Science of the Total Environment 852 (2022) 158503

Contents lists available at ScienceDirect

Science of the Total Environment

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

Polystyrene microplastics mitigate the embryotoxic damage of metformin and guanylurea in *Danio rerio*

Gustavo Axel Elizalde-Velázquez [a], Leobardo Manuel Gómez-Oliván [a,*], Sandra García-Medina [b], Misael Hernández-Díaz [b], Hariz Islas-Flores [a], Marcela Galar-Martínez [b], Alba Lucero García-Medina [b], José Jorge Chanona-Pérez [c], Josué David Hernández-Varela [c]

[a] *Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico*

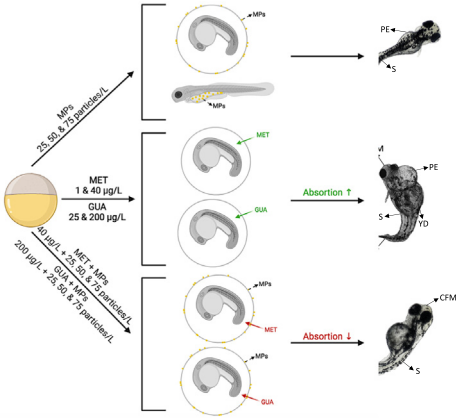
[b] *Laboratorio de Toxicología Acuática, Departamento de Farmacia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, Ciudad de México CP 07700, Mexico*

[c] *Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, Ciudad de México CP 07700, Mexico*

HIGHLIGHTS

- Plastic particles remained gathered in the chorion of the embryos.
- Microplastics avoid the entrance of both contaminants into the embryo.
- Contaminants' toxicity was mitigated by the presence of microplastics.
- Microplastics prompted the generation of body malformations in the embryos.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
Microplastics
Antidiabetic drug
Embryotoxic effect
Oxidative stress
Zebrafish

ABSTRACT

Microplastics (MPs) alone may endanger the health and fitness of aquatic species through different mechanisms. However, the harmful effects of these when mixed with other emerging contaminants require additional research. Herein, we aimed to determine whether a mixture of MPs with metformin (MET) or guanylurea (GUA) might induce embryotoxicity and oxidative stress in *Danio rerio*. Upon exposure to mixtures, our results showed MPs reduced the mortality rate of MET and GUA in embryos. Moreover, the severity and the rate of malformations were also decreased in all mixtures with MPs. Concerning oxidative stress, our findings indicated MET, GUA, MPs, and the mixtures increased the levels of lipoperoxidation, hydroperoxide content, and protein carbonyl content in *D. rerio* larvae. However, the oxidative damage induced in all mixtures was lower than that produced by both drugs alone. Thus, it is likely that the accumulation of MPs avoided the entrance of MET and GUA into the embryos. Once the embryo

* Corresponding author at: Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico.
E-mail address: lmgomezo@uaemex.mx (L.M. Gómez-Oliván).

http://dx.doi.org/10.1016/j.scitotenv.2022.158503
Received 20 June 2022; Received in revised form 29 August 2022; Accepted 30 August 2022
Available online 2 September 2022
0048-9697/© 2022 Elsevier B.V. All rights reserved.

hatched, MPs did only remain accumulated in the yolk sac of larvae and did not translocate to other organs. Our risk assessment analysis confirmed that MPs shrunk the damage produced by MET and GUA. In a nutshell, MPs mitigate the embryotoxic damage of metformin and guanylurea in *D. rerio* by blocking their entrance.

1. Introduction

Metformin (MET) is the main-oral drug for the treatment of type two diabetes (Foretz et al., 2019). However, in the last decade, its therapeutic usage has been expanded to treat other diseases such as cancer, epilepsy, COVID-19, and polycystic ovary syndrome (Saraei et al., 2019; Vazifehkhah et al., 2020; Bramante et al., 2021). As a result of the above, the consumption of MET has increased considerably in the last decade, and thus, this drug has reached and polluted multiple sources of water (Elizalde-Velázquez and Gómez-Oliván, 2020).

Previous to the discharge of MET into worldwide surface waters, this drug enters wastewater treatment plants (WWTPs), where it is biotransformed into guanylurea (GUA) (Trautwein et al., 2014; Briones et al., 2018; Poursat et al., 2019). GUA is the main transformation product of the biotic degradation of MET, and it occurs in higher concentrations in surface waters than the parental drug. Up to date, authors have reported the occurrence of MET in WWTPs effluents (0.0075–82.7 μg/L), surface waters (0.0002–33.6 μg/L), groundwater (0.01–107 μg/L), and drinking water (0.001–0.008 μg/L) (Kot-Wasik et al., 2016; Meador et al., 2016; Elliott et al., 2017, Asghar et al., 2018; Lesser et al., 2018). However, the scientific community has only assessed the presence of GUA in WWTPs effluents (0.02–810 μg/L) and surface waters (0.001–222 μg/L) (Kosma et al., 2015; Tisler & Zwiener, 2018; Posselt et al., 2018). Thus, we encourage future studies to ascertain the levels of GUA in other water matrices. Besides its higher occurrence in water settings, GUA also generates a more harmful and lethal response in aquatic organisms. Several authors, for instance, have reported that MET and GUA produce oxidative stress (Lee et al., 2019), embryotoxicity (Ussery et al., 2018;Ussery et al., 2019; Elizalde-Velázquez et al., 2021a; Elizalde-Velázquez et al., 2021b), genotoxicity (Ussery et al., 2021), and neurotoxicity (Elizalde-Velázquez et al., 2022a) in fish, but the metabolite prompts them at lower concentrations.

Microplastics (MPs) are defined as plastics of <0.2 in. (<5 mm) and are classified into two categories: primary and secondary (Iyare et al., 2020). Primary MPs are those designed for commercial use, such as the microbeads added to cosmetics and the microfibers shed from clothing and other textiles (Chang, 2015). Secondary MPs are the ones that end up in the environment as a result of the breakdown of larger plastics, such as water bottles (Vega et al., 2021). MPs concentrations range from near 0 to 5.4 × 10^5 particles/m^3 in surface, sub-surface, and deep freshwater (Lu et al., 2021a, 2021b) and from about 1 to 2.4 × 10^6 particles/m^3 along the seawater column (Ricciardi et al., 2021). Moreover, these emerging contaminants have been detected in lake and river sediments (1 to 9.5 × 10^4 particles/m^3), deep-sea sediments (0.6 to 6.6 × 10^4 particles/kg), sea-ice (2 to 17 particles/L), sea-food (0.03 to 21 items/individual), and drinking water (0.1 to 6.3 × 10^3 particles/L) (Lu et al., 2021a, 2021b; Bessa et al., 2018; Giani et al., 2019; Savoca et al., 2019; Kanhai et al., 2020; Elizalde-Velázquez and Gómez-Oliván, 2021; Piyawardhana et al., 2022). Therefore, MPs may pose real harm to aquatic organisms and human beings.

Currently, it is well known that MPs generate a lethargic swimming and feeding behavior, oxidative stress, histopathological damage, biochemical and hematological disruption, and embryotoxicity in nematodes, mussels, and fish (Chen et al., 2017; Lei et al., 2018a, 2018b; Yin et al., 2018; Lu et al., 2018; Yu et al., 2018; Elizalde-Velázquez et al., 2020; Li et al., 2020; Yin et al., 2020; Solomando et al., 2020; Rios-Fuster et al., 2021). Nonetheless, it is paramount to indicate that the effects of MPs depend upon the interaction of them with concurrent pollutants, and the dose-response relationships are altered by interacting pollutants (Agathokleous et al., 2021). For example, a huge number of articles have shown that MPs can increase the toxicity of various other emerging contaminants such as bisphenol A, herbicides, heavy metals, hydrocarbons, and polybrominated diphenyl ethers in various aquatic organisms (Bussolaro et al., 2019; Gu et al., 2020; Liu and Wang, 2020; Qiao et al., 2019; Zocchi and Sommaruga, 2019). However, several other studies have indicated that either MPs do not significantly affect the toxicity of other pollutants, or reduce the harmfulness of various hydrocarbons, metals, ions, organic pollutants, and pesticides in fresh- and sea-water organisms (Horton et al., 2018; Magara et al., 2019; Ziajahromi et al., 2019; Bartonitz et al., 2020; Sun et al., 2020; Tang et al., 2020; Zhu et al., 2020). The reasons by which co-exposure of MPs and other pollutants can generate antagonism include: 1) adsorption of contaminants onto MPs, 2) adherence of MPs to the cell surface, 3) MPs homo- and hetero- aggregation, 4) chelating capacity of MPs against some heavy metals (Sun et al., 2020; Thiagarajan et al., 2019; Wakkaf et al., 2020; Zhu et al., 2020; Ziajahromi et al., 2019).

Bearing in mind all the above data, and considering that no studies have evaluated the effects that co-exposure of MPs and MET or GUA may induce in fish, herein, we aimed to determine whether or not MPs may increase or decrease the toxicity of MET and GUA in *D. rerio*. For this purpose, we exposed *D. rerio* embryos to several concentrations of MET, GUA, MPs, and their mixtures and evaluated the mortality, malformations, and degree of oxidative damage these pollutants, alone and in combination, may generate in zebrafish embryos.

2. Method

2.1. Ethical statement

All methods and techniques described herein were approved by The Ethics and Research Committee of the Autonomous University of the State of Mexico (approval ID: 2021-135).

2.2. Compounds

Polystyrene microplastics (CAS number: 9003-53-6) and Fluorescent Polystyrene microplastics were purchased from Spherotech (Lake Forest, IL). Metformin hydrochloride (CAS number: 1115-70-4) was purchased from Toronto Research Chemicals (Toronto, ON). N-Guanylurea sulfate salt hydrate (CAS number: 207300-86-5) and all other reagents were acquired from Sigma-Aldrich (St. Louis, MO).

2.3. Confocal Laser Scanning Microscopy (CLSM)

For the study of polystyrene beads (PB) without fluorescence composition and yellow beads (YB) with noticeable fluorescence, an inverted confocal laser scanning microscope (CLSM) was used (LSM 880, Carl Zeiss, Germany). Samples were observed using a 63 × 1.4 Zeiss Plan Apochromat oil-immersion DIC M27 objective under the same methodology. A procedure based on two separated channels with a 405 nm laser for fluorescence microscopy and light transmitted microscopy for contrast observation was used. Images were acquired in a size of 2048 × 2048 pixels and stored in TIFF format. The particle size of PB and YB was evaluated by means of ImageJ software version 1.47 (http://imagej.nih.gov; National Institute of Health, Bethesda, MD, USA). Each particle was measured using the length tool of the software and the collected data was using for comparison.

2.4. Atomic Force Microscopy (AFM)

For AFM analysis, an Innova AFM was used (Veeco Instruments Inc., USA) to evaluate and compare the size of polystyrene beads (PB) and yellow beads (YB). Solutions with samples were deposited in glass slides and mounted onto an AFM stub with carbon tape for both samples. 2D and 3D height images were obtained in contact mode in air at ambient temperature using a DNP-10 tip and scan rate of 1 Hz. Image analysis was evaluated using the NanoScope Analysis v1.4 software(Bruker, USA). 2D height images were analyzed with a flattened at 0 order and the data scale was also manually adjusted according to the color bars presented in each image. All sample images were acquired in a scan size of 5 μm and values of each particle were measured using the sectioning tool in the software.

2.5. D. rerio upkeep

Several AB strain *D. rerio* adults (483 ± 10 mg, 3.5 ± 0.2 cm) were maintained in two aquaria of 50 L capacity provided with charcoal-filtered and UV-sterilized tap water. Male and female fish were kept in different aquaria, and each aquarium upheld the same temperature and light/dark cycles (27 ± 1 °C; 14 h:10 h ratio). Feeding was performed two times a day with *Artemia salina nauplii*. Water from each aquarium was renewed every other day, and quality parameters were ascertained before the restoration of the medium (Table 1).

2.6. Collection and exposure of D. rerio embryos

One night before spawning, 24 zebrafish adults were placed in four individual breeding chambers on a ratio of 2:1 (2 female:1 male). The next morning, after the onset of light, embryos were collected and rinsed according to the protocols of Varga, 2011. By using a stereomicroscope (Zeiss Stemi 305) and following the protocols of Kimmel et al., 1995, embryos were classified, and only sphere stage embryos (4 hpf) were selected for exposure.

To carry out the embryotoxicity test, 72 sphere stage embryos per group of treatment (Table 2) were selected an allocated into 24-well plates. One embryo was assigned to each well; thus, a total of three 24-well plates were used for each treatment group. All concentrations used here are environmentally relevant as they have been previously reported in water bodies (Kosma et al., 2015; Kot-Wasik et al., 2016; Meador et al., 2016; Elliott et al., 2017, Asghar et al., 2018; Lesser et al., 2018; Tisler & Zwiener, 2018; Posselt et al., 2018; Elizalde-Velázquez and Gómez-Oliván, 2020; Lu et al., 2021a, 2021b; Elizalde-Velázquez and Gómez-Oliván, 2021). All plates were kept at the same temperature (27 ± 1 °C) and light/dark (14 h:10 h ratio) conditions. All along the course of the exposure (12, 24, 48, 72, and 96 hpf), we reckon the number of dead and malformed embryos and calculated their rates. The malformation rate was set as the percentage of embryos with at least one deformation compared to the control group. A maximum-likelihood linear regression analysis was performed with mortality data to determine lethal concentration 50 (LC50) and effective concentration 50 of malformations (EC50m) with their 95 % confidence intervals ($p < 0.05$). Finally, using IBM SPSS Statistics 22 software, we carried out a plot with main alterations induced by each treatment group.

Table 1
Water quality parameters ascertained during the maintenance and reproduction of *D. rerio*.

Parameter	Value
Conductivity	370 ± 25 μS/cm
Dissolved Oxygen	9.5 ± 0.7 mg/L
Nitrite	0.026 ± 0.007 mg/L
Nitrate	2.5 ± 0.4 mg/L
pH	7.32 ± 0.08
Un-ionized ammonia	0.010 ± 0.002 mg/L

Table 2
Treatment groups evaluated herein.

Treatment	Concentration
Control	0 μg/L
MET 1	1 μg/L
MET 2	40 μg/L
GUA 1	25 μg/L
GUA 2	200 μg/L
MPs 1	25 particles/L
MPs 2	50 particles/L
MPs 3	75 particles/L
MET 2 + MPs 1	40 μg/L + 25 particles/L
MET 2 + MPs 2	40 μg/L + 50 particles/L
MET 3 + MPs 3	40 μg/L + 75 particles/L
GUA 2 + MPs 1	200 μg/L + 25 particles/L
GUA 2 + MPs 2	200 μg/L + 50 particles/L
GUA 2 + MPs3	200 μg/L + 75 particles/L

2.7. Oxidative stress assessment

For evaluation of oxidative stress biomarkers, fourteen systems, each with 1 g of sphere stage embryos, were allocated in aquaria of 5 L of capacity. Each of the fourteen systems represented each of the treatment groups used herein (Table 2). For all systems, temperature (27 ± 1 °C) and light/dark cycles (14 h:10 h ratio) were upheld without change during exposure. As at 72 hpf and 96 hpf, the antioxidant system of embryos is already working, we opted to assess the oxidative damage induced by each treatment group at those periods. Thus, half of the embryos in each aquarium were used at 72 hpf and the other half at 96 hpf. Embryos collected at 72 hpf and 96 hpf were treated according to the method of Elizalde-Velázquez et al., 2021a, 2021b. Briefly, embryos were homogenized in 1 mL of phosphate buffer solution (pH 7.4), and homogenate was split into two Eppendorf tubes. One of the tubes confined 300 μL of trichloroacetic acid (20 %) and 300 μL of homogenate (tube 1), and the other one only enclosed 700 μL of the latter (tube 2). For assessment of hydroperoxide content (HP), lipid peroxidation (LPO), and protein carbonyl content (PC), we used tube 1 and stuck to the methods described by Jiang et al., 1992, Buege and Aust, 1978, and Levine et al., 1994, respectively. Moreover, for the quantification of catalase (CAT) and superoxide dismutase (SOD), we employed the tube two and followed the techniques described by Radi et al., 1991, and Misra and Fridovich, 1972, respectively. The total protein content of each sample was determined through the Bradford method (Bradford, 1976) and used to express the results of oxidative stress biomarkers. For an in-depth description of the methods employed, see Table 1S in the supplementary material.

2.8. Compounds determination

MET and GUA concentrations were determined in water and embryos. Water samples were collected from both embryotoxicity test and oxidative stress experiment using the method described by Elizalde-Velázquez et al., 2021a, 2021b. Briefly, 140 μL of water from each of the 72 wells from each treatment group were collected and pooled to get a 10 mL sample for the embryotoxicity test. In the case of the oxidative stress experiment, 10 mL of water were directly collected from each of the fourteen formed systems. For both tests, samples were taken up at 0 and 96 hpf. Embryos from both experiments were taken up at the same endpoints as water samples and were treated following the procedure described by Łabuzek et al., 2010. Concisely, the homogenate was deproteinized with 400 μL of acetonitrile and 500 μL of MeOH added with 57 μmol/L of internal standard. It was then sieved using a 10 μm strainer and evaporated to dryness at 45 °C under a nitrogen stream. Next, we dissolved the samples with 100 μL of mobile phase and centrifuged them at 16000*g* for 15 min. Quantification was performed on an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS equipped with a Turbo V Ion spray source. Parameters of positive ESI were adjusted to the following conditions: ion source temperature 550 °C;

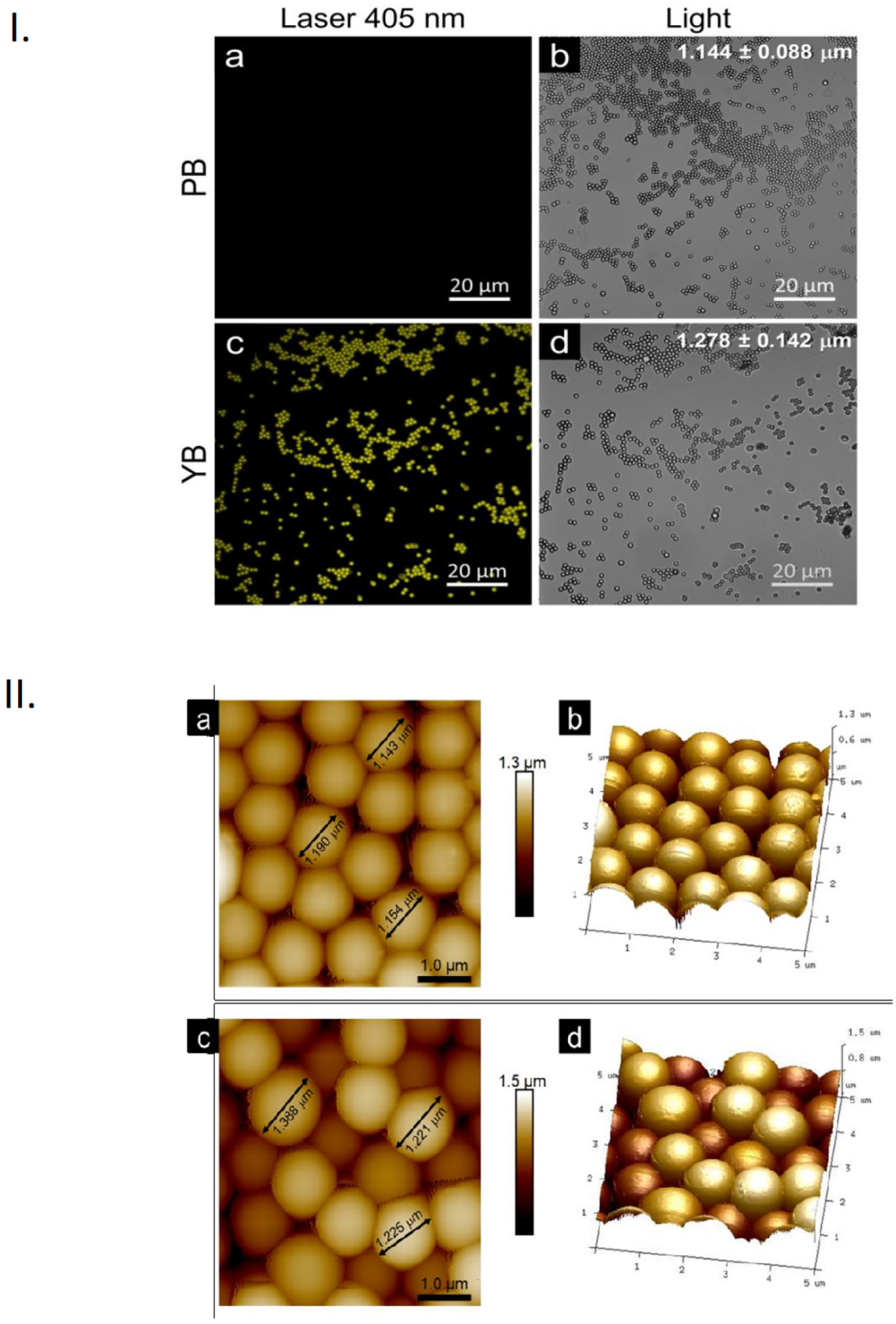


Fig. 1. CLSM images for polystyrene beads (PB) and yellow beads (YB), using a laser of 405 nm (a, c) and a light transmitted microscope (b, d). Sizes for particles are showed as insets in the images (I). Height images in 2D and 3D for polystyrene beads (a, b) and yellow beads (c, d), respectively (II).

collision gas: medium; ion spay voltage 4.5 kV; turbo gas 448 kPa; curtain gas 172 kPa; and nebulizer gas 310 kPa. Nitrogen was used as collision gas and desolvation nebulizer. An Xbridge Phenyl column (150 mm × 2.1 mm, particle size 3.5 μm) and a mobile phase consisting of water with 5 mM ammonium formate as eluent A and MeOH 100 % with 5 mM ammonium formate as eluent B were used to achieve separation. The injection volume was 50 μL and the rate was 100 μL/min. Data were evaluated with Analyst 1.6 software.

2.9. Statistical analysis

Results from all tests were uttered as the mean ± standard deviation (SD). A Shapiro-Wilk test was performed to evaluate the normality of data. Differences between means were ascertained by carrying out a Student Newman Keuls test. Moreover, differences between treatment groups and time endpoints were assessed through a two-way ANOVA test. By using R software, a principal component analysis was done to determine the correlation between variables. Using each treatment group's frequency of malformations and mortality, we performed a chi-square test to determine the relative risk of compounds alone and the mixtures. Results from compounds alone were used as the reference group for the chi-square test (PASW 18).

3. Results

3.1. CLSM and AFM images

Through CLSM, we observed MPs and confirmed the fluorescence of yellow beads. Moreover, according to our CLSM analysis, polystyrene and yellow beads reached a mean diameter of 1.144 ± 0.088 and 1.278 ± 0.142 μm, respectively (Fig. 1I). By AFM, we confirmed the bead shape of MPs. Unlike CLSM, our results of AFM indicated that the mean diameter of non-fluorescent and fluorescent were 1.132 ± 0.061 and 1.263 ± 0.130 μm, respectively (Fig. 1II). In both cases, the diameter of MPs reported by the manufacturer agrees with the ones reported herein.

3.2. Rate of MPs in D. rerio embryos

MPs did not cross the chorion of embryos; instead, these remained gathered on the surface (Fig. 2). Moreover, as the concentration of MPs increased, the higher the number of particles accumulated on the chorion. Once the embryos hatched, MPs only gathered on the yolk of fish and did not distribute to other organs. Negative control did not show any presence of MPs neither around the chorion nor in the yolk sac of embryos. Overall, embryos exposed to each of the treatment groups showed a much lower occurrence of MPs in the chorion and yolk sac of embryos regarding the positive control.

3.3. MET and GUA quantification

Concentrations of MET and GUA in the control and MPs groups remained below the limit of quantification in water gathered from both experiments and in embryos collected from oxidative stress experiments (Table 3). Overall, the concentration of MET and GUA in their respective individual treatment groups' water samples decreased between 15.6 and 18.8 % compared to the nominal concentration. However, for the mixtures, concentrations of both drugs only decreased between 13.7 and 15.7 % compared to the nominal concentration. Thus, we suggest MPs gathering in the chorion might reduce the absorption of MET and GUA into the embryos. The above mentioned agreed with our quantification of MET and GUA in

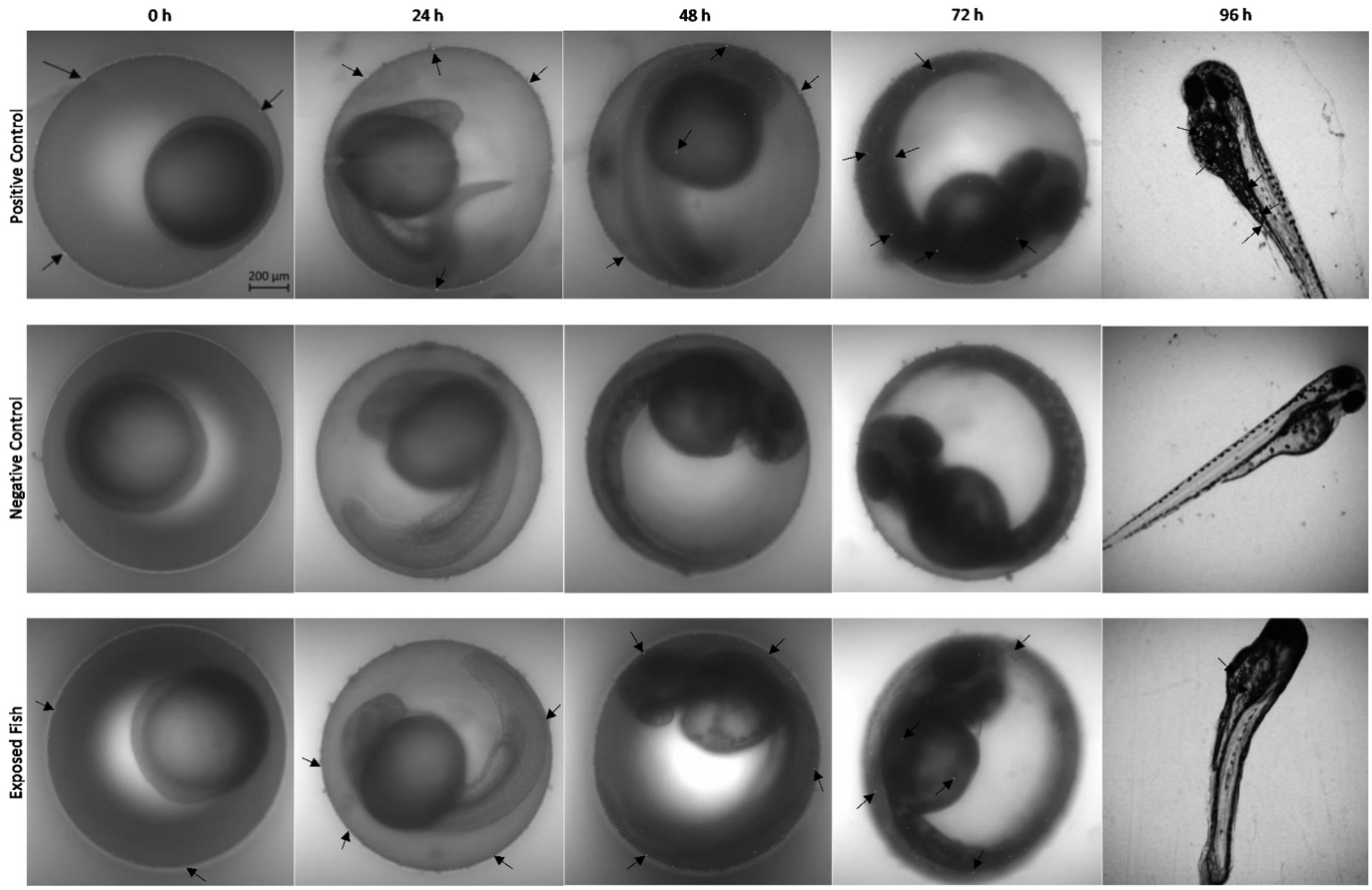


Fig. 2. Incidence of MPs in *D. rerio* embryos. Arrowheads indicate the spots where MPs accumulated in a higher proportion. To better appreciate the presence of MPs is necessary to zoom in on the photos.

Table 3
MET and GUA levels in water and embryos.

Matrix	Treatment	Nominal concentration	Real concentration (0 hpf)	Real concentration (96 hpf)
Water	Control	0 μg/L	<LOQ	<LOQ
F.E.T.	MET 1	1 μg/L	0.98 ± 0.05 μg/L	0.83 ± 0.06 μg/L
	MET 2	40 μg/L	39.89 ± 0.27 μg/L	32.81 ± 0.35 μg/L
	GUA 1	25 μg/L	24.91 ± 0.23 μg/L	21.47 ± 0.29 μg/L
	GUA 2	200 μg/L	198.97 ± 1.37 μg/L	164.90 ± 2.12 μg/L
	MPs 1	25 particles/L	<LOQ	<LOQ
	MPs 2	50 particles/L	<LOQ	<LOQ
	MPs 3	75 particles/L	<LOQ	<LOQ
	MET 2 + MPs 1	40 μg/L + 25 particles/L	39.85 ± 0.19 μg/L	36.06 ± 0.21 μg/L
	MET 2 + MPs 2	40 μg/L + 50 particles/L	39.87 ± 0.25 μg/L	35.89 ± 0.18 μg/L
	MET 3 + MPs 3	40 μg/L + 75 particles/L	39.91 ± 0.21 μg/L	36.13 ± 0.25 μg/L
	GUA 2 + MPs 1	200 μg/L + 25 particles/L	199.14 ± 1.39 μg/L	179.96 ± 1.12 μg/L
	GUA 2 + MPs 2	200 μg/L + 50 particles/L	198.91 ± 1.34 μg/L	179.88 ± 1.19 μg/L
	GUA 2 + MPs 3	200 μg/L + 75 particles/L	199.05 ± 1.30 μg/L	180.24 ± 1.10 μg/L
Water	Control	0 μg/L	<LOQ	<LOQ
O.S.	MET 1	1 μg/L	0.98 ± 0.03 μg/L	0.81 ± 0.04 μg/L
	MET 2	40 μg/L	39.94 ± 0.19 μg/L	32.42 ± 0.13 μg/L
	GUA 1	25 μg/L	24.93 ± 0.18 μg/L	20.73 ± 0.16 μg/L
	GUA 2	200 μg/L	199.31 ± 0.84 μg/L	162.14 ± 0.79 μg/L
	MPs 1	25 particles/L	<LOQ	<LOQ
	MPs 2	50 particles/L	<LOQ	<LOQ
	MPs 3	75 particles/L	<LOQ	<LOQ
	MET 2 + MPs 1	40 μg/L + 25 particles/L	39.91 ± 0.10 μg/L	34.16 ± 0.06 μg/L
	MET 2 + MPs 2	40 μg/L + 50 particles/L	39.97 ± 0.09 μg/L	33.91 ± 0.08 μg/L
	MET 3 + MPs 3	40 μg/L + 75 particles/L	39.92 ± 0.13 μg/L	33.85 ± 0.05 μg/L
	GUA 2 + MPs 1	200 μg/L + 25 particles/L	199.35 ± 0.69 μg/L	175.09 ± 0.54 μg/L
	GUA 2 + MPs 2	200 μg/L + 50 particles/L	199.43 ± 0.78 μg/L	174.89 ± 0.59 μg/L
	GUA 2 + MPs 3	200 μg/L + 75 particles/L	199.37 ± 0.74 μg/L	175.17 ± 0.51 μg/L
Embryos	Control	0 μg/L	<LOQ	<LOQ
O.S.	MET 1	1 μg/L	<LOQ	0.02 ± 0.01 μg/L
	MET 2	40 μg/L	<LOQ	0.81 ± 0.07 μg/L
	GUA 1	25 μg/L	<LOQ	0.34 ± 0.03 μg/L
	GUA 2	200 μg/L	<LOQ	7.29 ± 0.16 μg/L
	MPs 1	25 particles/L	<LOQ	<LOQ
	MPs 2	50 particles/L	<LOQ	<LOQ
	MPs 3	75 particles/L	<LOQ	<LOQ
	MET 2 + MPs 1	40 μg/L + 25 particles/L	<LOQ	0.29 ± 0.02 μg/L
	MET 2 + MPs 2	40 μg/L + 50 particles/L	<LOQ	0.31 ± 0.04 μg/L
	MET 3 + MPs 3	40 μg/L + 75 particles/L	<LOQ	0.26 ± 0.03 μg/L
	GUA 2 + MPs 1	200 μg/L + 25 particles/L	<LOQ	2.05 ± 0.11 μg/L
	GUA 2 + MPs 2	200 μg/L + 50 particles/L	<LOQ	1.89 ± 0.14 μg/L
	GUA 2 + MPs 3	200 μg/L + 75 particles/L	<LOQ	2.01 ± 0.12 μg/L

<LOQ: below limit of quantification.

the embryos. For example, we observed that the concentrations of both compounds in the embryos significantly decreased compared to quantities observed in the embryos exposed to individual compounds.

3.4. Mortality, malformation, and hatching rates

Overall, mortality and malformation rates were higher in embryos exposed to MET or GUA alone than in organisms exposed to MPs and the mixtures (Fig. 3I). In the case of MET, the rate of dead and malformed embryos was 35.5 and 57.7 % for 1.0 μg/L and 60 and 75.5 % for 40 μg/L, respectively. Meanwhile, for GUA, the percentage of not living and deformed embryos was 28.8 and 44.4 for 25 μg/L and 71.1 and 82.2 for 200 μg/L, correspondingly. As can be seen, in both treatment groups, the results were concentration-dependent, and GUA, at the highest concentration, produced more deaths and malformations in the embryos than MET. In the treatments with MPs alone and the mixtures, the number of dead and malformed embryos was kept almost constant. For example, for MPs alone, we got death rates of 42.2, 35.5, and 33.3 % for 25, 50, and 75 particles/L, respectively; while, for the MPs mixed with MET, the death rates were 42.3, 37.8, and 35.6 % for 25, 50, 75 particles/L plus 40 μg/L of MET, correspondingly. Moreover, for the mixtures with MPs and GUA, our results indicated that for 25, 50, 75 particles/L plus 200 μg/L of GUA, the mortality rates were 48.9, 42.3, and 39.1, respectively. Thus, from mixtures and MPs alone, we can observe that the number of dead embryos decreased as the concentration of MPs increased in the medium. The control group reached the highest number of hatched embryos after 96 h of exposure, followed by 1 and 40 g/L of MET alone (96.5 % and 94.4 %), 25 g/L of GUA (87.5 %), 40 g/L of MET plus 25 and 50 particles/L of MPs (84.6 and 82.1 %), 200 g/L of GUA (76.9), 40 g/L of MET plus 75 particles/L of MPs (75.8 %), 25 particles/L of MPs (65,3 %), 200 g/L of GUA plus 25 and 50 particles/L of MPs (60.8 and 68 %), 50 particles/L of MPs (51.7 %), 200 g/L of GUA plus 75 particles/L of MPs (44.7 %), and 75 particles/L (43.3 %). It is paramount to indicate that the hatching rate in all treatment groups was concentration-dependent. The foremost malformations observed in embryos exposed to all treatments were scoliosis, tail deformation, pericardial edema, yolk deformation, delay of the hatching process, and craniofacial malformation. However, the severity of malformations was different with each treatment (Fig. 3II). Bearing in mind the aforementioned, the more severe malformations were observed in fish exposed to GUA, followed by those exposed to MET, the mixtures, and MPs. In the mixtures, it is paramount to indicate that as the concentration of MPs increased in the medium, the severity of malformations decreased.

3.5. Oxidative stress test

MET and GUA exposed fish showed a significant increase in the activity of CAT and SOD, as well as in the levels of LPO, PC, and HP (Fig. 4I–V). The increase in all oxidative stress biomarkers was time- and concentration-dependent; thus, we observed significant differences between treatment groups, except for 40 g/L of MET and 25 g/L of GUA and between time

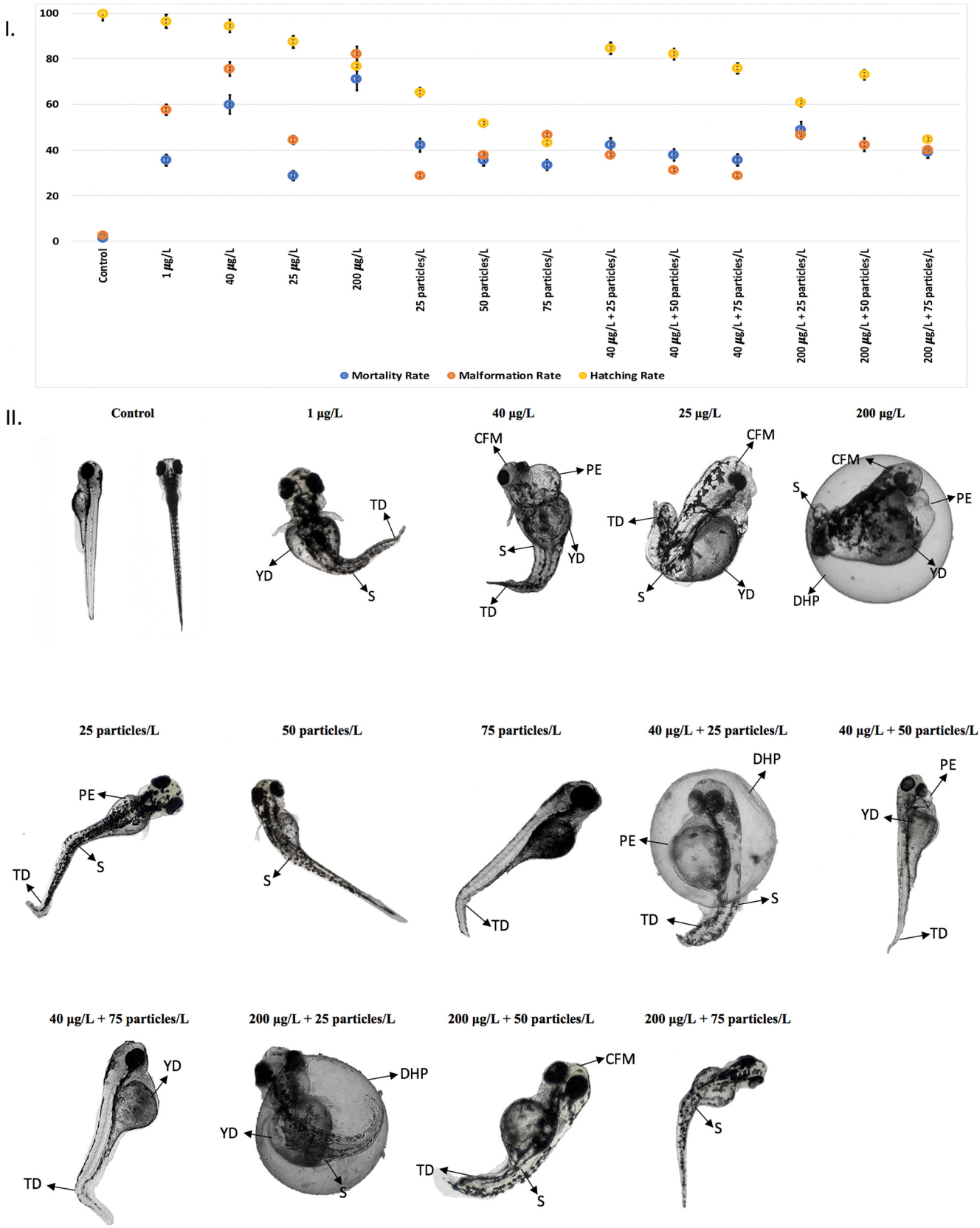
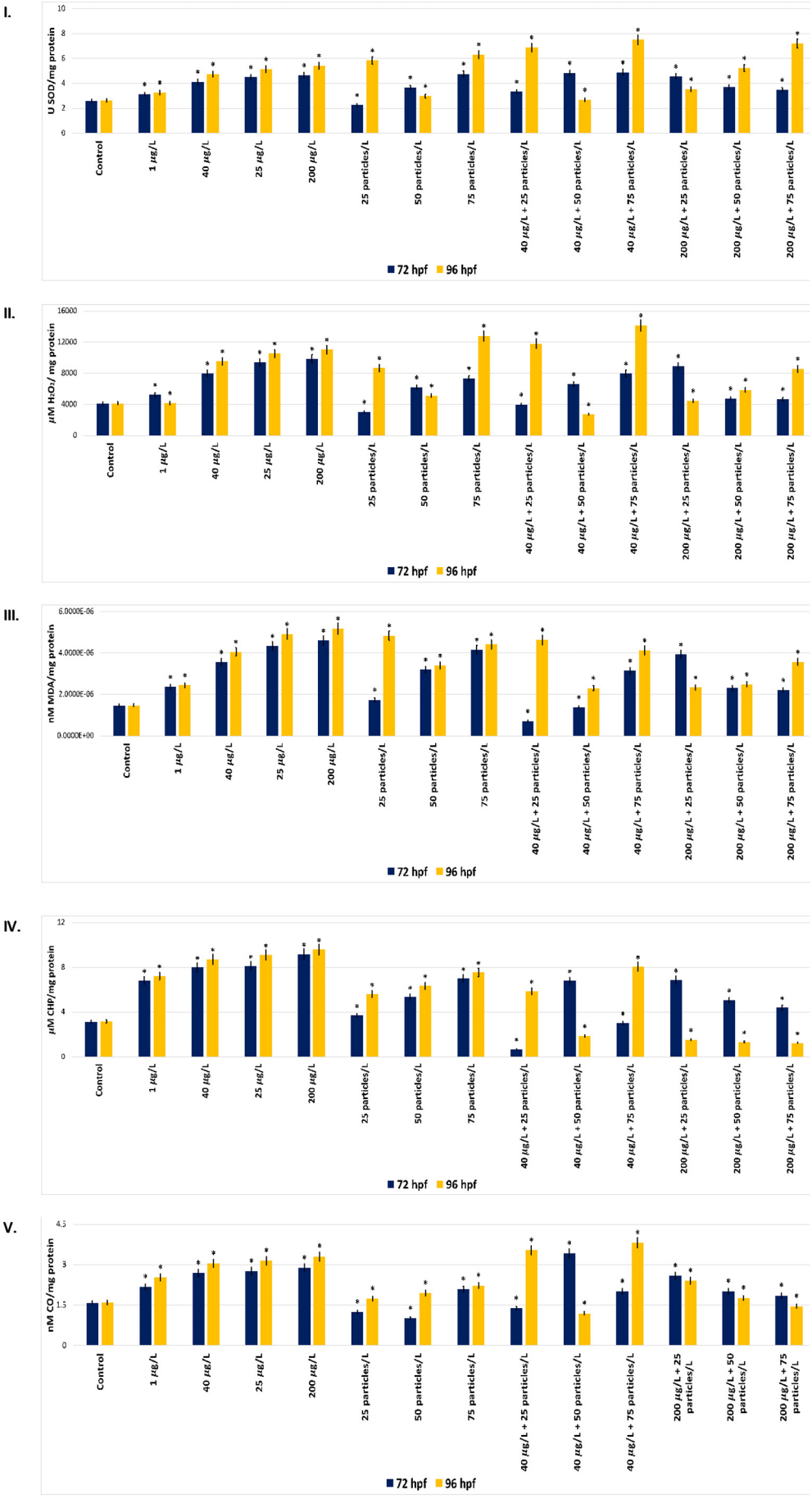


Fig. 3. Mortality, malformation, and hatching rates of *D. rerio* embryos after exposure to MPs, MET, GUA, and their mixture (I). Foremost malformations found in *D. rerio* embryos after treatment (II). TD: tail deformation, S: scoliosis, YD: yolk deformation, CFM: craniofacial malformation, PE: pericardial edema, DHP: delay of the hatching process.


I.
U SOD/mg protein
II.
µM H2O2/ mg protein
III.
nM MDA/mg protein
IV.
µM CHP/mg protein
V.
nM CO/mg protein
Control
1 µg/L
40 µg/L
25 µg/L
200 µg/L
25 particles/L
50 particles/L
75 particles/L
40 µg/L + 25 particles/L
40 µg/L + 50 particles/L
40 µg/L + 75 particles/L
200 µg/L + 25 particles/L
200 µg/L + 50 particles/L
200 µg/L + 75 particles/L
72 hpf
96 hpf

endpoints (SOD (F(13, 224) = 210.892;$p < 0.001$), CAT (F(13, 224) = 207.987; $p < 0.001$), LPO (F(13, 224) = 269.043; $p < 0.001$), PC (F(13, 224) = 146.167; $p < 0.001$), and HP (F(13, 224) = 433.168; $p < 0.001$)). Organisms exposed to MPs showed a significant reduction in the activity of CAT and levels of PC compared to embryos exposed to MET and GUA at both endpoints (CAT (F(13, 224) = 207.987; $p < 0.001$) and PC (F(13, 224) = 146.167; $p < 0.001$)). On the other hand, the activity of SOD and levels of LPO and HP, particularly after 96 h of exposure, in embryos exposed to this treatment was significantly higher than in organisms exposed to the control group (SOD (F(13, 224) = 210.892; $p < 0.001$), LPO (F(13, 224) = 269.043; $p < 0.001$), and HP (F(13, 224) = 433.168; $p < 0.001$)). Mixtures of MET and GUA showed different tendencies. In the treatments with MET and MPs, we found that the oxidative damage biomarkers augmented more at 96 hpf than at 72 hpf. For example, in the mixtures with MET and MPs, we did find these augmented the oxidative damage biomarkers more at 96 hpf than at 72 hpf. However, for GUA mixtures, most biomarkers were increased at 72 hpf. Similar to fish exposed to MPs alone, MPs with MET, at the lowest and middle concentration, reduced the activity of PC, HP, LPO, and CAT in comparison to the control group (CAT (F(13, 224) = 207.987; $p < 0.001$), LPO (F(13, 224)=; $p < 0.001$), PC (F(13, 224) = 146.167; $p < 0.001$), and HP (F(13, 224) = 433.168; $p < 0.001$)). Moreover, in the case of fish exposed to MPs and GUA, we observed a significant depletion in the levels of HP compared to the control group (HP (F(13, 224) = 433.168; $p < 0.001$)).

3.6. MET and GUA toxic effect' relative risk associated to MPs presence

Our chi-square test demonstrated that the relative risk of mixtures was significantly reduced compared to MET and GUA alone. Nonetheless, our findings did indicate there was not a significant difference between mixtures and MPs alone (Table 4).

3.7. Principal Component Analysis (PCA)

According to our PCA, the variables that contributed more in Dim. 1 were all antioxidant enzymes and oxidative damage biomarkers (see supplementary material). Moreover, for Dim. 2, the mortality and malformation rate were the variables with the highest contribution. In Dim. 3, PCA analysis indicated MET and GUA uptake had a more prominent contribution concerning other variables. Finally, HP, uptake of MET, and SOD had the most noticeable contribution in Dim. 4 (Fig. 5).

4. Discussion

MPs occurrence in surface waters is a worldwide concern issue because it threatens aquatic ecosystems, and although, in the last decade, more studies have evaluated their toxicity on marine and freshwater biota, the information on the detrimental effects of MPs and their mixture with drugs is still limited (De Sá et al., 2018; Ma et al., 2020; Agathokleous et al., 2021). Herein, we aimed to determine whether or not environmentally relevant concentrations of MPs might boost the embryotoxic effect of MET and GUA in *D. rerio* embryos. Previously, several studies demonstrated MET and GUA, at realistic concentrations, generated oxidative stress, numerous body malformations, and dead of a considerable number of embryos (Ussery et al., 2018; Ussery et al., 2019; Elizalde-Velázquez et al., 2021a, 2021b; Phillips et al., 2021). In agreement with those findings, our results demonstrated both compounds induced scoliosis, tail deformation, pericardial edema, yolk malformation, and craniofacial malformation. Concerning MPs, embryos also showed the presence of the first three abovementioned body malformations. However, it is paramount to indicate that those malformations were less frequent and severe than in embryos exposed to MET and GUA. In a previous study, De Marco et al., 2022 demonstrated that 10 μm spherical PS-MPs (200 particles/mL) altered the normal development of *D. rerio* by generating in them different malformations, mainly at the level of column and tail. Moreover, Lu et al., 2021b and Chen et al., 2022 indicated that PS-MPs round in shape and size of 327 nm (10 μg/L) and 5 μm (500 μg/L), respectively, prompted malformations at multiple

Table 4
Relative risk assessment of MET, GUA, and their mixture with MPs in *D. rerio* embryos.

	Concentration	Mortality	RR	Raw model IC 95 %	p
Metformin	40	MET	1.00		0.03
	40 + 25	MIXTURE	0.51	0.28–0.92	
	25	MPs	1.00		1.00
	40 + 25	MIXTURE	1.00	0.55–1.80	
	40	MET	1.00		0.005
	40 + 50	MIXTURE	0.42	0.23–0.77	
	50	MPs	1.00		0.76
	40 + 50	MIXTURE	1.10	0.60–2.02	
	40	MET	1.00		0.002
	40 + 75	MIXTURE	0.39	0.21–0.70	
	75	MPs	1.00		0.75
	40 + 75	MIXTURE	1.10	0.60–2.04	

	Concentration	Malformations	RR	Raw model IC 95 %	p
	40	MET	1.00		**<0.001**
	40 + 25	MIXTURE	0.20	0.10–0.37	
	25	MPs	1.00		0.21
	40 + 25	MIXTURE	1.49	0.80–2.79	
	40	MET	1.00		**<0.001**
	40 + 50	MIXTURE	0.15	0.08–0.28	
	50	MPs	1.00		0.35
	40 + 50	MIXTURE	0.74	0.40–1.38	
	40	MET	1.00		**<0.001**
	40 + 75	MIXTURE	0.13	0.07–0.25	
	75	MPs	1.00		0.01
	40 + 75	MIXTURE	0.46	0.25–0.86	

	Concentration	Mortality	RR	Raw model IC 95 %	p
Guanylurea	200	GUA	1.00		0.002
	200 + 25	MIXTURE	0.39	0.21–0.72	
	25	MPs	1.00		0.37
	200 + 25	MIXTURE	1.31	0.72–2.36	
	200	GUA	1.00		**<0.001**
	200 + 50	MIXTURE	0.30	0.16–0.55	
	50	MPs	1.00		0.36
	200 + 50	MIXTURE	1.32	0.73–2.42	
	200	GUA	1.00		**<0.001**
	200 + 75	MIXTURE	0.27	0.15–0.50	
	75	MPs	1.00		0.35
	200 + 75	MIXTURE	1.33	0.73–2.45	

	Concentration	Mortality	RR	Raw model IC 95 %	p
Guanylurea		GUA	1.00		**<0.001**
		MIXTURE	0.19	0.10–0.37	
		MPs	1.00		0.01
		MIXTURE	2.15	1.16–3.99	
		GUA	1.00		**<0.001**
		MIXTURE	0.14	0.07–0.29	
		MPs	1.00		0.76
		MIXTURE	1.10	0.60–2.00	
		GUA	1.00		**<0.001**
		MIXTURE	0.12	0.06–0.24	
		MPs	1.00		0.13
		MIXTURE	0.63	0.35–1.15	

P<0.001 associated to chi-square test

Fig. 4. Enzymatic activity of SOD (I) and CAT (II) and oxidative damage levels of LPO (III), HP (IV), and PC (V) in *D. rerio* embryos exposed to MPs, MET, GUA, and their mixtures. * indicates a significant difference against control group.

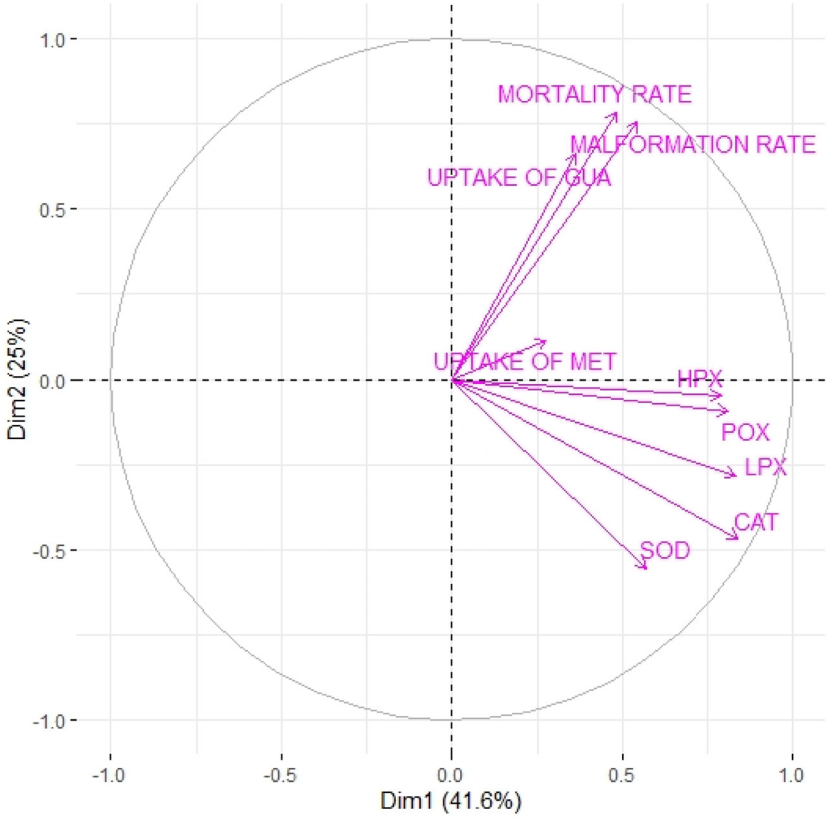


Fig. 5. Principal Component Analysis of biomarkers evaluated in *D. rerio* embryos.

regions and stages of zebrafish development. Nonetheless, in disagreement with our results and those reported by other authors, previous studies indicated that PS-MPs did not influence the development on ELS of *Salmo trutta f. fario* and *Oryzias melastigma* (Jakubowska et al., 2020; Schmieg et al., 2020; Kammann and Scharsack, 2021). Differences in results might be due to the sensitivity of species. Thomas et al., 2020, for instance, provided evidence for lesser sensitivity on ELS of *Paracentrotus lividus* to MPs compared to *Sphaerechinus granularis* and highlighted the importance of choosing a highly sensitive specie when evaluating mildly harmful materials. Even though the mechanism of embryotoxicity of MPs remains unclear, recent findings demonstrate that the adsorption of MPs to the chorion surface contributes to noticeable hypoxia, which in turn prompts malformations (Xia et al., 2022). All vertebrates rely on flow for gas exchange and respiratory gas transport during the early stages of development (De Guzman et al., 2020). PS-microbeads used herein measured 1.0 μm in size; meanwhile, the diameter of the chorionic pores in *D. rerio* measures <1.0 μm (Kim and Tanguay, 2014). Potential adherence of PS-MPs in the chorionic membrane of *D. rerio* during embryogenesis may have caused clogged pores, hindered gas exchange, and consequently, insufficient oxygen supply. Another mechanism by which PS-MPs may induce embryotoxicity in fish is the migration of toxic residual or unreacted quantities of styrene monomers (Martínez-Gómez et al., 2017). Moreover, other authors have pointed out that malformations produced by PS-MPs are due to toxic chemical additives, such as flame retardants, plasticizers, UV stabilizers, and pigments, intentionally incorporated into their surface (Gallo et al., 2018).

Reactive oxygen species (ROS) play a vital role in organisms, as they act as second messengers in cells; nevertheless, when ROS-scavenging system cannot successfully neutralize the excess of ROS, oxidative damage occurs (Colín-García et al., 2022). Recently, few authors provided evidence related to PS-MPs capacity to induce oxidative stress in different aquatic species (Lu et al., 2016; Chen et al., 2017; Wan et al., 2019; Qiao et al., 2019; Usman et al., 2021; Kaloyianni et al., 2021; Qiang and Cheng, 2021; De Marco et al., 2022; Chen et al., 2022; Solomando et al., 2022). For instance, Usman et al. (2021) showed that PS-MPs beads (5 μm) induced oxidative stress in the brain and intestine of *Oryzias javanicus* after 21 days of exposure. Moreover, in their results, the authors also indicated activities of SOD and CAT in fish exposed to the lowest concentration of MPS (100 μg/L) were significantly higher than in those exposed to the middle (500 μg/L) and high (1000 μg/L) concentrations. We observed a similar situation in our findings, as fish exposed to 25 particles/L had higher CAT and SOD activities than organisms exposed to 50 particles/L. These findings may be due to the fact that as the concentration of MPs increases, the production of ROS increases and, consequently, the antioxidant response, until it reaches a point where the organism is unable to respond. Thus, once the oxidative stress in the embryos in response to PS-MPs reaches a certain threshold, it may cause the suppression of antioxidant enzyme activity and ROS metabolism in fish. Heredia-García et al. (2022) indicated that ROS overproduction damage the histidine residues, which are essential for the activation of SOD and lead to its inhibition. The above mechanism may also explain the differences in enzymatic activity we observed at 72 and 96 hpf, specifically in the mixtures. Results in fish embryos still being inconclusive, as some authors pointed out that PS-MPs significantly decreased the activity of CAT and GSH (Wan et al., 2019); meanwhile, others indicated that either the levels of CAT (Chen et al., 2017) or the gene expression of sod1, sod2, cat, and gst augmented on larvae of *D. rerio* (De Marco et al., 2022; Chen et al., 2022). Our results agree with the latest, as we observed that PS-MPs significantly increased the activity of CAT and SOD. Moreover, we also showed that PS-MPs boosted the formation of lipid peroxides, hydroperoxides, and carbonyls. Thus, PS-MPs are capable of generating oxidative stress on ELS of fish. Up to date, data related to the mechanism by which PS-MPs prompt the production of ROS on ELS of fish is scarce; nonetheless, authors have suggested it might be the result of an immune response (Veneman et al., 2017) or a disruption in the catabolism of several oxidative stress-related metabolites (Wan et al., 2019). Regardless of the

pathway that leads to the production of ROS, further investigation is needed to clarify how PS-MPs prompt oxidative stress in fish embryos. Concerning MET and GUA oxidative stress response, our results indicated both compounds prompted the production of ROS, which in turn led to high levels of LPX, HP, and PC in embryos. Concordantly with our findings, previous studies have indicated both substances can disrupt the redox status of embryos and fish, triggering an oxidative stress response (Lee et al., 2019; Elizalde-Velázquez et al., 2021a, 2021b; Elizalde-Velázquez et al., 2022a, 2022b). In addition, Lee et al., 2019 suggested that the inhibition of mitochondrial complex I by MET may disrupt the electron flow and cause superoxide generation by an FMN reduction. However, further studies are needed to comprehend the mechanism by which this drug prompts the production of superoxide in the mitochondria.

Overall, our findings demonstrate that PS-MPs significantly reduced the embryotoxic response of MET and GUA. Moreover, we also observed MPs considerably prevent the entrance of MET and GUA into the embryos. Therefore, we suggest MPs aggregation in the chorion surface led to an antagonist response in the embryos. This hypothesis agrees with previous findings, which demonstrated MET did not sorb to PS, polyamide (PA), and polypropylene (PP) microplastics (Goedecke et al., 2017). According to the authors, it might occur due to the double positive charge MET carries at pH below <9. The above leads to an increased solubility in water and inhibits interaction with non-polar microplastics. However, the literature does not refer to the behavior of GUA, so it is necessary to carry out studies to clarify what happens with this metabolite. Previous studies have been inconclusive concerning whether or not PS-MPs enhance or decrease the toxic response of other contaminants during co-exposure. For example, recently, Chen et al., 2022 showed PS-MPs can boost the harmful effects of Cd on growth, oxidative damage, and apoptosis in ELS of *D. rerio*. However, other prior studies indicated that PS-MPs mitigate or decrease the toxic effects of phenanthrene, triphenyl phosphate, and sulfamethoxazole in *D. rerio* and *Oryzias melastigma* embryos (Li et al., 2020; Zhang et al., 2021; Lu et al., 2021b). Overall our results highlight that environmentally relevant concentrations of PS-MPs (1 μm) alone can disrupt the development and oxidative status of *D. rerio* embryos. Nonetheless, when zebrafish embryos are co-exposed to MPs and MET or GUA, PS-MPs decrease the toxicity of the latter.

5. Conclusion

The present study assessed the detrimental effects of MPs alone and in combination with MET or GUA. In summary, all treatment groups prompted the generation of body malformations in *D. rerio* embryos. Scoliosis, tail deformation, and pericardial edema were the main malformations observed after 96 h of exposure. Besides the alterations in the normal development of the embryos, our findings also showed all treatment groups induced oxidative stress by disrupting the levels of SOD, CAT, LPO, PC, and HP. Interestingly, MPs significantly decreased the embryotoxic response of MET and GUA after co-exposure. According to our data, MPs remain gathered on the chorion surface, preventing the uptake of MET and GUA into the embryo. Therefore, we suggest aggregation of MPs leads to mitigation of the toxic response of these two compounds.

CRediT authorship contribution statement

Gustavo Axel Elizalde-Velázquez, Misael Hernández-Díaz, and Josué David Hernández-Varela performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception.

Leobardo Manuel Gómez-Oliván, Gustavo Axel Elizalde-Velázquez, Sandra García-Medina and Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from Marcela Galar-Martínez, Alba Lucero García-Medina and José Jorge Chanona-Pérez.

Data availability

Data will be made available on request.

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.

Acknowledgements

This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Project 300727).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158503.

References

Agathokleous, E., Iavicoli, I., Barceló, D., Calabrese, E.J., 2021. Micro/nanoplastics effects on organisms: a review focusing on 'dose'. J. Hazard. Mater. 417, 126084.
Asghar, M.A., Zhu, Q., Sun, S., Shuai, Q., 2018. Suspect screening and target quantification of human pharmaceutical residues in the surface water of Wuhan, China, using UHPLC-Q-Orbitrap HRMS. Sci. Total Environ. 635, 828–837.
Bartonitz, A., Anyanwu, I.N., Geist, J., Imhof, H.K., Reichel, J., Graßmann, J., Drewes, J.E., Beggel, S., 2020. Modulation of PAH toxicity on the freshwater organismG. Roeseli by microparticles. Environ. Pollut. 260, 1139999.
Bessa, F., Barría, P., Neto, J.M., Frias, J.P., Otero, V., Sobral, P., Marques, J.C., 2018. Occurrence of microplastics in commercial fish from a natural estuarine environment. Mar. Pollut. Bull. 128, 575–584.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.
Bramante, C.T., Buse, J., Tamaritz, L., Palacio, A., Cohen, K., Vojta, D., Tignanelli, C.J., 2021. Outpatient metformin use is associated with reduced severity of COVID-19 disease in adults with overweight or obesity. J. Med. Virol. 93 (7), 4273–4279.
Briones, R.M., Zhuang, W.Q., Sarmah, A.K., 2018. Biodegradation of metformin and guanylurea by aerobic cultures enriched from sludge. Environ. Pollut. 243, 255–262.
Buege, J.A., Aust, S.D., 1978. [30] Microsomal lipid peroxidation. Methods in Enzymology. 52, pp. 302–310 Academic press.
Bussolaro, D., Wright, S.L., Schnell, S., Schirmer, K., Bury, N.R., Arlt, V.M., 2019. Co-Chang, M. (2015). Reducing microplastics from facial exfoliating cleansers in wastewater through treatment versus consumer product decisions. Mar. Pollut. Bull. 101 (1), 330–333.
Chang, M., 2015. Reducing microplastics from facial exfoliating cleansers in wastewater through treatment versus consumer product decisions. Mar. Pollut. Bull. 101 (1), 330–333.
Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Sci. Total Environ. 584, 1022–1031.
Chen, X., Peng, L.B., Wang, D., Zhu, Q.L., Zheng, J.L., 2022. Combined effects of polystyrene microplastics and cadmium on oxidative stress, apoptosis, and GH/IGF axis in zebrafish early life stages. Science of The Total Environment 813, 152514 chronic toxicity of three antimicrobial agents to Daphnia magna in the presence/ absence of microplastyrene. Environ. Pollut. 263, 114551.
Colín-García, K., Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., Islas-Flores, H., García-Medina, S., Galar-Martínez, M., 2022. Acute exposure to environmentally relevant concentrations of sucralose disrupts embryonic development and leads to an oxidative stress response in Danio rerio. Sci. Total Environ. 829, 154689.
De Marco, G., Conti, G.O., Giannetto, A., Cappello, T., Galati, M., Iaria, C., Maisano, M., 2022. Embryotoxicity of polystyrene microplastics in zebrafish Danio rerio. Environ. Res. 208, 112552.
De Guzman, M.C., Chua, P.A.P., Sedano, F.S., 2020. Embryotoxic and teratogenic effects of polyethylene microbeads found in facial wash products in Zebrafish (Danio rerio) using the Fish Embryo Acute Toxicity Test. bioRxiv.
De Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029–1039.
Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., 2020. Occurrence, toxic effects and removal of metformin in the aquatic environments in the world: recent trends and perspectives. Sci. Total Environ. 702, 134924.
Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., 2021. Microplastics in aquatic environments: a review on occurrence, distribution, toxic effects, and implications for human health. Sci. Total Environ. 780, 146551.

Elizalde-Velázquez, A., Carcano, A.M., Crago, J., Green, M.J., Shah, S.A., Cañas-Carrell, J.E., 2020. Translocation, trophic transfer, accumulation and depuration of polystyrene microplastics in Daphnia magna and Pimephales promelas. Environ. Pollut., 113937

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., García-Medina, S., Islas-Flores, H., Hernández-Navarro, M.D., Galar-Martínez, M., 2021a. Antidiabetic drug metformin disrupts the embryogenesis in zebrafish through an oxidative stress mechanism. Chemosphere 285, 131213.

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., Islas-Flores, H., Hernández-Navarro, M.D., García-Medina, S., Galar-Martínez, M., 2021b. Oxidative stress as a potential mechanism by which guanylurea disrupts the embryogenesis of Danio rerio. Sci. Total Environ. 799, 149432.

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., Rosales-Pérez, K.E., Hernández, J.M.O., García-Medina, S., Islas-Flores, H., Galar-Martínez, M., 2022a. Chronic exposure to environmentally relevant concentrations of guanylurea induces neurotoxicity of Danio rerio adults. Sci. Total Environ. 153095

Elizalde-Velázquez, G.A., Gómez-Oliván, L.M., García-Medina, S., Rosales-Pérez, K.E., Orozco-Hernández, J.M., Islas-Flores, H., Galar-Martínez, M., Hernández-Navarro, M.D., 2022b. Chronic exposure to realistic concentrations of metformin prompts a neurotoxic response in Danio rerio adults. Sci. Total Environ. 157888

Elliott, S.M., Brigham, M.E., Lee, K.E., Banda, J.A., Choy, S.J., Gefell, D.J., Jorgenson, Z.G., 2017. Contaminants of emerging concern in tributaries to the Laurentian Great Lakes: I. Patterns of occurrence. PloS One 12 (9), e0182868 Exposure to polystyrene plastic beads and polycyclic aromatic hydrocarbon contaminants in fish gill (RTgill-W1) and intestinal (RTgutGC) epithelial cells derived from rainbow trout (Oncorhynchus mykiss). Environ. Pollut. 248, 706–714.

Foretz, M., Guigas, B., Viollet, B., 2019. Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus. Nat. Rev. Endocrinol. 15 (10), 569–589.

Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., Nadal, A., Romano, D., 2018. Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. Environ. Sci. Eur. 30 (1), 1–14.

Giani, D., Baini, M., Galli, M., Casini, S., Fossi, M.C., 2019. Microplastics occurrence in edible fish species (Mullus barbatus and Merluccius merluccius) collected in three different geographical sub-areas of the Mediterranean Sea. Mar. Pollut. Bull. 140, 129–137.

Goedecke, C., Mülow-Stollin, U., Hering, S., Richter, J., Piechotta, C., Paul, A., Braun, U., 2017. A first pilot study on the sorption of environmental pollutants on various microplastic materials. J. Environ. Anal. Chem. 4 (1), 1000191.

Gu, H., Wei, S., Hu, M., Wei, H., Wang, X., Shang, Y., Li, L., Shi, H., Wang, Y., 2020. Microplastics aggravate the adverse effects of BDE-47 on physiological and defense performance in mussels. J. Hazard. Mater. 398, 122909. https://doi.org/10.1016/j.jhazmat.2020.122909.

Heredia-García, G., Gómez-Oliván, L.M., Elizalde-Velázquez, G.A., Cardoso-Vera, J.D., Orozco-Hernández, J.M., Rosales-Pérez, K.E., García-Medina, S., Islas-Flores, H., Galar-Martínez, M., Dublán-García, O., 2022. Multi-biomarker approach and IBR index to evaluate the effects of bisphenol A on embryonic stages of zebrafish (Danio rerio). Environ. Toxicol. Pharmacol. 94, 103925.

Horton, A.A., Vijver, M.G., Lahive, E., Spurgeon, D.J., Svendsen, C., Heutink, R., van Bodegom, P.M., Baas, J., 2018. Acute toxicity of organic pesticides to Daphnia magna is unchanged by co-exposure to polystyrene microplastics. Ecotoxicol. Environ. Saf. 166, 26–34.

Iyare, P.U., Ouki, S.K., Bond, T., 2020. Microplastics removal in wastewater treatment plants: a critical review. Environ. Sci.: Water Res. Technol. 6 (10), 2664–2675.

Jakubowska, M., Białowąs, M., Stankevičiūtė, M., Chomiczewska, A., Pažusienė, J., Jonko-Sobuś, K., Urban-Malinga, B., 2020. Effects of chronic exposure to microplastics of different polymer types on early life stages of sea trout Salmo trutta. Sci. Total Environ. 740, 139922.

Jiang, Z.Y., Hunt, J.V., Wolff, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. 202 (2), 384–389.

Kaloyianni, M., Bobori, D.C., Xanthopoulou, D., Malioufa, G., Sampsonidis, I., Kalogiannis, S., Feidantsis, K., Kastrinaki, G., Dimitriadi, A., Koumoundouros, G., Lambropoulou, D.A., 2021. Toxicity and functional tissue responses of two freshwater fish after exposure to polystyrene microplastics. Toxics 9 (11), 289.

Kanhai, L.D.K., Gardfeldt, K., Krumpen, T., Thompson, R.C., O'Connor, I., 2020. Microplastics in sea ice and seawater beneath ice floes from the Arctic Ocean. Sci. Rep. 10 (1), 1–11.

Kammann, U., Scharsack, J.P., 2021. Exposure to microplastic fibers does not change fish early life stage development of three-spined sticklebacks (Gasterosteus aculeatus). Microplastics Nanoplastics 1 (1), 1–9.

Kim, K.T., Tanguay, R.L., 2014. The role of chorion on toxicity of silver nanoparticles in the embryonic zebrafish assay. Environ. Health Toxicol. 29.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203 (3), 253–310.

Kosma, C.I., Lambropoulou, D.A., Albanis, T.A., 2015. Comprehensive study of the antidiabetic drug metformin and its transformation product guanylurea in Greek wastewaters. Water Res. 70, 436–448.

Kot-Wasik, A., Jakimska, A., Śliwka-Kaszyńska, M., 2016. Occurrence and seasonal variations of 25 pharmaceutical residues in wastewater and drinking water treatment plants. Environ. Monit. Assess. 188 (12), 1–13.

Łabuzek, K., Suchy, D., Gabryel, B., Bielecka, A., Liber, S., Okopień, B., 2010. Quantification of metformin by the HPLC method in brain regions, cerebrospinal fluid and plasma of rats treated with lipopolysaccharide. Pharmacol. Rep. 62 (5), 956–965.

Lee, J.W., Shin, Y.J., Kim, H., Kim, H., Kim, J., Min, S.A., Park, K., 2019. Metformin-induced endocrine disruption and oxidative stress of Oryzias latipes on two-generational condition. J. Hazard. Mater. 367, 171–181.

Lei, L., Liu, M., Song, Y., Lu, S., Hu, J., Cao, C., He, D., 2018. Polystyrene (nano) microplastics cause size-dependent neurotoxicity, oxidative damage and other adverse effects in Caenorhabditis elegans. Environ. Sci.: Nano 5 (8), 2009–2020.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. Sci. Total Environ. 619, 1–8.

Lesser, L.E., Mora, A., Moreau, C., Mahlknecht, J., Hernández-Antonio, A., Ramírez, A.I., Barrios-Piña, H., 2018. Survey of 218 organic contaminants in groundwater derived from the world's largest untreated wastewater irrigation system: Mezquital Valley, Mexico. Chemosphere 198, 510–521.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. [37] Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology. 233, pp. 346–357 Exposure to polystyrene plastic beads and polycyclic aromatic hydrocarbon contaminants in fish gill (RTgill-W1) and intestinal (RTgutGC) epithelial cells derived from rainbow trout (Oncorhynchus mykiss). Environ. Pollut. 248, 706–714.

Li, Y., Wang, J., Yang, G., Lu, L., Zheng, Y., Zhang, Q., Ru, S., 2020. Low level of polystyrene microplastics decreases early developmental toxicity of phenanthrene on marine medaka (Oryzias melastigma). J. Hazard. Mater. 385, 121586.

Liu, X., Wang, J., 2020. Algae (Raphidocelis subcapitata) mitigate combined toxicity of microplastic and lead on Ceriodaphnia dubia. Front. Environ. Sci. Eng. 14, 1–10.

Lu, J., Struewing, I., Vereen, E., Kirby, A.E., Levy, K., Moe, C., Ashbolt, N., 2016. Molecular Detection of Legionella spp. and their associations with Mycobacterium spp., Pseudomonas aeruginosa and amoeba hosts in a drinking water distribution system. J. Appl. Microbiol. 120 (2), 509–521.

Lu, K., Qiao, R., An, H., Zhang, Y., 2018. Influence of microplastics on the accumulation and chronic toxic effects of cadmium in zebrafish (Danio rerio). Chemosphere 202, 514–520.

Lu, H.C., Ziajahromi, S., Neale, P.A., Leusch, F.D., 2021. A systematic review of freshwater microplastics in water and sediments: recommendations for harmonisation to enhance future study comparisons. Sci. Total Environ. 781, 146693.

Lu, J., Wu, J., Gong, L., Cheng, Y., Yuan, Q., He, Y., 2021. Combined toxicity of polystyrene microplastics and sulfamethoxazole on zebrafish embryos. Environ. Sci. Pollut. Res. 1–10.

Ma, J., Niu, X., Zhang, D., Lu, L., Ye, X., Deng, W., Li, Y., Lin, Z., 2020. High levels of microplastic pollution in aquaculture water of fish ponds in the Pearl River Estuary of Guangzhou, China. Sci. Total Environ. 744, 140679.

Magara, G., Khan, F.R., Pinti, M., Syberg, K., Inzirillo, A., Elia, A.C., 2019. Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (Mytilus edulis). J. Toxicol. Environ. Health Part A Curr. 82, 616–625.

Martínez-Gómez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Mar. Environ. Res. 130, 69–76.

Meador, J.P., Yeh, A., Young, G., Gallagher, E.P., 2016. Contaminants of emerging concern in a large temperate estuary. Environ. Pollut. 213, 254–267.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247 (10), 3170–3175.

Phillips, J., Akemann, C., Shields, J.N., Wu, C.C., Meyer, D.N., Baker, B.B., Pitts, D.K., Baker, T.R., 2021. Developmental phenotypic and transcriptomic effects of exposure to nanomolar levels of metformin in zebrafish. Environ. Toxicol. Pharmacol. 87, 103716.

Piyawardhana, N., Weerathunga, V., Chen, H.S., Guo, L., Huang, P.J., Ranatunga, R.R.M.K.P., Hung, C.C., 2022. Occurrence of microplastics in commercial marine dried fish in Asian countries. J. Hazard. Mater. 423, 127093.

Posselt, M., Jaeger, A., Schaper, J.L., Radke, M., Benskin, J.P., 2018. Determination of polar organic micropollutants in surface and pore water by high-resolution sampling-direct injection-ultra high performance liquid chromatography- tandem mass spectrometry. Environ. Sci. Process Impacts 20 (12), 1716–1727.

Poursat, B.A., van Spanning, R.J., Braster, M., Helmus, R., de Voogt, P., Parsons, J.R., 2019. Biodegradation of metformin and its transformation product, guanylurea, by natural and exposed microbial communities. Ecotoxicol. Environ. Saf. 182, 109414.

Qiang, L., Cheng, J., 2021. Exposure to polystyrene microplastics impairs gonads of zebrafish (Danio rerio). Chemosphere 263, 128161.

Qiao, R., Deng, Y., Zhang, S., Wolosker, M.B., Zhu, Q., Ren, H., Zhang, Y., 2019. Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish. Chemosphere 236, 124334.

Radi, R., Turrens, J.F., Chang, L.Y., Bush, K.M., Crapo, J.D., Freeman, B.A., 1991. Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266 (32), 22028–22034.

Ricciardi, M., Pironti, C., Motta, O., Miele, Y., Proto, A., Montano, L., 2021. Microplastics in the aquatic environment: occurrence, persistence, analysis, and human exposure. Water 13 (7), 973.

Rios-Fuster, B., Arechavala-Lopez, P., García-Marcos, K., Alomar, C., Compa, M., Álvarez, E., Deudero, S., 2021. Experimental evidence of physiological and behavioral effects of microplastic ingestion in Sparus aurata. Aquat. Toxicol. 231, 105737.

Saraei, P., Asadi, I., Kakar, M.A., Moradi-Kor, N., 2019. The beneficial effects of metformin on cancer prevention and therapy: a comprehensive review of recent advances. Cancer Manag. Res. 11, 3295.

Savoca, S., Capillo, G., Mancuso, M., Bottari, T., Crupi, R., Branca, C., Spanò, N., 2019. Microplastics occurrence in the tyrrhenian waters and in the gastrointestinal tract of two congener species of seabreams. Environ. Toxicol. Pharmacol. 67, 35–41.

Schmieg, H., Burmester, J.K., Krais, S., Ruhl, A.S., Tisler, S., Zwiener, C., Triebskorn, R., 2020. Interacting effects of polystyrene microplastics and the antidepressant amitriptyline on early life stages of brown trout (Salmo trutta f. fario). Water 12 (9), 2361.

Solomando, A., Capó, X., Alomar, C., Álvarez, E., Compa, M., Valencia, J.M., Sureda, A., 2020. Long-term exposure to microplastics induces oxidative stress and a pro-inflammatory response in the gut of Sparus aurata linnaeus, 1758. Environ. Pollut. 266, 115295.

Solomando, A., Cohen-Sánchez, A., Box, A., Montero, I., Pinya, S., Sureda, A., 2022. Microplastic presence in the pelagic fish, Seriola dumerili, from Balearic Islands (Western Mediterranean), and assessment of oxidative stress and detoxification biomarkers in liver. Environ. Res. 212, 113369.

Sun, C., Zhang, W., Ding, R., Wang, J., Yao, L., 2020. Mechanism of low concentrations of polystyrene microplastics influence the cytotoxicity of ag ions to Escherichia coli. Chemosphere 253, 126705.

Tang, Y., Zhou, W., Sun, S., Du, X., Han, Y., Shi, W., Liu, G., 2020. Immunotoxicity and neurotoxicity of bisphenol a and microplastics alone or in combination to a bivalve species, Tegillarca granosa. Environ. Pollut. 265, 115115.

Thiagarajan, V., Iswarya, V., P, A.J., Seenivasan, R., Chandrasekaran, N., Mukherjee, A., 2019. Influence of differently functionalized polystyrene microplastics on the toxic effects of P25 TiO2 NPs towards marine algae Chlorella sp. Aquat. Toxicol. 207, 208–216.

Thomas, P.J., Oral, R., Pagano, G., Tez, S., Toscanesi, M., Ranieri, P., Lyons, D.M., 2020. Mild toxicity of polystyrene and polymethylmethacrylate microplastics in Paracentrotus lividus early life stages. Mar. Environ. Res. 161, 105132.

Tisler, S., Zwiener, C., 2018. Formation and occurrence of transformation products of metformin in wastewater and surface water. Sci. Total Environ. 628, 1121–1129.

Trautwein, C., Berset, J.D., Wolschke, H., Kümmerer, K., 2014. Occurrence of the antidiabetic drug metformin and its ultimate transformation product guanylurea in several compartments of the aquatic cycle. Environ. Int. 70, 203–212.

Usman, S., Abdull Razis, A.F., Shaari, K., Amal, M.N.A., Saad, M.Z., Mat Isa, N., Nazarudin, M.F., 2021. Polystyrene microplastics exposure: an insight into multiple organ histological alterations, oxidative stress and neurotoxicity in Javanese Medaka Fish (Oryzias javanicus Bleeker, 1854). Int. J. Environ. Res. Public Health 18 (18), 9449.

Ussery, E., Bridges, K.N., Pandelides, Z., Kirkwood, A.E., Bonetta, D., Venables, B.J., Holdway, D., 2018. Effects of environmentally relevant metformin exposure on japanese medaka (Oryzias latipes). Aquat. Toxicol. 205, 58–65.

Ussery, E., Bridges, K.N., Pandelides, Z., Kirkwood, A.E., Guchardi, J., Holdway, D., 2019. Developmental and full-life cycle exposures to guanylurea and guanylurea-metformin mixtures results in adverse effects on japanese medaka (Oryzias latipes). Environ. Toxicol. Chem. 38 (5), 1023–1028.

Ussery, E.J., Nielsen, K.M., Simmons, D., Pandelides, Z., Mansfield, C., Holdway, D., 2021. An 'omics approach to investigate the growth effects of environmentally relevant concentrations of guanylurea exposure on japanese medaka (Oryzias latipes). Aquat. Toxicol. 232, 105761.

Varga, Z.M., 2011. Aquaculture and husbandry at the zebrafish international resource center. Methods Cell Biol. 104, 453–478.

Vazifehkhah, S., Khanizadeh, A.M., Mojarad, T.B., Nikbakht, F., 2020. The possible role of progranulin on anti-inflammatory effects of metformin in temporal lobe epilepsy. J. Chem. Neuroanat. 109, 101849.

Vega, G.C., Gross, A., Birkved, M., 2021. The impacts of plastic products on air pollution-a simulation study for advanced life cycle inventories of plastics covering secondary microplastic production. Sustain. Prod. Consum. 28, 848–865.

Veneman, W.J., Spaink, H.P., Brun, N.R., Bosker, T., Vijver, M.G., 2017. Pathway analysis of systemic transcriptome responses to injected polystyrene particles in zebrafish larvae. Aquat. Toxicol. 190, 112–120.

Wakkaf, T., Allouche, M., Harrath, A.H., Mansour, L., Alwasel, S., Mohamed Thameemul Ansari, K.G., Beyrem, H., Sellami, B., Boufahja, F., 2020. The individual and combined effects of cadmium, polyvinyl chloride (PVC) microplastics and their polyalkylamines modified forms on meiobenthic features in a microcosm. Environ. Pollut. 266, 115263.

Wan, Z., Wang, C., Zhou, J., Shen, M., Wang, X., Fu, Z., Jin, Y., 2019. Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. Chemosphere 217, 646–658.

Xia, B., Sui, Q., Du, Y., Wang, L., Jing, J., Zhu, L., Xing, B., 2022. Secondary PVC microplastics are more toxic than primary PVC microplastics to oryzias melastigma embryos. J. Hazard. Mater. 424, 127421.

Yin, L., Chen, B., Xia, B., Shi, X., Qu, K., 2018. Polystyrene microplastics alter the behavior, energy reserve and nutritional composition of marine jacopever (Sebastes schlegelii). J. Hazard. Mater. 360, 97–105.

Yin, C., Yang, X., Zhao, T., Watson, P., Yang, F., Liu, H., 2020. Changes of the acute and.

Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile Eriocheir sinensis and oxidative stress effects in the liver. Aquat. Toxicol. 200, 28–36.

Zhang, Y.T., Chen, M., He, S., Fang, C., Chen, M., Li, D., Mu, J., 2021. Microplastics decrease the toxicity of triphenyl phosphate (TPhP) in the marine medaka (Oryzias melastigma) larvae. Sci. Total Environ. 763, 143040.

Zhu, X., Zhao, W., Chen, X., Zhao, T., Tan, L., Wang, J., 2020. Growth inhibition of the microalgae Skeletonema costatum under copper nanoparticles with microplastic exposure. Mar. Environ. Res. 158, 105005.

Ziajahromi, S., Kumar, A., Neale, P.A., Leusch, F.D.L., 2019. Effects of polyethylene microplastics on the acute toxicity of a synthetic pyrethroid to midge larvae (Chironomus tepperi) in synthetic and river water. Sci. Total Environ. 671, 971–975.

Zocchi, M., Sommaruga, R., 2019. Microplastics modify the toxicity of glyphosate on Daphnia magna. Sci. Total Environ. 697, 134194. https://doi.org/10.1016/j. scitotenv.2019.134194.