopus californicus*,

Table 3
Summary of the acute and chronic toxicities of 4-MBC in *T. japonicas*.

μg L^{-1}	72 h acute toxicity	One generation (F0) life-cycle test			Multigenerational toxicity test	
	Mortality	Lethal toxicity	Decrease of N-C/N-A duration	Reduced reproduction	Decrease of N-C/N-A duration	Reduced reproduction
LC_{50}	92.9	8.5	–	–	–	–
LOEC	10	5	10	10	0.5	0.5
NOEC	< 10	1	5	5	< 0.5	< 0.5

Table 4
Preliminary risk assessment of 4-MBC on marine ecosystems using the chronic toxicity data from the multigenerational test on *T. japonicas*.

Development toxicity	Reproduction toxicity	AF	PNEC[b]	MEC	MEC/PNEC	Risk classification
0.5 μg L^{-1} (LOEC)	0.5 μg L^{-1} (LOEC)	50[a]	0.01 μg L^{-1}	0.379 μg L^{-1} (Hong Kong) [c]	37.9	Significant potential for adverse effects

[a] According to European Chemicals Bureau (ECB, 2003).
[b] PNEC = LOEC/AF.
[c] Data was from Tsui et al. (2014b).

which showed adaption to tributyltin oxide exposure (Sun et al., 2014). We therefore surmised that the *T. japonicas* population might have undergone transgenerational genetic and/or epigenetic changes during the chronic exposure to 4-MBC, consequently maintaining the symptoms during development.

5. Conclusion

The organic ultraviolet filter 4-MBC induced the transcription of genes related to oxidative stress response, apoptosis and ecdysis in the marine copepod *T. japonicas*, which significantly affected its survival, development and reproduction. The lethal effect was more pronounced in the first generation (F0), while the developmental and reproductive toxicity of 4-MBC were more significant after multigenerational exposure. A preliminary risk assessment using the chronic toxicity data suggested that 4-MBC had significant potential for adverse effects on marine crustaceans and marine ecosystems.

Acknowledgements

The present study was supported by the Natural Science Foundation of Fujian Province, China (No. 2015J01174); the National Natural Science Foundation of China (No. 41206090, 41676094); and MEL Internal Research Funding.

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