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Comparative toxicity of UV-filter Octyl methoxycinnamate and its photoproducts on zebrafish development

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ABSTRACT

In the present study, we explored the adverse effects of Octyl methoxycinnamate (OMC), and its photoproducts, namely 2-ethylhexanol (2-EH) and 4-methoxybenzaldehyde (4-MBA) on the developmental stages of zebrafish using various biomarkers such as developmental toxicity, oxidative stress, antioxidant response, neurotoxicity and histopathological changes. The effective concentrations (EC_{50}) of OMC, 2-EH and 4-MBA were found to be 64.0, 34.0 and 3.5 µg/ml, respectively in the embryo toxicity tests. Embryos exposed to the EC_{50} of OMC, 2-EH and 4-MBA showed time-dependent increases in the malformation, heart rate and hatching delay. The lipid peroxidation (LPO) level was significantly ($p<0.05$) increased and both induction and inhibition of SOD, CAT, GPx and GST activities were observed in the zebrafish larvae exposed to OMC, 2-EH and 4-MBA. GSH activity was significantly ($p<0.05$) decreased in the highest exposure groups, for all the exposed compounds when compared with the control. AChE activity was increased in lower concentrations of OMC, 2-EH and 4-MBA exposed embryos whereas, the activity was found to be decreased in highest concentration. Moreover, the histopathological studies showed severe damage to the muscle fibres and yolk sac regions of the larvae with 4-MBA treatment. The photoproducts 4-MBA has the highest toxic effect, following by 2-EH and OMC. Our results provide useful insights into the impacts of OMC and its photoproducts on zebrafish development.

Keywords: UV filter, embryotoxicity, antioxidants, histopathology, zebrafish, photoproducts.

1. Introduction

UV filters (UV-Fs) have emerged as environmental contaminants of great concern in the recent years (Molins-Delgado et al., 2016). UV-Fs constitute a large and heterogeneous group of chemicals that are widely used as cosmetic ingredients in several personal care products (PCPs) such as shampoos, soaps, lipsticks, after-shave lotions and sunscreens (body lotions) that offer protection from sunburn. In addition, UV-Fs have been used to enhance the light stability of sunscreen products, pharmaceutical products, as well as vehicle maintenance and food packaging materials to prevent their degradation (Zucchi et al., 2011; Gackowska et al., 2016; Zhou et al., 2019). The widespread application of UV-Fs in personal care products and incomplete removal in wastewater treatment plants leads to entry of these compounds into the environment (Balmer et al., 2005). Their presence poses a major threat since they are generally resistant to degradation in the wastewater treatment plants (WWTPs) (Liu et al., 2011; Gao et al., 2013; Gago-Ferrero et al., 2015; Ramos et al., 2016).

UV-Fs can reach surface waters (rivers, lakes and coastal sea waters) through wastewater treatment (WWT) processes. Besides, these chemicals can be washed off from the skin and released into the aquatic ecosystem during based-water activities such as swimming and bathing (Lambropoulou et al., 2002; Poiger et al., 2004; Balmer et al., 2005; Ramos et al., 2016). Due to their lipophilic nature, UV-Fs can bioaccumulate in the biota and may cause adverse effects in both aquatic organisms and humans (Gago-Ferrero et al., 2013; Stein et al., 2017; Quintaneiro et al., 2019) and also display hormonal activity in fish (Molins-Delgado, 2018). In recent years, the endocrine disrupting effects of these emerging contaminants has become the hot topic (Wang et al., 2016; Zhu et al., 2018).

Due to their continuous discharge in to the environment these compounds are considered pseudo-persistent and grouped as emerging contaminants (Gago-Ferrero et al., 2013; Du et

al., 2017). Recently, US Environmental Protection Agency (EPA) has included UV-Fs in the contaminant candidate list (CCL-3) (Montesdeoca-Esponda et al., 2018). Available data reveal that UV-Fs containing sunscreen products have the ability to block vitamin D synthesis, or act as endocrine disruptors that lead to developmental toxicity (Ruszkiewicz et al., 2017). In addition, UV-Fs are capable of causing acute effects, developmental toxicity and reproductive toxicity in different organisms like rat, zebrafish and *Daphnia magna* (Axelstad et al., 2011; Kinnberg et al., 2015; Hui et al., 2015; Molins-Delgado et al., 2016).

In the environment UV-Fs are susceptible to degradation and their transformations products may pose a high risk to aquatic organisms (Molins-Delgado et al., 2016). These compounds may bioaccumulate in the target organs or metabolized to form new compounds (Molins-Delgado et al., 2018). Furthermore, the physicochemical and toxicological properties of degradation products (DPs) and their behavior in the environment may greatly differ from those of the parent compounds. Therefore, critical assessment is needed to understand the complete fate of these compounds (Villaverde et al., 2018). UV-Fs are susceptible to degradation through sunlight, and this process can occur in chlorine-containing media (swimming pools) thereby resulting in the subsequent release of chlorinated or photoproducts that often exert more adverse effects than the parental compound itself (Diaz-Cruz et al., 2008; Santos et al., 2012). The DPs are moderately stable in the environment and have a high degree of solubility property that can be more pronounced than that of the parental compounds (Görner, 2003; Sayre et al., 2005; Donner et al., 2013).

Octyl methoxycinnamate (OMC), also known as ethylhexyl methoxycinnamate (EHMC), is one of the members of the organic UV-Fs family. The compound is frequently used worldwide in over 90% of the commercially available sunscreens and cosmetic formulations that are topically applied (Díaz-Cruz et al., 2008; Stein et al., 2017). In the United States and European Union, OMC has been approved as a cosmetic ingredient at a concentration of 7.5-

10% (Krause et al., 2012; Celeiro et al., 2019). OMC was first added in the European watch list for potential water contaminants under the Environmental Quality Standards Directive 2008/105/EC to evaluate its future consideration (European Commission, 2015; Loos et al., 2018; Celeiro et al., 2019). Although OMC is an effective organic ultraviolet filter chemical (UV-FC), it is also susceptible to photo-degradation and can hence lead to the formation of potentially hazardous photoproducts (Santos et al., 2012). In addition to that, when OMC is exposed to UV light, it can form DPs, such as 2-ethylhexanol (2-EH) and 4-methoxybenzaldehyde (4-MBA) (Stein et al., 2017). OMC has been detected in various concentrations in different environments ranging between 0.01-0.1 mg/L (treated wastewater), 0.26-5.61 µg/L (drinking water), 390 ng/L in coastal seawater and a maximum concentration (19 mg/L) was noted in raw municipal wastewater (Balmer et al., 2005; Fent et al., 2010; Loraine and Pettigrove, 2006; Langford and Thomas, 2008). OMC can easily penetrate through the skin, and it is detectable in blood, urine and breast milk samples upon continuous usage of OMC containing cosmetic products. This observation indicates that humans are systemically exposed to this compound (Janjua et al., 2004; Schlumpf et al., 2008; Huang et al., 2019).

It has been reported that OMC has an estrogenic, anti-androgenic, and anti-thyroid activity to different organisms (Lorigo et al., 2018). Acute toxicity of OMC has previously reported by Fent *et al*. (2010) in *Daphnia magna.* The combined toxicities of OMC and benzophenone-3 (BP-3) UV-Fs have been studied by Jang et al. (2016) in *D. magna* and *Danio rerio*. In fathead minnows, OMC is known to induce histological changes in the testes and ovaries (Christen et al., 2011). More recently, Zhou et al. (2019) have reported the parental transfer of OMC and its biochemical responses in zebrafish after chronic exposure. In spite of the prevalence of many studies, there is a dearth of information on the complete embryo toxicity of OMC and its photoproducts (2-EH and 4-MBA) in *Danio rerio*. In recent

119 years, among the various vertebrate models, zebrafish (*Danio rerio*) has become one of the
120 most popular and powerful ones employed in developmental and toxicological studies owing
121 to its advantages such as transparency of the embryos, high fecundity, rapid embryonic
122 development, etc. (Shi et al., 2017; Parolini et al., 2018; Félix et al., 2018). The oxidative
123 stress indices and alterations in antioxidant enzymes serve as important molecular biomarkers
124 in toxicological studies involving a range of aquatic organisms. Oxidative stress is
125 counteracted by antioxidant enzymes such as SOD, CAT, GPx, GST and GSH. Besides,
126 AChE activity has been established to be a crucial biomarker in the neurotoxicological
127 studies concerning aquatic organisms (Muthulakshmi et al., 2018).

128 The demand for personal care products which contain UV filters and their release into the
129 environment represent a new class of emerging contaminants. To our best knowledge data on
130 acute and chronic toxicity of these contaminants on various physiological biomarkers of
131 aquatic organisms particularly on fish are limited (Liu et al., 2015). Furthermore, a
132 comparative toxicity of UV filters and their photoproducts on fish are very limited.
133 Therefore, the present work was aimed to determine the EC_{50} value of OMC and its
134 photoproducts such as 2-EH and 4-MBA and to evaluate the ecotoxicity of these compounds
135 on the developmental stages of zebrafish by employing embryo toxicity, oxidative stress
136 biomarkers (LPO), antioxidant defense systems (SOD, CAT, GPx, GST and GSH), and
137 AChE activity as indicators of neurotoxicity and histopathological changes.

138

139 **2. Materials and methods**

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141 *2.1. Test compound*

142

Octyl methoxycinnamate (CAS No: 5466-77-3; purity: 98%), ethylhexanol (CAS No: 5466-77-3; purity: 98%) and 4-methylbenzaldehyde (4-MBA) (CAS No: 5466-77-3; purity: 98%) were purchased from Sigma Aldrich, (Tokyo, Japan). The compounds were dissolved in 100% methanol to obtain stock solutions of 10 mg/mL and stored in the dark environment at 4 °C.

2.2. Zebrafish maintenance

Adult wild-type zebrafish (*Danio rerio*), were maintained in the laboratory condition as reported by Maharajan et al. (2018). During acclimation, zebrafish were fed with blood-worm and protein-rich food twice a day, and the water renewal was performed once a day.

2.3. Embryo collection

Healthy males and females were selected and placed in the breeding tank for spawning in the ratio of 2:1. The tanks were maintained in a 14 h light and 10 h dark cycle to induce spawning. The fertilized embryos were collected and washed with sterile double distilled water followed by culture solution to remove the debris. Later, the embryos were staged under the microscope (Optika XDS-2, Italy) according to the standard methods of Kimmel et al. (1995); the unfertilized and dead embryos were discarded.

2.4. Exposure

The short-term developmental effects of OMC, 2-EH and 4-MBA were conducted in the embryos at 3 - 96 hpf and FET was done according to the Organization for Economic Co-

operation and Development (OECD Test No. 236) guidelines for short-term toxicity testing (OECD, 2013). The culture media (294 mg/L of $CaCl_2$ in $2H_2O$, 63.0 mg/L $NaHCO_3$, 123.3 mg/L $MgSO_4$ in $7H_2O$ and 5.5 mg/L KCl) was prepared freshly before use (ISO, 2007; Ku et al., 2015). The embryos at 3 hpf were randomly exposed to the control and different concentrations of OMC, 2-EH and 4-MBA, in a clean plate. The exposure medium was renewed every 24 h to maintain appropriate concentrations of OMC, 2-EH, 4-MBA. The EC_{50}-was determined as per the OECD guidelines. The exposure concentrations were selected based on 96h EC_{50} of OMC, 2-EH and 4-MBA (6.2, 3.4, 0.35) such that the least was $1/10^{th}$ of EC_{50}, the second was $1/5^{th}$ of EC_{50} and the highest was equal to EC_{50} concentration (supplementary information, Table S1) which corresponds to 6.2, 12.4 and 62 µg/ml for OMC; 3.4, 6.8 and 34 µg/ml for 2-EH and 0.35, 0.7 and 3.5 µg/ml for 4-MBA. Three replicates were performed for each concentration with the respective reference groups. The exposure plates were examined regularly, and any dead embryos if present were removed immediately to avoid contamination.

2.5. Developmental toxicity, heart rate and hatching rate

Embryonic development of the treated and control embryos was monitored daily, to observe the morphological development and detect the anomalies using a light microscope (Olympus BH-2). The dead embryos were recorded daily and removed in a timely manner. The heart rate was measured (at 48, 72 and 96 hpf) as per the method of Ahmad et al. (2015). Briefly, the heart rates of the embryos were counted at 48, 72 and 96 hpf by visual observation at 60-s intervals under the stereomicroscope. For the embryos of the treated and control zebrafish were anaesthetized with 0.016% tricaine. The hatching delay in the zebrafish embryos exposed to OMC, 2-EH and 4-MBA was examined upto 96 hpf.

193

194 *2.6. Oxidative stress and antioxidant enzyme*

195

196 In the present study, 96 hpf larvae were used to evaluate the oxidative stress and
197 antioxidant enzyme levels after exposure to OMC, 2-EH and 4-MBA. The lipid peroxidation
198 (LPO) level was determined by the procedure of Adeyemi et al. (2015). Marklund and
199 Marklund (1974) method was followed to quantify SOD activity. Catalase (CAT) activity
200 was estimated according to the process described by Maharajan et al. (2018). Glutathione
201 peroxidase *(*GPx) activity was measured by following the protocol of Muthulakshmi et al.
202 (2018). Glutathione S-transferase (GST) activity was measured as per the Habig and Jakoby
203 (1981) method, with a slight modification for adapting it to the microplate reader method
204 (Frasco and Guilhermino, 2002). Reduced glutathione (GSH) activity was measured using the
205 protocol of Ganie et al. (2011) with some alterations. The protein levels were measured
206 according to Lowry et al. (1951) adapting the protocol to the microplate method (Fryer et al.,
207 1986). For all biochemical assays, microplate readers (Synergy H1, BioTek) with the
208 respective absorbance ranges were used.

209

210 *2.7. AChE*

211

212 Total AChE activity was estimated by following the method of Ellman et al. (1961),
213 with some modifications adapted to microplate method by Guilhermino et al. (1996).
214 Embryos from each treatment and control were homogenized on ice with potassium
215 phosphate buffer (0.1 M, pH 7.2). The supernatant was taken after the centrifugation (4 °C,
216 3000 g, and 4 min) was used for AChE activity determination, using 50 µL of homogenate
217 sample and 250 µL of the reaction mixture (1 mL of 10 mM 5,5-dithiobis-2-nitrobenzoic acid

solution with sodium hydrogen carbonate, 0.2 mL of 0.075 M acetylcholine solution and 30 mL of 0.1 M phosphate buffer). The absorbance was measured at 414 nm in microplate reader.

2.8. Histopathology

At the end of 96 hpf, the embryos from OMC, 2-EH and 4-MBA exposed and control groups were washed and fixed in 4% paraformaldehyde. Then, Bouin's solution was used for fixation, and the dehydration process was done by using the ascending graded sequence of ethanol. The embryos were cleaned twice in xylene and embedded in paraffin wax. Embryos sections of 5 µm thickness were prepared using a rotatory microtome, and stained with hematoxylin-eosin. The histopathological changes were examined at 40x magnification with a light microscope (Optika XDS-2, Italy).

2.9. Statistical analysis

Statistical analysis was carried out by using GraphPad Prism (version 5.0). To compare the control and treatment groups, one-way ANOVA was performed using Tukey's post hoc test for all the experiments, except for heart rate and hatching delay which involved two-way ANOVA followed by Bonferroni post-tests. The data were presented as mean ± SE (n=3). The statistical acceptance level was $p<0.05$ for the heart rate, whereas the survival rate, hatching delay and antioxidant assays were measured at both significance levels ($p<0.01$ and $p<0.05$).

3. Results and Discussion

Developmental toxicity of the various types of UV-Fs on aquatic organisms has not yet been clearly understood (Fong et al., 2016). Adequate information on the adverse effects of the parent compound as well as the metabolites or degraded products of UV-Fs on the embryonic development of aquatic vertebrates is still lacking. Hence, this research was undertaken to evaluate the adverse effects of OMC and its photoproducts (4-MBA and 2-EH) by scrutinizing the embryo toxicity, oxidative stress and antioxidant responses, neurotoxicity and histopathological changes in zebrafish embryos.

3.1. Developmental toxicity

The developmental toxicity of OMC and its photoproducts was determined based on EC_{50}. According to the 96h EC_{50} values of the embryo toxicity test, 4-MBA (3.5 µg/ml) was the toxic to the embryos, followed by 2-EH (32 µg/ml) and OMC (64 µg/ml), which suggests that the photoproducts (2-EH and 4-MBA) are more dangerous than the parental compound (OMC). When compared with the previously found OMC levels in the environment, the EC_{50} value is quite high. Besides, OMC, 2-EH and 4-MBA have been observed to cause developmental toxicity in a dose-dependent manner. As per the previous data, the EC_{50} of OMC in *Daphnia magna* was 0.57 mg/L in the acute toxicity test, which asserts that the compound is more toxic to *Daphnia magna* than *D. rerio* (Martins et al., 2007; Sieratowicz et al., 2011). Moreover, the 24-h EC_{50} of OMC to *Tetrahymena thermophila* has been reported to be >15 mg/L (Gao et al., 2013). These results suggest that the effective concentration of OMC is species-specific and time dependent.

Developmental deformities have been considered as the indicators of toxicity assessment in the zebrafish embryo (Pamanji et al., 2015). In the present investigation, it was

noted that the control embryo showed normal structure and spine axis. In contrast, the OMC (12.4, 62 µg/ml) and 2-EH (6.8, 34 µg/ml) exposed embryos exhibited pericardial edema, scoliosis and tail malformation (Fig.1, 2 and S1). However, at lower concentrations of OMC (6.2 µg/ml) and 2-EH (3.4 µg/ml) the embryos displayed a normal spine axis. In addition, 4-MBA induced yolk sac edema, spinal curvature, tail deformity and pericardial edema were witnessed in the higher concentrations (0.7 and 3.5 µg/ml). Nonetheless, at a concentration of 0.35 µg/ml concentration, considerable deformities were not observed when compared with the control group (Fig.3 and S1). In our study, the most commonly observed malformations were pericardial edema, scoliosis and tail malformation in all higher concentrations of the OMC, 2-EH and 4-MBA exposed groups, while yolk sac edema was observed only in the 4-MBA exposed embryos. Recently, Zhou et al. (2019) has stated that the parental transfer of OMC to the offspring at high concentrations could significantly reduce the development ability, hatchability and growth of zebrafish, thereby leading to a hike in the malformation and mortality rates. These changes were also perceived in our present results, as the embryos had developmental deformities and their hatching was delayed.

A similar study by Li et al. (2016) has reported abnormal axial curvature or scoliosis in the zebrafish embryos exposed to the UVF 4-methylbenzylidene camphor (4-MBC). Moreover, Li et al. (2018a) have also noticed abnormalities in the embryos of zebrafish exposed to a mixture of UV-Fs consisting of BP-3, OMC and octocrylene (OC). In addition, the photo-isomerization of OMC after exposure to UV radiation may enhance the toxicity of OMC photoproducts (Hanson et al., 2006; Duale et al., 2010). The present findings are in accordance with the studies of Balázs et al. (2016) in which tail deformation, pericardial edema, and yolk sac edema have been reported in zebrafish embryos exposed to benzophenone-3. The spine deformation observed by us might be associated with a decrease in myosin which is essential for normal development (Cheng et al., 2000). The pericardial

293 edema perceived in the embryos might be attributed to swelling and fluid accumulation
294 around the heart chambers as well as circulation faults; which might have resulted in
295 decreased or absent blood flow in zebrafish after exposure to the compounds.
296
297 *3.2. Hatching delay*
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299 Hatching is a natural process in embryogenesis by which the embryo is transformed
300 into larvae. The hatching rate has been widely reported as an important endpoint in
301 developmental toxicity (Torres et al., 2016; Samaee et al., 2015; Si et al., 2019). In the
302 present study, OMC exposure did not affect the hatching rate in any of the exposed groups
303 (Fig. 4). However, at 96 hpf, significant ($p<0.01$) delay in hatching was noticed when 2-EH
304 was used at a concentration of 96 hpf in 34.0 µg/ml. In the 4-MBA treatment, a significant
305 decrease in the hatching rate was observed at 0.35, 0.70 and 3.50 µg/ml ($p<0.05$)
306 concentrations. Likewise, delayed hatching has been reported by other researchers upon
307 exposing the zebrafish embryos to the UV-Fs 4-MBC (Torres et al., 2016), triclosan (Oliveira
308 et al., 2010), benzophenone-3 (Balázs et al., 2016) and UV-234 (Liang et al., 2019). In our
309 study, the detected hatching delay was due to spinal curvature or inhibition of the hatching
310 gland, which prevents the embryo from hatching out of the chorion (Sun and Liu, 2017).
311 Further, in-depth molecular studies are needed to ascertain the role of the proteolytic enzyme
312 in the hatching delay (Trikić et al., 2011; Kawaguchi et al., 2017).
313
314 *3.3. Heart rate*
315
316 The heart rate has been used as an endpoint analysis in the developmental toxicity
317 assay (Suvarchala and Philip, 2016). In our study, the zebrafish embryos exposed to OMC

318 (Fig. 5a), 2-EH (Fig. 6a) and 4-MBA (Fig. 7a) showed a decrease in the heart rate with an
319 increase in concentration and time. For instance, at 48 hpf, significant ($p<0.05$) lowering of
320 the heart rate was observed at 62.0 µg/ml in the OMC, and 0.70 and 3.50 µg/ml in the 4-
321 MBA exposed groups. Further, at 72 and 96 hpf the highest exposed groups of OMC, 2-EH
322 & 4-MBA exhibited significant ($p<0.05$) reductions in their heart rates. The embryos exposed
323 to 12.4 µg/ml of OMC and 0.70 µg/ml of 4-MBA were also inferred to have a significantly
324 ($p<0.05$) reduced heart rate. Jang et al. (2016) observed decreased heart rate in the wild type
325 zebrafish after exposed to the OMC which is dose and time dependent. In the present study,
326 decreased heart rate observed in the embryos exposed to highest concentration of OMC and
327 2-EH at 72, 96 hpf and highest concentration 4-MBA at 48, 96 hpf might be related with
328 pericardial edema observed during development stages. Similarly, a previous study has
329 highlighted that benzotriazole UV stabilizer can decrease the heart rate of zebrafish embryos
330 (Damalas et al., 2018). Furthermore, Torres et al. (2016) have also stated that exposure to 4-
331 MBC affects the heart rate of zebrafish embryos. Besides, Li et al. (2018a) have established
332 that deformities occur in the heart and pericardium; and that pericardial edema may affect the
333 cardiac function, resulting in heartbeat irregularities and blood flow failure.
334
335 *3.4. Oxidative stress and antioxidant response*
336
337 Developmental and teratogenic toxicities of many xenobiotics are highly associated
338 with oxidative stress (Mu et al., 2015). Organisms activate various antioxidant defence
339 enzymes, including SOD, CAT, GPx and GST against the damaging effects of activated ROS
340 (Alak et al., 2017; Jiang et al., 2019). UV-filters can induce oxidative stress through
341 production of ROS which may leads to activation of antioxidant enzymes to prevent the
342 oxidative damage (Liu et al., 2015). Hence, we determined the oxidative stress and

antioxidant enzyme status of the zebrafish embryos exposed to OMC, 2-EH and 4-MBA. LPO acts as an important indicator of oxidative stress in the aquatic organisms (Chen et al., 2011). After treatment with OMC, the LPO levels were significantly ($p<0.01$) increased in the zebrafish embryos at the highest concentration of 62 µg/ml (Fig. 5b). Likewise, in the 2-EH and 4-MBA exposed groups, a significant ($p<0.05$ and $p<0.01$) induction of the LPO levels was discerned at the highest exposure concentrations (Fig. 6b & 7b). In our previous study, we observed a significant increase in LPO level in a freshwater fish *Labeo rohita* exposed to triclosan at 0.039 and 0.078 mg L^{-1} concentrations (Hemalatha et al., 2019). The elevated MDA levels might be due to ROS induced toxicity and the biotransformation of these compounds through redox cycling (Islas-Flores et al., 2014).

The SOD and CAT enzymes constitute the first line of defense against free radicals and are the best indicators of exposure to pollutants causing oxidative stress (Zheng et al., 2016; Zhou et al., 2019). SOD plays a vital role in maintaining the oxidant-antioxidant balance and the routine physiological functions (Mi et al., 2018). The results obtained for SOD activity (Fig. 5c) reveals that the OMC treated zebrafish embryos showed a significant ($p<0.05$ and $p<0.01$) increase in all the exposure concentrations. Nevertheless, the 2-EH treated embryos exhibited a significant ($p<0.05$) increase in SOD activity only in 3.4 µg/ml treated group (Fig. 6c). In contrast to the OMC and 2-EH treatments, a significant ($p<0.01$) reduction in the SOD activity was noticed in the embryos treated with 3.5 µg/ml of 4-MBA (Fig. 7c).

Hu et al. (2009) have documented both increase and decrease in SOD activity when the zebrafish embryos were exposed to tetrabromo bisphenol A and hexabromocyclododecane, respectively. In our study, the variations noted in the activity of SOD enzymes upon exposure to different concentrations of OMC and its photoproducts (2-EH and 4-MBA) suggest that the observed changes could be an adaptive response to ROS.

368 The declined SOD activity implies the failure of the antioxidant system to scavenge the
369 excessive ROS produced in the exposed embryos (Muthulakshmi et al., 2018). Therefore, the
370 remarkable increase in SOD activity in embryos of zebrafish might be an adaptive defense
371 mechanism in response to the oxidative stress exerted by the compounds (OMC and 2-EH).

372 In this study, the OMC exposure caused a significant ($p<0.05$ for the 6.2 and 12.4
373 µg/ml groups; $p<0.01$ for the 62 µg/ml group) induction of CAT activity at all the
374 concentrations (Fig. 5d). While the 2-EH exposure did not lead to any significant changes in
375 CAT activity (Fig. 6d), we discerned an increase in the embryos treated with 0.7 µg/ml of 4-
376 MBA (Fig. 7d). A significant increase in CAT activity was also noted in zebrafish embryos
377 exposed to mixtures of three UV filters (BP-3, OMC and OC) (Li et al., 2018a). In our study
378 the increase in the CAT activity suggested an adaptive response to OMC and its
379 photoproducts stress and a compensatory mechanism to defend against oxidative stress.
380 Further, Torre et al. (2018) have noted an increase in the enzyme activity in the zebrafish
381 embryos exposed to fullerene C60. A hike was observed in the study indicating that the
382 antioxidant system is affected and to withstand such a stress, the anti-oxidative capacity of
383 zebrafish was activated (Li et al., 2018b). Another study has reported an elevated CAT
384 activity in adult zebrafish after being exposed to OMC (1, 10, 100 µg/L), which indicates that
385 the compound can induce excessive ROS production (Zhou et al., 2019).

386 GPx is an enzyme that is involved in the decomposition of hydrogen peroxide along
387 with GSH which acts as a co-substrate to overcome the stress generated by ROS (Binelli et
388 al., 2011; Park et al., 2017). This activity was found to be significantly ($p<0.01$ and $p<0.05$)
389 increased in the zebrafish embryos at the highest exposure concentrations of OMC (62 µg/ml)
390 and 2-EH (34 µg/ml) (Fig. 5e, 6e). In contrast, a significant ($p<0.05$) decrease in the enzyme
391 activity was noted at the highest exposure concentration of 4-MBA (Fig. 7e). An increase in

GPx activity was noticed in Nile tilapia (*Oreochromis niloticus*) after exposed to parabens (Silva et al., 2018). In contrast, Shi et al. (2018) has recorded reported a fall in the GPx activity of zebrafish embryos exposed to 6:2 fluorotelomer sulfonamide alkylbetaine. The reason for this decline is the excessive amount of oxidized glutathione (Li et al., 2013). Hence, in the present study, GPx and GSH are involved in the detoxification of hydrogen peroxide produced in the zebrafish embryos exposed to OMC and its photoproducts (2-EH and 4-MBA).

GST is an essential enzyme in the phase II detoxification mechanism that promotes the conjugation of GSH with the electrophilic compounds and converts the latter into easily extractable hydrophilic metabolites (Van der Oost et al., 2003). A significant ($p<0.05$) increase in GST activity was observed in the embryos exposed to 62 µg/ml of OMC (Fig. 5f), as well as 0.7 and 3.5 µg/ml of 4-MBA (Fig. 7f). Decreased activity was noticed only in at lower concentration of 2-EH (Fig. 6f). Quintaneiro et al. (2019) has documented a significant increase in GST activity in zebrafish embryos exposed to 4-MBC at highest concentration (0.44mg/L). Likewise, the activity of GST was significantly increased after exposed to pharmaceutical drug gabapentin at high exposure concentrations (Li et al., 2018b). Induction of GST activity was noted in *Pelophylax perezi* (Martins et al., 2017) and in *C. riparius* (Campos et al., 2017) after exposed to 4-MBC. Overall, the results from GST activity obtained for zebrafish and other species suggest the activation of phase II detoxification system to cope with 4-MBC induced stress. In the present study, we observed that the changes in GST activity might be correlated with the reduced GSH level. The increased GST activity suggests the development of an adaptive response against OMC and its photoproducts induced stress.

GSH is one of the most important ROS scavengers, and its ratio to oxidized glutathione (GSSG) can indicate the oxidative status of the cells (Almeida et al., 2017). The present study

417 reveals that GSH level was significantly ($p<0.05$) increased in the embryos treated with 6.2
418 µg/ml of OMC, while the level was decreased in those exposed to 62 µg/ml of OMC (Fig.
419 5g). Furthermore, a significant ($p<0.05$) decrease in the GSH level was observed in the 2-EH
420 (34 µg/ml), and 4-MBA (0.7, 3.5 µg/ml) exposed embryos (Fig. 6g and 7g). Similar to our
421 findings Liu et al. (2015) has reported that GSH level was increased significantly in the fish
422 after exposed to BP-1 and BP-3 even in the lower concentration. However, BP-3 caused a
423 reduction in GSH content in *T. thermophila* (Gao et al., 2013). Increased GSH level in
424 present study, indicating an adaptive and protective role against the oxidative stress induced
425 by OMC. The reduction in the GSH level reveals that the enzyme rapidly binds to the ROS to
426 reverse the adverse effect of the toxicant (Yu, 1994). Moreover, changes in the antioxidant
427 levels also contribute to developmental abnormalities (Rodríguez-Fuentes et al., 2015). We
428 conclude that OMC, 2-EH and 4-MBA may induce oxidative stress by generation of ROS
429 which may affect the antioxidant system resulting decrease in GSH content, reduced SOD
430 activity, and increased MDA contents.
431
432 *3.5. Neurotoxicity*
433
434 Alteration in the AChE activity was used as an indicator to detect the neurotoxicity of
435 several exogenous compounds (Gholami-Seyedkolaei et al., 2013; Xie et al., 2016; Zhou et
436 al., 2019). The result presented in Fig. 5h, 6h and 7h indicates no inhibition of AChE activity
437 in the OMC (6.2 and 12.4 µg/ml) and 2-EH (6.8 and 34.0 µg/ml) exposed embryos. However,
438 a significant ($p<0.05$) lowering of the activity was noted in the 62 µg/ml of OMC and 3.5
439 µg/ml of 4-MBA exposed groups. Furthermore, a significant ($p<0.05$) increase in the AChE
440 activity was observed in the 2-EH (3.4 µg/ml) and 4-MBA (0.35, 0.7 µg/ml) treated embryos.
441 Similar to our findings, Quintaneiro et al. (2019) noticed significant increase in AChE

activity in zebrafish embryos exposed to 4-MBC. In contrast, a significant decrease in AChE activity was observed in zebrafish embryos exposed to (4-MBC) (Li et al., 2016). Araujo et al. (2018) have noted a significantly increased AChE activity in the embryos of *Solea senegalensis* after being exposed to the UV Filter 4-methylbenzylidene camphor. Increased of AChE activity might lead to cholinergic neurotransmission impairment through ACh high degradation contributing to neurological dysfunctions (Mushtaq et al., 2014). It has been reported by Behra et al. (2002) that the inhibition of AChE is mainly due to the accumulation of acetylcholine (ACh) and the saturation of acetylcholine receptor (AChR), which leads to its continuous stimulation of the receptor and ultimately leading to its inactivation. In our study, inhibition of AChE may lead to developmental deformities in embryo exposed to OMC and 4-MBA.

3.6. Histopathological examination

Chemical exposure leads to changes in the cellular structures which can be assessed by histopathological examination, and also serves as an indicator for assessing the quality of the aquatic environment (Meyers and Hendricks, 1982; Osterauer et al., 2010; Maharajan et al., 2018). In this study, when compared with the control group (Fig. 8A), the embryos treated with the higher concentration of OMC (Fig. 8C, D), and 2-EH (Fig. 8F, G), exhibited moderate changes such as elongated yolk sac, abnormal muscle, swim bladder inflammation and yolk sac edema. Besides, changes were noticed in the embryos treated with the lower concentrations of OMC (Fig. 8B), and 2-EH (Fig. 8E), as well. In the 4-MBA exposed embryos, pronounced structural changes were observed at 3.5 µg/ml (Fig. 8J), while the

lower concentrations (0.35 and 0.7 µg/ml) caused moderate effects on the muscle cells (Fig. H, I). Fig 8 (A) is meant as a common control for all the experimental groups.

Comparable patterns of histological lesions have been observed by Ghobadian et al. (2017) in the zebrafish larvae after their exposure to MgO nanoparticles. Zhang et al. (2017) has obtained similar results in the embryos of zebrafish exposed to fine particulate matter. Hence, the histopathological changes observed in the treated embryos (muscle cell degeneration and yolk sac) are supported by the developmental deformities observed in the malformation study and vice versa. In our study, the alterations noted in the muscle fibers emphasize the harmful effects of OMC and its photoproducts (2-EH and 4-MBA). AChE is quite beneficial in the muscular and neuronal development and its absence could therefore affect either the arrangement or the integrity of myofibers (Meyers and Hendricks, 1982; Sun and Liu, 2017). Hence, alterations in the muscle fibers may be correlated with the decreased AChE activity in the exposed embryos.

Our overall results are comparable with those of Damalas et al. (2018), in which no apparent adverse effects were perceived in the zebrafish embryos exposed to the parent compound benzotriazoles UV stabilizers at lower concentrations. However, it has been reported that the biotransformation products causes developmental anomalies such as pericardial edema in the zebrafish embryos when present in higher concentrations. Li et al. (2016) have asserted that acesulfame and its photodegraded transformation products lead to adverse effects such as edema formation, decrease in heart rate, and hatching rate during the fish embryo development. A recent study indicates that the cytotoxicity of OMC may differ from their photoproducts (Stein et al., 2017). Overall, among the three compounds, the 4-MBA exposed embryos showed the most severe damage, followed by 2-EH and OMC. Besides, concentration dependent alterations were noticed in all the groups.

4. Conclusions

The EC_{50} value of OMC, 2-EH and 4-MBA in embryo toxicity was found to be 64.0, 34.0 and 3.5 µg/ml, respectively. 4-MBA was found to be more toxic followed by 2-EH and OMC, which suggests that photoproducts were highly toxic to zebrafish embryos when compared to their parental compound. The behaviour of DPs in terms of physicochemical and toxicological properties may greatly differ from their parent compounds in the environment. In the present study, we unearthed that the photo products of OMC such as 2-EH and 4-MBA exert more adverse effects on the zebrafish embryo development than the parental compound OMC. The photoproducts directly affected the zebrafish embryos, caused developmental deformities and elicited various physiological responses such as oxidative stress, changes in antioxidant enzymes, neurotoxicity and histopathological changes at higher concentrations. The alterations of these parameters can be effectively used to monitor the impact of these emerging contaminants on aquatic organisms. Further, based on the results, it was evident that the photoproduct 4-MBA exerted the highest impact on the zebrafish embryo development followed by 2-EH and the parental compound OMC. This study further highlights the necessity of in-depth study to gain insights on synergistic effects of these photoproducts at chronic and molecular level.

Declaration

Authors declare no conflict of interest

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861

862 **Figure captions**

863 **Fig. 1.** Photomicrographs showing malformations in zebrafish embryos and larvae of the
864 OMC exposed groups when compared with the control at 24, 48, 72 and 96 hpf time
865 intervals. **PE**– Pericardial Edema, **SC**– Scoliosis, **NS**–Normal Spine axis and **TM**–Tail
866 Malformation.

867 **Fig. 2.** Photomicrographs showing malformations in the zebrafish embryos and larvae of the
868 2-EH exposed groups when compared with the control at 24, 48, 72 and 96 hpf time intervals.
869 **PE**– Pericardial Edema, **SC**–Scoliosis, **NS**–Normal Spine axis and **TM**–Tail
870 Malformation.

871 **Fig. 3.** Photomicrographs depicting malformations in the zebrafish embryos and larvae of the
872 4-MBA exposed (0.35, 0.7 and 3.5 µg/ml) groups when compared with the control at 24, 48,
873 72 and 96 hpf time intervals. **PE**–Pericardial Edema, **SC**–Scoliosis, **NS**–Normal Spine axis,
874 **YSE**-Yolk Sac Edema, **DH**-Delayed Hatching and **TM**–Tail Malformation.

875 **Fig. 4.** Hatching ratio of the zebrafish embryos exposed to different concentrations of OMC
876 (6.2, 12.4, 62.0 µg/ml), 2-EH (3.4, 6.8, 34.0 µg/ml) and 4-MBA (0.35, 0.70, 3.50 µg/ml)
877 concentrations at 96 hpf. The data are presented as mean ± SE (n=3). Two-way ANOVA was
878 performed followed by a Bonferroni post-test. * and ** indicate the significance level at $p <$
879 0.05 and $p<0.01$ between the control and treated embryos.

880 **Fig. 5.** Heart rate (a), LPO (b), SOD (c), CAT (d), GPx (e), GST (f), GSH (g) and AChE (h)
881 response in the zebrafish embryos (96 hpf) exposed to OMC. The data are presented as mean
882 ± SE (n=3). One way ANOVA with Tukey's post hoc test was used; * indicates $p<0.05$ and
883 ** indicates $p<0.01$.

884 **Fig. 6.** Heart rate (a), LPO (b), SOD (c), CAT (d), GPx (e), GST (f), GSH (g) and AChE (h)
885 response in the zebrafish embryos (96 hpf) exposed to 2-EH. The data are presented as mean
886 ± SE (n=3). One way ANOVA with Tukey's post hoc test was used; * indicates $p<0.05$ and
887 ** indicates $p<0.01$.

888 **Fig. 7.** Heart rate (a), LPO (b), SOD (c), CAT (d), GPx (e), GST (f), GSH (g) and AChE (h)
889 response in the zebrafish embryos (96 hpf) exposed to 4-MBA. The data are presented as
890 mean ± SE (n=3). One way ANOVA with Tukey's post hoc test was used; * indicates $p<0.05$
891 and ** indicates $p<0.01$.

892 **Fig. 8.** Histopathological pattern of the 96hpf embryos after exposure to OMC (a), 2-EH (b)
893 and 4-MBA (c). A- show the normal development of the control; B, C and D depict the larvae
894 exposed to 6.2, 12.4 and 62.0 D µg/ml of OMC, respectively; E, F and G correspond to the
895 larvae exposed to 3.4, 6.8 and 34.0 µg/ml of 2-EH, respectively; H, I and J signify the larvae
896 treated with 0.35, 0.7 and 3.5 µg/ml of 4-MBA, respectively. (**SBI-**swim bladder
897 inflammation; **NM**-Normal muscle; **YS-**yolk sac; **EYS-**elongated yolk sac; **AM-**abnormal
898 muscle; **YSE-**yolk sac edema).

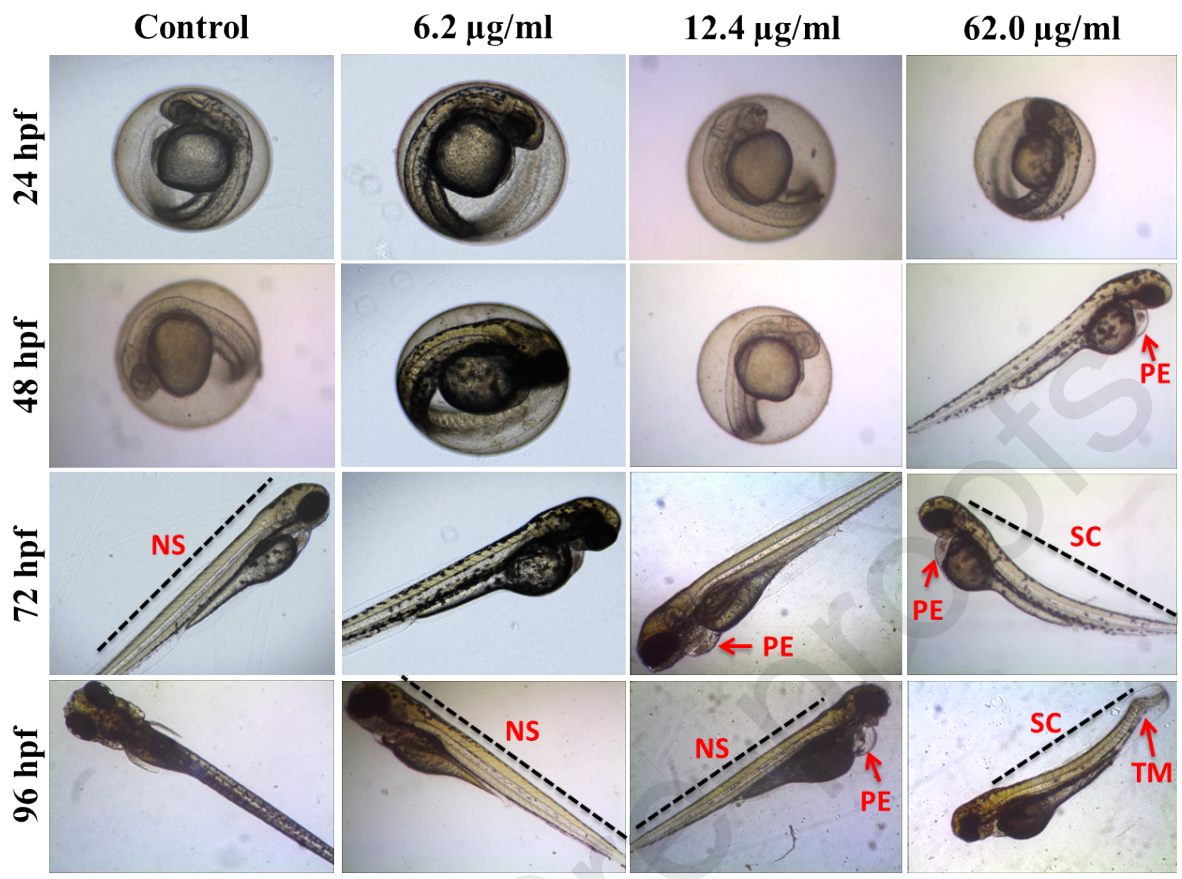
899

900

901 **Highlights**

902 • The adverse effect of OMC and its photoproducts (2-EH and 4-MBA) were studied
903 • 4-MBA were highly toxic than 2-EH and OMC in zebrafish embryo development
904 • 2-EH and 4-MBA disturbs antioxidant balance by inducing oxidative stress
905 • OMC and its photoproducts inhibits AChE and cause histopathological damage
906 • Photoproducts were highly toxic compared to parental compound (OMC)

907



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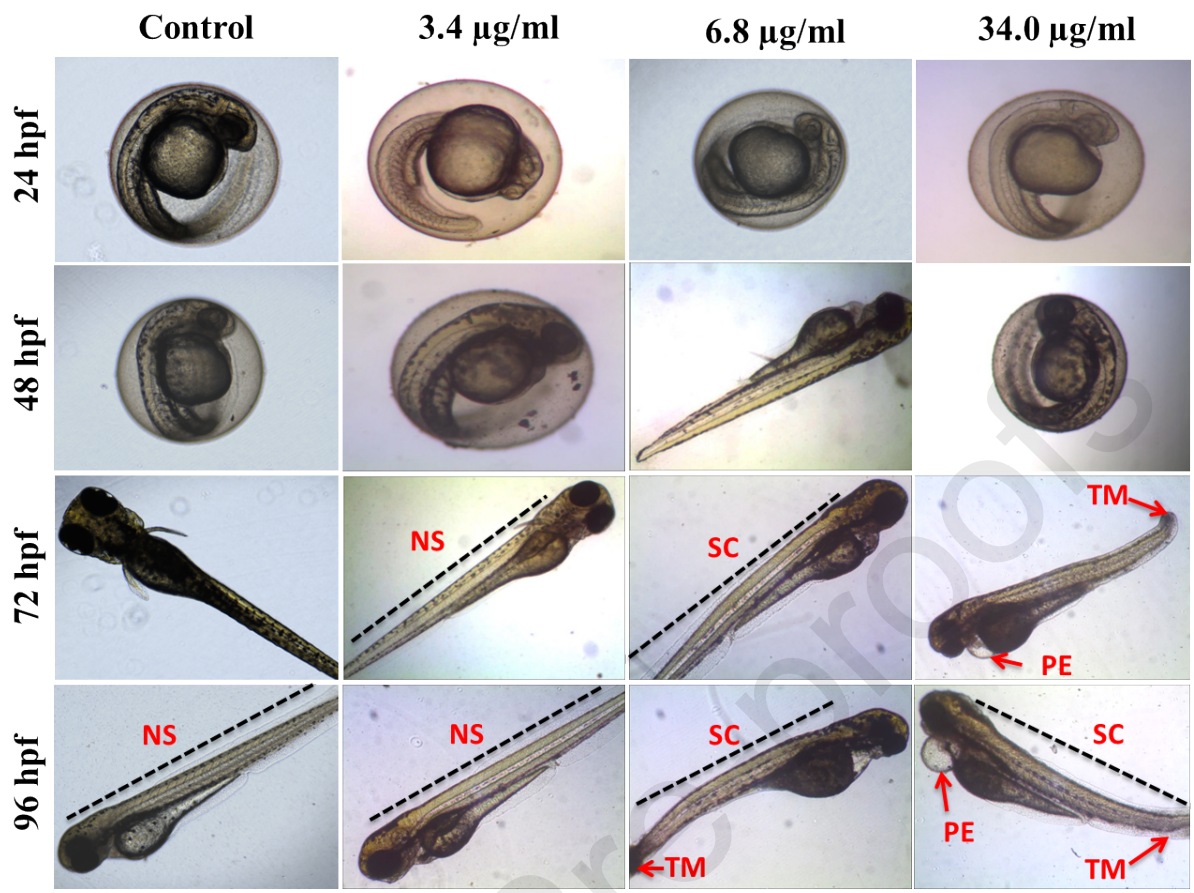

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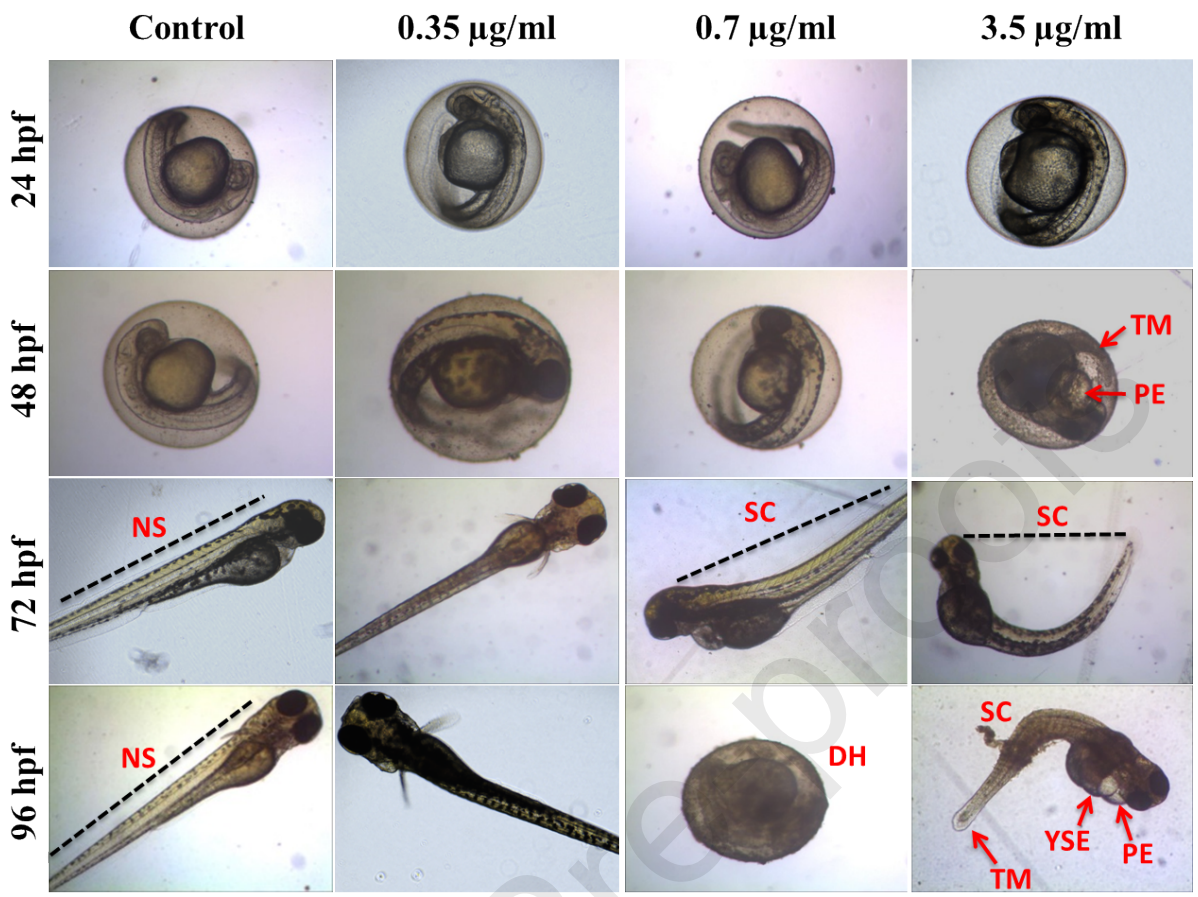
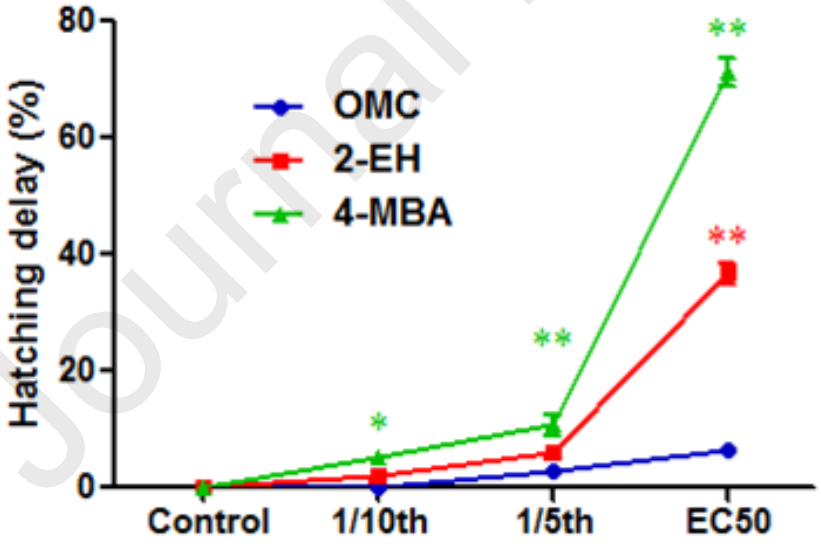
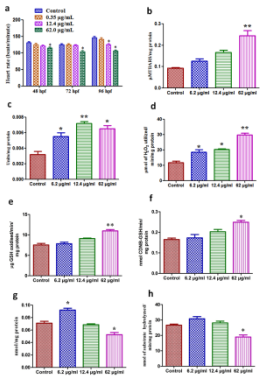

Control
0.35 µg/ml
0.7 µg/ml
3.5 µg/ml
24 hpf
48 hpf
72 hpf
96 hpf
TM
PE
NS
SC
SC
NS
DH
SC
YSE
PE
TM

910


Hatching delay (%)
80
60
40
20
0
OMC
2-EH
4-MBA
Control
1/10th
1/5th
EC50

911

912



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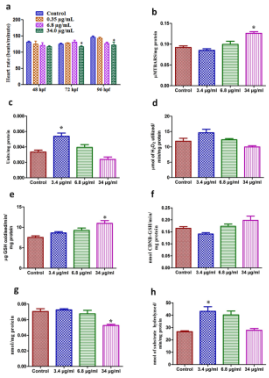

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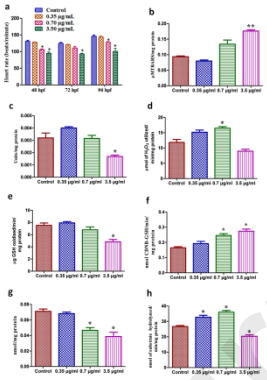

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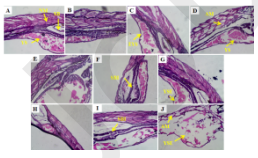

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