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Toxicological effects induced on early life stages of zebrafish (*Danio rerio*) after an acute exposure to microplastics alone or co-exposed with copper

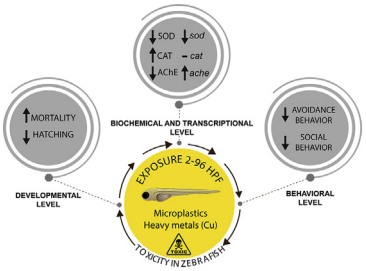
Dércia Santos [a, *], Luís Félix [a, b], Ana Luzio [a], Susana Parra [a], Edna Cabecinha [a], Juan Bellas [c], Sandra M. Monteiro [a]

[a] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, CITAB, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5000-801, Vila Real, Portugal
[b] Institute for Research and Innovation in Health, Laboratory Animal Science, Institute of Molecular and Cell Biology, University of Porto, Rua Alfredo Allen, nº 208, 4200-135, Porto, Portugal
[c] Centro Oceanográfico de Vigo, Instituto Español de Oceanografía, Subida a Radio Faro 50, 36390, Vigo, Spain

HIGHLIGHTS

- Toxicological effects of MPs and Cu were studied on zebrafish early life stages.
- Co-exposure of MPs and Cu induced oxidative stress.
- Antagonism between MPs and Cu was observed in glutathione levels and gene expression.
- An inhibition of AChE was observed in Cu and Cu + MPs exposed larvae.
- Co-exposure of MPs with Cu induced an avoidance behavior disruption.

GRAPHICAL ABSTRACT



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ABSTRACT

Data about the toxicological interactions of MPs and heavy metals in biota is limited, particularly in fish early life stages. This study aimed to evaluate the toxicological effects of MPs and copper (Cu), alone or combined, in zebrafish early life stages. Embryos were exposed from 2 until 96-h post-fertilization (hpf) to MPs (2 mg/L), three sub-lethal concentrations of Cu (15, 60 and 125 μg/L) and binary mixtures containing Cu and MPs (Cu15+MPs, Cu60+MPs, Cu125+MPs). Lethal and sub-lethal parameters, histopathological changes, biochemical biomarkers, gene expression and behavior were assessed. Our findings showed that Cu and Cu + MPs decreased embryos survival and hatching rate. Increased ROS levels were observed in larvae exposed to the two lowest Cu and Cu + MPs groups, suggesting an induction of oxidative stress. An increased CAT and GPx activities were observed in Cu and Cu + MPs, implying a response of the antioxidant defense system to overcome the metal and MPs stress. The *sod1* expression was downregulated in all Cu groups and in the two highest Cu + MPs exposed groups. AChE was significantly inhibited in Cu and Cu + MPs groups, indicating neurotoxicity. A disruption of avoidance and social behaviors were also noticed in the Cu125 and Cu125+MPs exposed larvae. Evidences of Cu-toxicity modulation by MPs were observed in some endpoints. Overall, the findings of this study highlight that Cu alone or co-exposed with MPs lead to oxidative stress, neurotoxicity and ultimately

* Corresponding author. Centre for the Research and Technology of Agro-Environment and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD), Quinta de Prados, 5001-801, Vila Real, Portugal.
E-mail address: dsantos@utad.pt (D. Santos).

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behavioral alterations in early life stages of zebrafish, while MPs alone do not produce significant effects on zebrafish larvae.

1. Introduction

Microplastics (MPs), defined as small plastic particles with a size lower than 5 mm, have emerged as a global environmental problem (Alimba and Faggio, 2019; Auta et al., 2017; Pacheco et al., 2018; Wagner et al., 2014). Given their persistence and long-range transport, MPs have been detected in marine (Browne et al., 2011), estuarine (Browne et al., 2010), and freshwater (Free et al., 2014; Mani et al., 2015) ecosystems and even in polar regions (Kanhai et al., 2018). In fact, it has been shown that MPs are readily consumed by a wide variety of freshwater and marine fish (Markic et al., 2019; Wagner et al., 2014). Several studies have reported that MPs can elicit ecotoxicological effects in fish, including reduced body weight (Zhao et al., 2020), oxidative stress (Barboza et al., 2018a; Choi et al., 2018; Lu et al., 2016; Qiao et al., 2019b; Wang et al., 2019), neurotoxicity (Barboza et al., 2018a; Miranda et al., 2019; Roda et al., 2020), endocrine and reproductive disruption (Wang et al., 2019), histopathological lesions (Lu et al., 2016; Qiao et al., 2019b; Wang et al., 2019) and altered behavior patterns (Barboza et al., 2018b; Choi et al., 2018).

Most of the studies were focused on adult fishes, while embryos and larval fish have been less studied, particularly freshwater species. Investigations have shown that the impact of pollution on early life stages can compromise later life stages, consequently resulting in reduced survivorship and reproduction rate of a variety of animal species, including fish, ultimately affecting their fitness (Pechenik, 2006). To date, only a few studies reported that exposure of early life stages of freshwater and marine fish to MPs impair survival, hatching, swimming behavior, gene expression and energy metabolism (Karami et al., 2017; LeMoine et al., 2018; Pannetier et al., 2020, 2019; Qiang and Cheng, 2019; Wan et al., 2019). Taking this into consideration, the consequences of MPs exposure and its toxicological effects on fish early life stages are not negligible, demanding an urgent risk assessment of these small particles in fish early life stages.

Due to their large surface area and hydrophobic properties, MPs can adsorb waterborne contaminants, such as heavy metals (mercury, copper, cadmium, aluminum, silver and lead) (Brennecke et al., 2016; Godoy et al., 2019; Rivera-Hernández et al., 2019; Turner and Holmes, 2015). Laboratory studies have investigated the effects of mixtures of MPs and heavy metals on fish (Barboza et al., 2018b; Lu et al., 2018; Miranda et al., 2019; Qiao et al., 2019a; Roda et al., 2020), with some of them highlighting that MPs can influence the bioaccumulation of metals (Barboza et al., 2018a). Copper (Cu), an essential micronutrient, but toxic to organisms at high levels (Sorensen, 1991), is commonly found in aquatic ecosystems at concentrations ranging from 0.04 to 560 μg/L (Oliveira et al., 2008; USEPA, 2007). Moreover, a wide variety of adverse effects, including biochemical, physiological and behavioral changes have been reported in fish exposed to Cu (Haverroth et al., 2015; Sonnack et al., 2015). Cu is ubiquitous in rivers (Couto et al., 2018; Milivojevic et al., 2016), estuaries (Couto et al., 2018) and coastal ecosystems (Rossi and Jamet, 2008), where MPs pollution also has a high occurrence (e.g. average of 892.777 particles km^{-2} reported in Rhine River, Europe) (Frias et al., 2016; Lasee et al., 2017; Mani et al., 2015), which makes the exposure of fish early life stages to the combination of MPs and Cu inevitable. In fact, some studies have reported very high concentrations of metals, including Cu (e.g. 188 μg/g), recovered from the plastics debris in coastal (Munier and Bendell, 2018; Rochman et al., 2014) and freshwater (Wang et al., 2017) ecosystems.

However, there is a considerable gap of knowledge regarding the interactions between MPs and Cu, and their toxicological effects on fish early life stages. The assessment of the associated risks of MPs and sorbed contaminants in aquatic biota is crucial to understand the impact of plastics in the aquatic ecosystems and for the development of effective mitigation measures. Within this conceptual framework, the aim of the present study was to evaluate the potential effects of an acute exposure to MPs, alone or co-exposed with Cu, on early developmental stages of zebrafish (*Danio rerio*), a freshwater vertebrate model. Two questions were defined in the present study: (1) does the presence of MPs alone in the water induce toxicological effects on zebrafish early life stages? and (2) does MPs influence Cu toxicity on zebrafish embryogenesis? For this purpose, several lethal and sub-lethal (enzymatic, genetic and behavioral) endpoints were evaluated in zebrafish embryos and larvae. A set of enzymatic biomarkers including enzymes involved in oxidative damage response (ROS, SOD, CAT, GPx, GST, GSH, GSSG and LPO), metabolic activity (LDH), metal detoxification (MT) and neurotoxicity (AChE), as well the respective genes involved in the biosynthesis or regulation of oxidative stress (*sod1*, *cat*, *gstp1*, *gclc* and *mt2*) and neurotoxicity (*ache*), were chosen to cover the oxidative stress response pathway. Behavioral endpoints related with locomotor, avoidance and social responses were also assessed to evaluate potential defective brain functions and, therefore, neurotoxicity induced by MPs and Cu on larvae.

2. Material and methods

2.1. Ethics statement on animal experiments

The experimental procedures were performed in agreement with the European (Directive 2010/63/EU) and Portuguese (Directive 113/2013) legislations on animal experimentation and welfare.

2.2. Chemicals

Red fluorescent spherical polymer particles (proprietary polymer of undisclosed composition, melt point ~290 °C, Lot number: 171025-1038), with sizes of 1–5 μm, were purchased from Cospheric LLC (Santa Barbara, CA, USA) and provided as a dry powder. These particles are red, having a density of 1.3 g/cm^3, an excitation wavelength of 575 nm and emission wavelength of 607 nm. Following the information of the manufacturer regarding the density, the number of polymer particles per mg was determined according to the following formula: $(3\pi r^3\delta)/4$, with r corresponding to radius and δ to the density of the particle. According to this, 1 mg of MPs has approximately 5.44E+07 spheres (estimate made for an average of 3 μm diameter). These polymer particles were chosen as MPs model due to their size, which is close to the range of the chorionic pores of fish embryos, and their fluorescence which allows to observe their biodistribution in the embryos. Besides, this type and size of MPs was already used in previous studies that reported toxicity of these particles, both alone or in mixture

with other pollutants, in fish species (Batel et al., 2016; Ferreira et al., 2016; Fonte et al., 2016; Luís et al., 2015; Oliveira et al., 2013).

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was purchased from Merck (Darmstadt, Germany). Stock solutions of $CuSO_4 \cdot 5H_2O$ were prepared in ultrapure water (Milli-Q, Millipore). All other chemicals were purchased from science reagents suppliers, at the highest purity available.

The exposure solutions of Cu, MPs or their mixtures were freshly made with embryo water [28 ± 0.5 °C, 200 mg/L Instant Ocean Salt (Aquarium Systems Inc., France) and 100 mg/L sodium bicarbonate; UV sterilized, pH 7.5–8] prepared from filtered-tap water.

2.3. Zebrafish maintenance and embryo collection

Adult AB wild type zebrafish (*Danio rerio*) were purchased from a local pet shop and maintained under standard conditions in the fish facilities of the University of Trás-os-Montes and Alto Douro (Vila Real). Briefly, zebrafish adults were kept at 28 ± 1 °C under a 14:10 h (light: dark) photoperiod, in a flow-through system with dechlorinated, aerated, charcoal filtered and UV sterilized water (pH 7.5 ± 0.5). Adults were fed twice a day with a standard diet (Westerfield, 2000), supplemented with *Artemia* sp. *nauplii*.

Zebrafish embryos were obtained from mass crosses of spawning adults (in a proportion of 2 males for each female) grouped in tanks overnight with a net bottom cover with glass marbles. In the following morning, spawning activity was induced by the onset of the light cycle and fertilized eggs were collected within 1 h after spawning, rinsed several times with embryo water, bleached and rinsed again (Westerfield, 2000).

2.4. Developmental toxicity experiments

The experiments were carried out following the OECD 236 guideline on fish embryo toxicity test (FET) (OECD, 2013), with minor modifications. Briefly, fertilized eggs were randomly distributed into 50 mL glass beakers and incubated under semi-static conditions, at 28 ± 0.5 °C with a photoperiod of 14:10 h (light: dark). Zebrafish embryos (100 embryos/group/replicate) were exposed from ~2 to 96 h post-fertilization (hpf), to eight treatments: control (embryo water), MPs (2 mg/L), three sub-lethal concentrations of Cu (Cu15, 15 μg/L; Cu60, 60 μg/L; and Cu125, 125 μg/L) and three binary mixtures containing Cu and MPs (Cu15+MPs, 15 μg Cu/L + 2 mg MP/L; Cu60+MPs, 60 μg Cu/L + 2 mg MP/L; and Cu125+MPs, 125 μg Cu/L + 2 mg MP/L).

Additional experiments, following the same experimental set, with 250 viable zebrafish eggs were performed for the metal, biochemical, metallothionein and gene expression sampling. These embryos were from the same batch of fertilized eggs used for the toxicological assay and were exposed in the same conditions. Five replicates were established for each condition. The concentration of MPs (2 mg/L) was selected based on toxicity results of previous studies (Chen et al., 2017; LeMoine et al., 2018) and in recent reports that described concentrations of MPs from 0.64 ± 0.92 up to 5.51 ± 9.09 mg/L in playa wetlands (Lasee et al., 2017) and a number of particles varying from 1×10^{-2} to 10^8 #/m^3 in drinking and freshwaters (reviewed in Koelmans et al., 2019). According to the calculated number of particles per g (described above), 2 mg/L will correspond to 1.09×10^5 particles, which is in the range of values described in drinking and freshwaters. The concentrations of Cu were selected based on ~10% of 96-h LC_{50} value (0.636 mg Cu/L) determined in the present study, and on described environmentally relevant concentrations (Couto et al., 2018; Milivojevic et al., 2016). The details of the LC_{50} determination are provided in the Supplementary data (Fig. S1). The exposure solutions were renewed daily to maintain the compounds concentrations. The mixtures solutions were prepared 24 h before exposure to allow Cu sorption to MPs (Chen et al., 2017).

Along the experiment, lethal and sub-lethal morphological parameters were determined. Mortality was recorded daily and the hatching rate was determined at 48, 56, 72 and 96 hpf. For the heartbeat rate evaluation at 48 and 96 hpf, 10 embryos/larvae from each group, per replicate, were placed on a Petri dish, under an inverted microscope and the heartbeat was counted during 15 s, at least three times, and expressed as beats per minute. Prior heartbeat evaluation, fish were acclimated for 5 min in the Petri dish. At 96 hpf, 15 larvae from each group/replicate were euthanized by overdose with neutralized tricaine methanesulfonate (MS-222) and body length, eye diameter and morphological malformations were then screened and photographed under an inverted microscope (IX 51, Olympus, Antwerp, Belgium). Images were acquired using Cell^A software (Olympus, Antwerp, Belgium), and processed with Adobe Photoshop CS6 (Adobe Systems, USA). Measurements of body length and eye diameter were made using digital image analysis software (Digimizer version 4.1.1.0, MedCalc Software, Belgium). To evaluate the internal distribution of the fluorescent MPs, 10 embryos or larvae were randomly selected from each treatment, at 24 and 96 hpf, for observation and evaluation. The selected fish were rinsed with ultrapure water (Milli-Q, Millipore), immobilized in 1% agarose, placed under a fluorescent inverted microscope (Fluorescein-Isothiocyanate (FITC) filter; IX 51, Olympus, Antwerp, Belgium) and images were taken for analysis. The developmental stages were identified according to Kimmel et al. (1995).

2.5. Analysis of metals

At the beginning and at the end of the experiments, water samples from the exposure solutions were collected for metal analysis. The samples were acidified (65% HNO_3, Merck, Darmstadt, Germany) and stored at 4 °C until analysis. For the analysis of Cu uptake by zebrafish, pools of 80 larvae were collected from each treatment, rinsed in ultrapure water (Milli-Q, Millipore) and stored in acid-washed 1.5 mL centrifuge tubes at −20 °C until analysis.

Before the analysis of Cu concentrations, tissue digestion of zebrafish larvae was conducted. For this, 2 mL of nitric acid (65% p.a., Merck, Darmstadt, Germany) and 1 mL of hydrogen peroxide (30% H_2O_2, Merck, Darmstadt, Germany) were added to each sample, incubated at room temperature for 24 h, and then heated until the solution was clear. Then, the samples were dried at 155 °C and cooled to room temperature. Finally, 5 mL of HNO_3 matrix solution was added to the digested samples and stirred. The Cu content in the water and in the digested samples was quantified using electrothermal atomic absorption spectrometry (Unicam 939 Spectrometer, GF90 furnace). All samples were analyzed in duplicate. The Cu concentrations in water and larvae samples are shown as the means ± standard deviation (SD).

2.6. Histological analysis

For the histopathological analysis, 15 larvae per group were randomly sampled at 96 hpf, fixed in 4% buffered formaldehyde (Panreac, Barcelona, Spain) for 24 h, dehydrated in ascending series of ethanol (70–100%), cleared with xylene and embedded in paraffin wax (Merck, Darmstadt, Germany) (Luzio et al., 2015). Blocks were then sectioned in a rotary microtome (Leica RM 2135, Nussloch, Germany) into serial 3 μm tick sagittal sections. The tissue sections were stained with hematoxylin-eosin (H&E stain), mounted with Entellan® and coverslipped to analysis under an inverted microscope (IX 51, Olympus, Antwerp, Belgium). The

presence of the histopathological changes was registered and the prevalence index of each type of change was determined as the percentage of its occurrence on larvae of each treatment, according to the following formula.

Prevalence of histological alteration (PP%) = (F/N) × 100, where:

$F_{(Frequency\ of\ larvae)}$ = number of larvae with the alteration

N = total number of larvae

The changes severity was then assessed using a score value ranging from 0 to 4, depending on the degree and extent of alteration: 0 − no histopathological changes, 1 − minimal, 2 − mild, 3 − moderate, and 4 − severe and widely distributed pathological alterations (Beker van Woudenberg et al., 2014).

2.7. Oxidative stress, antioxidant and biochemical assays

For the biochemical analysis, pools of 50 larvae per group/replicate were randomly sampled at 96 hpf as previously reported by Félix et al. (2016). The larvae were homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4) and centrifuged at 15 000×*g* at 4 °C for 20 min (Sigma model 3K30, Osterode, Germany). The supernatant was collected and stored at −80 °C until analysis. The enzymatic determinations were performed, at 30 °C, in a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The samples were analyzed in duplicate. Bradford's method at 595 nm (Bradford, 1976) was used to perform protein determination, with bovine serum albumin being used as a standard (0–2 mg/mL).

Superoxide dismutase (SOD) activity was determined according to the method of Durak et al. (1993), by measuring the inhibition of the nitroblue tetrazolium (NBT) reduction, at 560 nm. A standard curve of bovine SOD (0–60 U/mL) was used to quantify SOD activity (U/mg protein). Catalase (CAT) activity was assayed based on a method by Claiborne (1985), that measures H_2O_2 consumption, at 240 nm. The activity was normalized using bovine catalase as standard (0–6 U/mL) and expressed as U/mg protein. Glutathione-S-transferase (GST) activity was determined at 340 nm following the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) (Habig and Jakoby, 1981), and expressed as μmol/min.mg protein. Glutathione peroxidase (GPx) activity was assayed following the method described by Paglia and Valentine (1967), at 340 nm, and expressed as μmol NADPH/min.mg protein.

Acetylcholinesterase (AChE) activity was determined through the method of Rodriguez-Fuentes et al. (2015), which measures the conjugation of thiocoline with 5,5′- dithiobis-(2-nitrobenzoic acid) (DTNB), at 405 nm. The AChE activity was expressed as μmol TNB/min.mg protein. Lactate dehydrogenase (LDH) enzyme was assayed using the method of Domingues et al. (2010), at 340 nm, and expressed as μmol NADH/min.mg protein.

The glutathione levels, namely the reduced (GSH) and oxidized states (GSSG) (Gartaganis et al., 2007), were estimated using the fluorochrome *ortho*-phthalaldehyde (OPA), at 320 nm and 420 nm for excitation and emission wavelengths, in a Varian Cary Eclipse (Varian, USA) spectrofluorometer equipped with a microplate reader. GSH and GSSG concentrations were estimated with a GSH or GSSH standard curve (0–1000 μM), respectively, and expressed as μmol/mg protein. The oxidative-stress index (OSI), as the ratio between GSH/GSSG, was then calculated.

Reactive oxygen species (ROS) accumulation was determined following the methodology described by Deng et al. (2009), in a Varian Cary Eclipse (Varian, USA) spectrofluorometer equipped with a microplate reader. Total ROS were quantified using the fluorescent probe 2′,7′-dihlorofluorescein diacetate (DCFH-DA), at 485 nm and 530 nm for excitation and emission wavelengths. The ROS concentration was estimated based on a DCF standard curve (0–6.25 nM) and expressed as μmol DCF/mg protein. Lipid peroxidation (LPO) was analyzed through the thiobarbituric (TBA) acid-based methodology reported by Gartaganis et al. (2007), at an excitation wavelength of 535 nm and an emission wavelength of 550 nm in a Varian Cary Eclipse (Varian, USA) spectrofluorometer equipped with a microplate reader. The major oxidative product of phospholipids, malondialdehyde (MDA), was determined based on a standard curve (0–50 μM) of malondialdehyde bis(dimethyl acetal) and expressed as μmol MDA/mg protein.

2.8. Metallothionein determination

Metallothionein (MT) levels were determined following the methodology proposed by Viarengo et al. (1997), with some modifications. Briefly, pools of 50 larvae per group/replicate were randomly sampled at 96 hpf, homogenized in cold buffer (0.5 M sucrose, 20 mM Tris–HCl buffer (pH 8.6) with 0.5 mM phenylmethylsulphonylfluoride (PMSF) and β-mercaptoethanol 0.01%) and centrifuged at 15 000×*g* at 4 °C for 20 min (Sigma model 3K30, Osterode, Germany). The obtained supernatant was collected and protein determination (Bradford, 1976) performed, at 595 nm. Then, a solution of ethanol/chloroform was added to the sample supernatant and centrifuged at 6000×*g* for 10 min at 4 °C. Three volumes of ice-cold ethanol/HCl were added to the resulting supernatant and kept at −20 °C for 1 h. Subsequently, the sample was centrifuged at 6000×*g* for 10 min at 4 °C. The obtained pellet was resuspended with ethanol/chloroform in homogenization buffer (0.5 M sucrose, 20 mM Tris–HCl buffer, pH 8.6) and centrifuged again at 6000×*g* for 10 min at 4 °C. The supernatant was discarded and the pellet air dried. The pellet was then resuspended in 4 mM EDTA-HCl and 0.25 M NaCl and agitated. Then, 1 mL of Ellman's solution (2 M NaCl containing 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) in 0.2 M phosphate buffer, pH 8) was added and centrifuged at 3000×*g* for 5 min, at room temperature. MT was quantified at 412 nm and its concentration in samples was estimated with a reduced GSH standard solution (0–250 μM) and expressed as μmol GSH/mg protein.

2.9. Gene expression analysis

For the evaluation of gene expression, pools of 50 larvae per group, from three independent replicates, were randomly sampled at 96 hpf and stored in RNA Later (Sigma, Germany). Larvae were then homogenized in a TissueLyzer shaker (Qiagen, Hilden, Germany) for 2 min and total RNA was isolated using the "Illustra RNAspin Mini kit" (GE Healthcare, Munich, Germany), according to the manufacturer's instructions. RNA concentration and purity of each sample was determined spectrophotometrically at 260 and 280 nm (Powerwave XS2, BioTek Instruments, USA) and a 1% agarose gel, stained with Green Safe Premium (NZYtTech, Lisbon, Portugal), was used to assess RNA integrity and possible DNA contamination. RNA samples were stored at −80 °C until further analysis. Total RNA (1 μg) from all the samples was reverse transcribed into cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK) and stored at −20 °C.

The primer sequences for quantitative real-time PCR (qPCR) were selected from the literature and are listed on Table S1. The qPCR reactions were performed in triplicate using 1 μL of cDNA in a final volume of 20 μL reaction mixture containing 5x HOT FIREPol

EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) and 200 nM of each specific primer, on a PikoReal 96 Real-Time PCR System (Thermo Scientific, Massachusetts, USA). The thermal cycling conditions consisted on an initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing for 20 s (annealing temperatures in Table S1) and extension at 72 °C for 20 s. At the end of the amplification phase, a dissociation step (60 °C–95 °C) was carried out to confirm the presence of a single and specific product for each primer. Three-fold dilution series prepared from cDNA samples were used to generate the standard curves and calculate primer efficiencies. A no-template control was included on each plate. Each sample was run in duplicate. The normalized relative expression of each gene was performed following the method described by Hellemans et al. (2007), and which accounts the primer efficiencies (Pfaffl, 2001) and at least two normalizer genes which should be stable with minimal variation following chemical treatments. The stability of the reference genes *β-actin* (*actb2*), *eukaryotic translation elongation factor 1 alpha 1* (*eef1α1*) and *tubulin* (*tuba1b*) were analyzed using Bestkeeper software (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004). The two reference genes *actb2* and *tuba1b* were ranked as the best combination to normalize the data. For each gene, the mean of the cycle quantification value (*Cq*) of each sample was calculated and then converted into Relative Quantity (RQ) using the gene amplification efficiency ($RQ = E^{[\Delta Cq = Cqcalibrator - Cqsample]}$). The geometric mean of RQ values for the selected reference genes (*actb2* and *tuba1b*) was calculated and then used as a Normalization Factor (NF). Normalized Relative Quantities (NRQ) for each sample were determined by dividing the RQ value of the gene of interest by the Normalization Factor (NF). Results are expressed as normalized relative quantity (NRQ). The fold change relative to the control group was calculated by dividing the average NRQ value of each exposed group by the average NRQ of the control group.

2.10. Behavioral analysis

At 96 hpf, 5 larvae per treatment/replicate were used to assess behavioral parameters. Dead larvae or larvae showing malformations were excluded from the assay. Larval behavior parameters were monitored and quantified using a video-tracking system (TheRealFishTracker) (Buske and Gerlai, 2014), as previously described (Félix et al., 2017), and it was carried out in a temperature-controlled room (25 ± 1 °C). Briefly, larvae were randomly placed into 6-well agarose-coated plates (1 larva per well), with all experimental groups being analyzed at the same time. After 5 min of acclimation, the plates were monitored by a 14.2 megapixels Sony Nex-5 digital camera (APS-C CMOS sensor, Sony International) with a zoom lens (Sony SEL1855, E 18–55 mm, F3.5–5.6 OSS zoom) and a 15.6″ laptop LCD screen (1366 × 768 pixel resolution, an average brightness of 173.6 cd m^{-2} and a contrast of 208:1 with a black level of 0.83 cd m^{-2}) and recorded at 30 frames per second and at 1920 × 1080 pixels resolution.

The parameters mean speed, total distance moved, mean distance to the center zone of the well (i.e., to a 5 mm radius circle drawn in the center of the well), mean absolute turn angle (alterations in this parameter indicates a disorganized pattern of swimming) and the percentage of time active, were assessed in 10-min sessions, in the light period, to evaluate the exploratory behavior. For the avoidance response to the presence of a visual stimulus, larvae were tested by alternating 10 min period of a white background and 10 min period of a red bouncing ball (1.35 cm diameter and RGB 255, 0, 0) present at the upper half of the well and traveling from left-right-left at a speed of 1 cm/s over a straight 2 cm trajectory (Pelkowski et al., 2011). The time spent in the bottom area of the well without and with stimulus was then assessed. The social behavior was recorded for 10 min, using 5 larvae per treatment, according to established protocols (Richendrfer et al., 2012). The analysis was based on the average inter-individual distance (IID) and the nearest neighbor distance (NND) parameters, which are calculated based on the x-y coordinates quantified by the software TheRealFishTracker (Buske and Gerlai, 2014). The formulas involved in this quantification are described in Miller and Gerlai (2012).

Finally, the swimming behavior was evaluated in 5 larvae per treatment, by monitoring its response to sessions of light-to-dark transitions (10 min light–10 min dark), for 40 min, in a 940 nm-emitting LED constructed lightbox, as described by Frank et al. (2019). A 2-megapixel infrared (IR, 940 nm) camera (GENIUSPY, GS-NQ140CML) with a 3.6 mm lenses was used to record these sessions. The total distance moved (cm) was then analyzed.

2.11. Statistical analysis

Statistical analysis was performed using Prism 7.0 (GraphPad Software, Inc., CA, USA). All data were tested for ANOVA assumptions (normality with the Kolmogorov-Smirnov test and homogeneity of variances with the Bartlett's test). For each parameter, one-way analysis of variance (ANOVA) or two-way ANOVA with interactions, followed by the Tukey's multicomparison post-hoc test, were used to discriminate statistically significant differences among groups and assess if MPs presence influenced Cu toxicity. Significant differences between groups with different prevalence indices were assessed by the Mann–Whitney rank sum test. In the specific cases where the parametric ANOVA assumptions failed, data were submitted to a non-parametric ANOVA using the Mann-Whitney rank sum test or the Kruskal-Wallis on Ranks test followed by the post-hoc Dunn's test. The Student's *t*-test was used to discriminate statistically significant differences among groups in avoidance behavior. For gene expression, data (NRQ values) were *log2* transformed (*Cq'*) to reduce heterogeneity of variance, before performing the statistical analysis. Significant differences were considered when $p < 0.05$.

To determine the most important variables that could be the key factors for individual's response to MPs and Cu, a principal component analysis (PCA) was performed with the mortality, hatching, metallothionein levels and behavioral, biochemical and molecular parameters. Variables were standardized prior to all analyses in order to preserve the original scale. The PCA was carried out using CANOCO 5 (version 5.12) (Biometrics, Wageningen, Netherlands).

3. Results

3.1. Metal concentration analysis

The Cu concentrations of test solutions and larvae are shown in Table S2. The analysis of Cu show that, at the beginning of the experiments, embryos were exposed to 14.3 ± 0.6 μg/L, 51.8 ± 0.8 μg/L and 125.4 ± 4.8 μg/L of Cu at the lowest, intermediate and highest concentrations, respectively. Considering the Cu + MPs groups, the analysis of Cu showed values similar to the ones observed in the single exposures, namely, 17.7 ± 5.0 μg/L, 52.3 ± 5.2 μg/L and 122.9 ± 20.8 μg/L, respectively. With the test solutions renewal, the levels of Cu slightly increased, with the groups presenting, at 96 h, the following concentrations: 19.8 ± 0.3 μg/L, 52.0 ± 12.1 μg/L and 136.4 ± 8.0 μg/L for the single Cu exposures; and 20.3 ± 0.3 μg/L, 52.9 ± 8.0 μg/L and 127.7 ± 15.1 μg/L of Cu in the mixture groups. Since the variation between the nominal and measured Cu values is

not high, the results are presented and discussed relative to the nominal concentrations, i.e., 15, 60 and 125 μg/L.

3.2. Toxicological effects of MPs, Cu and their mixtures on early developing zebrafish

The mortality and hatching rates of the exposed groups are shown in Figs. 1 and 2, respectively. Exposure to MPs and Cu15 did not affected survival of zebrafish embryos ($p > 0.05$). However, in embryos exposed to Cu60 and Cu125 and respective mixtures, a lower survival rate was observed through the experiment, comparatively to the control group ($p < 0.01$). Concerning the hatching rate, there were significant differences among treatments. Except for embryos exposed to MPs, which showed similar hatching rates to the control ($p > 0.05$), in the remaining groups it was observed a dose-dependent decrease of hatching, that became evident from 72 hpf on ($p < 0.01$). In the control group, 94% of embryos were hatched at 96 hpf, whereas the lowest hatching rates, 30% and 28% respectively ($p < 0.001$), were observed in the Cu125 and Cu125+MPs groups. In both, mortality and hatching rates, no significant differences were observed between the groups with Cu and the corresponding mixtures ($p > 0.05$).

Body and eye size, heart and morphological malformations rates are shown in Table 1. Significantly lower body length and eye size ($p < 0.01$) were observed in 96 hpf larvae exposed to Cu60+MPs, Cu125 and Cu125+MPs, comparatively to the control larvae. The heart rate was measured at 48 and 96 hpf. Compared to controls, an increasing trend of the heart rate was noticed, at 48 hpf, in most of the exposed groups ($p > 0.05$). Despite transient, this increase was significant ($p < 0.05$) in zebrafish embryos exposed to Cu125+MPs.

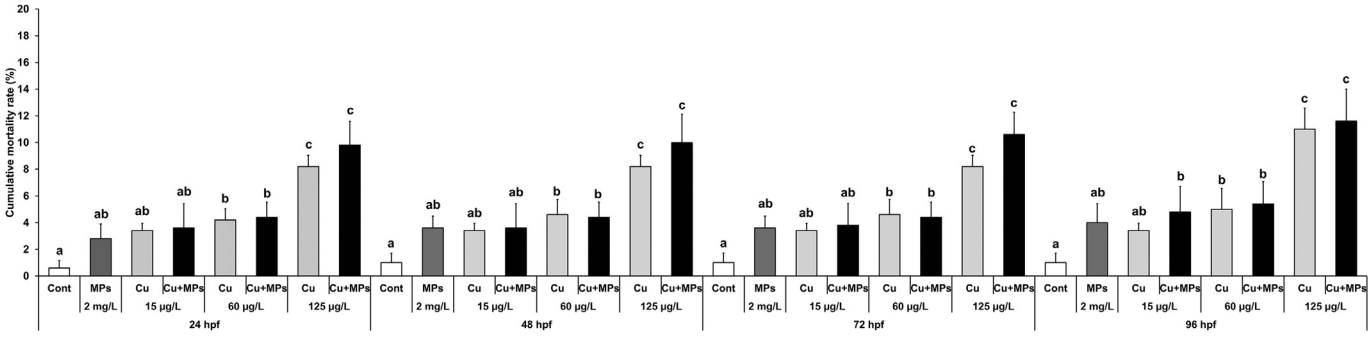

Fig. 1. Cumulative mortality rate of zebrafish embryos/larvae exposed to microplastics (MPs) and copper (Cu), alone or combined. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparison test. Data are expressed as mean ± S.D. Different lowercase letters indicate significant differences between groups, within each time period ($p < 0.05$). No significant differences between time, within the same group were observed.

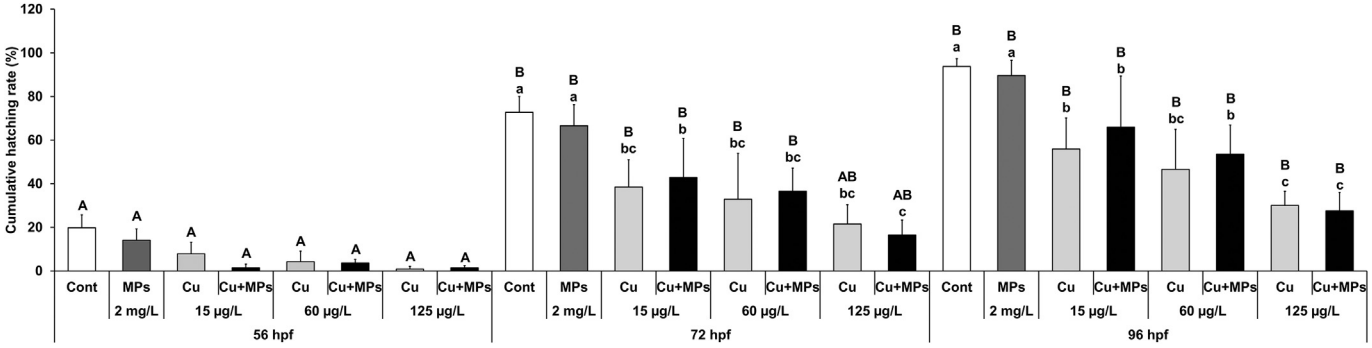

Fig. 2. – Cumulative hatching rate of zebrafish embryos/larvae exposed to microplastics (MPs) and copper (Cu), alone or combined. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple-comparison test. Data are expressed as mean ± S.D. Different lowercase letters indicate significant differences between groups, within each time period. Uppercase letters represent significant differences between time, within the same group ($p < 0.05$).

Table 1
Morphometric measurements, heart rates and morphological malformations of zebrafish embryos/larvae exposed to microplastics (MPs) and copper (Cu), alone or combined, for 96 h.

Treatment groups		Body length (mm)	Eye diameter (mm)	Heart rate (bpm) 48 hpf	Heart rate (bpm) 96 hpf	Morphological Abnormalities rate (%)
	Control	3.38 ± 0.21a	0.35 ± 0.02a	117 ± 8.7a	157 ± 9.3	0.00 ± 0.0a
2 mg/L	MPs	3.27 ± 0.26ac	0.35 ± 0.03ac	125 ± 6.5 ab	155 ± 9.7	0.21 ± 0.5 ab
15 μg/L	Cu	3.22 ± 0.36ac	0.34 ± 0.03ac	123 ± 9.8 ab	145 ± 8.8	2.48 ± 0.6 ab
	Cu + MPs	3.23 ± 0.29ac	0.33 ± 0.03 ab	127 ± 8.5 ab	147 ± 8.8	2.11 ± 1.9 ab
60 μg/L	Cu	3.18 ± 0.18 acd	0.32 ± 0.02 ab	125 ± 11.5 ab	144 ± 12.5	2.73 ± 1.6b
	Cu + MPs	3.07 ± 0.32bc	0.31 ± 0.03bc	130 ± 10.8 ab	149 ± 9.4	2.29 ± 1.1 ab
125 μg/L	Cu	2.91 ± 0.33b	0.30 ± 0.02b	129 ± 12.8 ab	158 ± 8.8	1.79 ± 1.3 ab
	Cu + MPs	2.96 ± 0.23bd	0.30 ± 0.02b	134 ± 11.0b	159 ± 10.9	2.23 ± 1.8 ab

Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Data are expressed as mean ± S.D. from five independent experiments. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

At 96 hpf, no significant changes were observed in the heart rate. Among the morphological malformations induced by the treatments, pericardial edema, yolk sac edema, abnormalities of body structure and axial spinal curvatures were the most common. A significant increase of malformations rate was observed in the larvae exposed to Cu60 ($p = 0.03$). Again, no significant differences between the groups with Cu and the Cu + MPs were observed in the above described parameters.

The distribution of MPs in zebrafish early life stages was analyzed at 24 and 96 hpf. It was found that the MPs on embryos were most abundant in the surface of the chorion (Fig. S2A). However, some MPs were observed inside the chorion of zebrafish embryos (high fluorescence signal within the yolk sac), indicating that the MPs with the lower diameter can pass through the chorionic pores. In larvae, MPs were also observed, particularly in the yolk sac and on the head (Fig. S2B), but also in their gastrointestinal tract.

3.3. Histopathological findings

The histopathological changes observed in larvae exposed to MPs, Cu and their mixtures are presented in Fig. 3 and Table S3. In the control group (Fig. 3B), the structural subdivisions of the larval brain, namely the forebrain, midbrain and hindbrain were well defined, with the cells presenting a normal arrangement. The retina of control larvae was also normal, with the ganglion cell, inner plexiform, inner nuclear, outer plexiform and photoreceptor cell layers, and the pigmented epithelium presenting a normal rearrangement and structure. In the exposed zebrafish embryos, the histological analysis revealed some signs of pathological changes, namely, disruption of retina layers (Fig. 3C), epithelial detachment (Fig. 3D), edema (Fig. 3E), changes in midbrain-hindbrain boundary (MHB) and cell death (Fig. 3F). Epithelial detachment was the histopathological change with the higher prevalence in exposed zebrafish embryos (Fig. 3A), being present in animals from all exposed groups. Considering the histological scoring approach, it shows a low incidence of the histopathological changes in brain and retina of zebrafish 96 hpf larvae ($p > 0.05$). The exception was observed for the epithelial detachment, which was more severe in Cu60+MPs group (Table S3) reaching significant values comparatively to control larvae ($p = 0.0007$).

3.4. Effects of MPs, Cu and their mixtures on oxidative stress, biochemical biomarkers and metallothioneins levels

The effects of MPs, Cu and their mixtures on oxidative stress, biochemical markers and MT are shown on Table 2. The ROS levels showed a significant increase in the Cu15, Cu60 and respective mixtures, comparatively to the control group ($p < 0.05$). Although not significant, a slight rise of ROS levels was also observed in larvae exposed to MPs alone ($p > 0.05$). Relatively to the oxidative damage, the LPO levels suffered a significant decrease in the Cu125 exposed larvae and in the Cu60+MPs and Cu125+MPs groups ($p < 0.001$), comparatively to the control group.

Considering the antioxidant biomarkers SOD, CAT, GPx and GST, the results showed that single exposure to MPs did not affect the activity of these enzymes ($p > 0.05$). In turn, SOD activity decreased significantly in the Cu125 and Cu125+MPs exposed larvae ($p < 0.05$), while GPx activity increased significantly ($p < 0.01$), in these same two groups. Moreover, a dose-dependent induction of CAT activity was observed in the Cu and mixture groups ($p < 0.01$), comparatively to the untreated larvae. GST activity was only affected in the Cu125+MPs exposed larvae, presenting significant higher values ($p < 0.05$), comparatively to control.

Regarding glutathione, a decrease of GSH levels was observed in Cu125 group, whereas in Cu60+MPs group it was observed an increase ($p < 0.01$), in comparison to the control group. GSSG levels significantly increased in Cu15+MPs exposed larvae ($p < 0.01$),

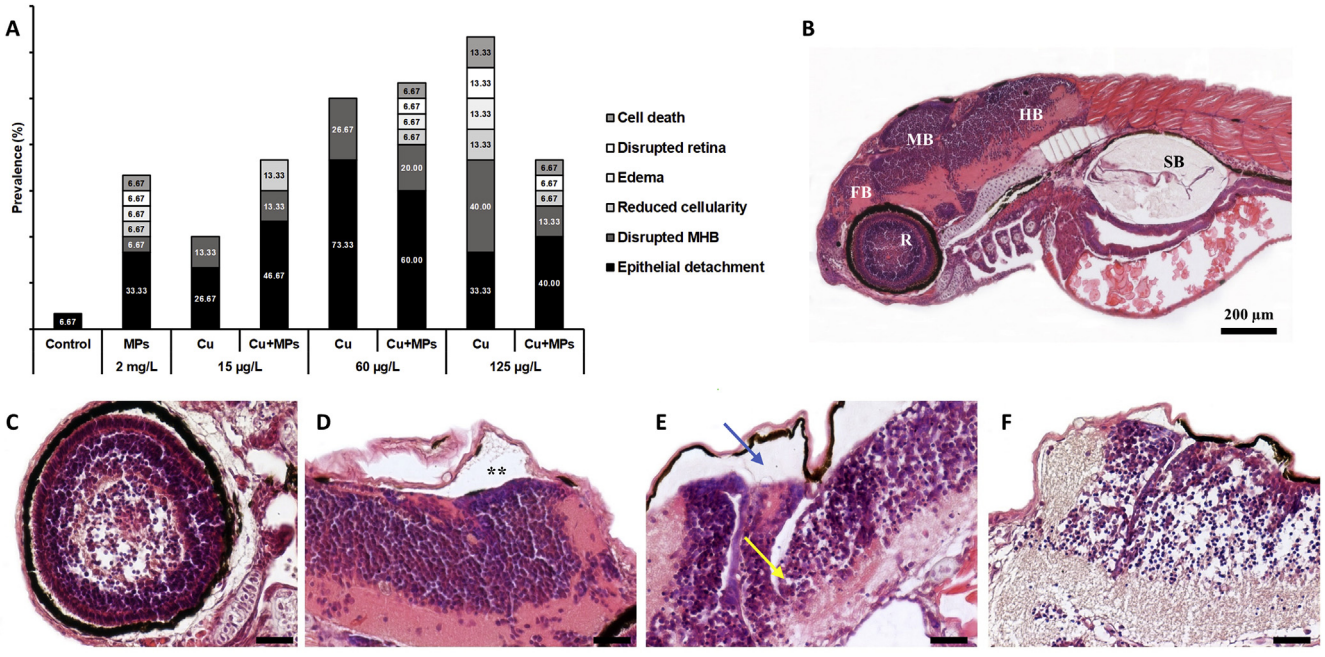

Fig. 3. Prevalence's of the histopathological changes (**A**). Within each bar, the percentage of animals from that group that evidenced each particular change, is numerically expressed. Illustrative images of histological sections of 96 hpf zebrafish larvae (**B**–**F**) exposed to microplastics (MPs) and copper (Cu), alone or combined. (B) Control larvae. (C) Larvae exposed to Cu125 showing alterations in the retina layers organization; (D) Larvae exposed to Cu125+MPs with epithelial detachment in brain (**); (E) Larvae exposed to MPs alone with epithelial detachment (blue arrow) and edema (yellow arrow) in brain; (F) Larvae exposed to Cu125 showing evidences of cell death. FB–Forebrain; MB–Midbrain; HB–Hindbrain; R–retina; SB–swimming bladder; On C–F, scale bar = 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Changes in oxidative stress indices, antioxidant enzymes, glutathione levels, oxidative stress index, acetylcholinesterase, lactate dehydrogenase and metallothioneins in zebrafish larvae (96 hpf) exposed to microplastics (MPs) and copper (Cu), alone or combined.

Biochemical parameters		Cont	2 mg/L	15 μg/L		60 μg/L		125 μg/L	
			MPs	Cu	Cu + MPs	Cu	Cu + MPs	Cu	Cu + MPs
Oxidative stress	ROS μmol DCF/mg of protein	62.9 ± 14.4ac	94.2 ± 15.4 ab	99.3 ± 10.2bd	131.2 ± 24.5d	99.3 ± 14.3bd	103.7 ± 18.9bd	55.4 ± 13.4c	61.4 ± 11.3c
	LPO μmol MDA/mg of protein	2.2 ± 0.2a	1.7 ± 0.4ac	1.7 ± 0.4ac	2.4 ± 0.3a	1.6 ± 0.2ac	0.9 ± 0.4b	1.0 ± 0.2b	1.2 ± 0.2bc
Antioxidant enzymes	SOD U/mg of protein	412.9 ± 62.9a	423.0 ± 47.9a	391.5 ± 54.9a	474.1 ± 50.9a	452.3 ± 54.7a	433.3 ± 86.3a	238.7 ± 72.4b	226.9 ± 43.4b
	CAT U/mg of protein	8.1 ± 1.6a	8.9 ± 2.2 ab	17.4 ± 4.6bce	11.4 ± 4.2abc	18.2 ± 4.2ce	30.2 ± 11.8de	43.0 ± 13.1df	58.8 ± 17.6f
	GPx μmol NADPH/min.mg of protein	14.8 ± 2.8a	15.1 ± 5.2a	11.6 ± 4.4a	12.2 ± 5.2a	11.1 ± 1.6a	11.1 ± 2.3a	30.9 ± 8.8b	44.2 ± 6.8b
	GST μmol CDNB/min.mg of protein	0.05 ± 0.01 ab	0.06 ± 0.01abc	0.05 ± 0.01 ab	0.07 ± 0.01abc	0.04 ± 0.02a	0.05 ± 0.01 ab	0.07 ± 0.02bc	0.08 ± 0.02c
Glutathione levels	GSH μmol/mg of protein	1815 ± 192.7ac	1888 ± 183.7ac	1658 ± 196.3a	2091 ± 196.4cd	1640 ± 151.1a	2390 ± 225.1d	1157 ± 98.3b	1536 ± 181.3 ab
	GSSG μmol/mg of protein	5670 ± 483.3a	5769 ± 597.4a	5751 ± 266.1a	7217 ± 276.6b	5229 ± 482.3a	2993 ± 378.0c	3066 ± 349.2c	2965 ± 325.7c
	GSH/GSSG	0.34 ± 0.01a	0.34 ± 0.02a	0.30 ± 0.05a	0.30 ± 0.02a	0.31 ± 0.02a	0.70 ± 0.09b	0.38 ± 0.05a	0.52 ± 0.10c
Neurotoxicity	AChE μmol TNB/min.mg of protein	0.09 ± 0.01a	0.07 ± 0.01 ab	0.06 ± 0.01b	0.06 ± 0.004b	0.03 ± 0.01c	0.03 ± 0.01c	0.05 ± 0.01b	0.06 ± 0.01b
Metabolic activity	LDH μmol NADH/min.mg of protein	0.41 ± 0.05 acd	0.38 ± 0.06 acd	0.32 ± 0.03a	0.47 ± 0.01c	0.35 ± 0.07ad	0.42 ± 0.07cd	0.06 ± 0.01b	0.06 ± 0.01b
Detoxification	MT μmol GSH/mg protein	4.5 ± 0.6a	3.5 ± 0.7 ab	3.6 ± 0.9 ab	4.7 ± 0.8a	3.2 ± 0.6b	2.9 ± 0.4b	2.4 ± 0.3b	3.5 ± 0.5 ab

Data, from at least four independent replicates, expressed as mean ± S.D. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

while in Cu60+MPs, Cu125 and Cu125+MPs groups a significant decrease ($p < 0.001$) was observed when compared to control group. For both GSH and GSSG, significantly higher values were observed in Cu15+MPs and Cu60+MPs groups, in comparison to the respective Cu group ($p < 0.05$), evidencing an antagonistic behavior of MPs in relation to Cu. For the oxidative stress index (GSH/GSSG), a significant increase was observed in the larvae exposed to Cu60+MPs and Cu125+MPs ($p < 0.05$).

The neurotoxicity biomarker AChE was significantly inhibited in all Cu and mixture groups ($p < 0.05$). Zebrafish embryos exposed to MPs alone tended to have a slight decrease of AChE activity, but this effect was not statistically significant relative to the control group ($p > 0.05$). In the Cu125 and Cu125+MPs exposed larvae, a significant decrease was also found for the LDH activity ($p < 0.001$), an anaerobic metabolism biomarker. For most of the enzymes, no significant differences between the groups with Cu and the respective mixtures were observed.

The MT levels did not differ among the control larvae and the groups exposed to MPs, Cu15, Cu15+MPs and Cu125+MPs ($p > 0.05$). However, exposure to the Cu60, Cu125 and Cu60+MPs showed significantly lower MT levels, comparatively to the control group ($p < 0.05$). No significant differences between the groups with Cu and Cu + MPs were observed ($p > 0.05$).

3.5. Effects of MPs, Cu and their mixture on oxidative and neurotoxicity related genes expression

The transcriptional levels of the selected target genes of zebrafish larvae are shown in Figs. 4 and 5. At 96 hpf, the *catalase* (*cat*) gene expression (Fig. 4A) showed no significant alterations in the exposed groups ($p > 0.05$). In contrast, *superoxide dismutase 1* (*Cu/Zn-sod* or *sod1*) expression (Fig. 4A) was significantly downregulated in all three Cu exposed groups, Cu60+MPs and Cu125+MPs groups ($p < 0.05$), comparatively to the control. Regarding *glutathione S-transferase pi 1* (*gstp1*) gene (Fig. 4B), its expression was significantly downregulated in the Cu exposed groups ($p < 0.01$), comparatively to the control group. However, in Cu + MPs groups, the *gstp1* expression was similar to the control ($p > 0.05$). In fact, it was observed that the *gstp1* expression was significantly higher in Cu15+MPs and Cu60+MPs, comparatively to the corresponding Cu groups ($p < 0.01$). The expression of *glutamate-cysteine ligase, catalytic subunit* (*gclc*) gene (Fig. 4B) increased 2.5-fold only in Cu60+MPs exposed larvae ($p < 0.05$), with respect to the control group. Similarly, the expression of *gclc* was higher in the Cu60+MPs, in comparison with the group exposed to Cu60 ($p < 0.05$). These findings indicate that the presence of MPs may have influenced the Cu toxicity, with MPs showing an antagonistic behavior in relation to Cu, in both genes *gstp1* and *gclc*. The expression of *acetylcholinesterase* (*ache*) (Fig. 5) increased significantly in MPs, Cu15, Cu15+MPs and Cu60+MPs groups ($p < 0.05$). The expression of *ache* was also higher in the Cu60+MPs, comparatively to Cu60 ($p < 0.05$). For the *mt2* gene (Fig. 5), which encodes *metallothionein 2*, no significant alterations were observed in the exposed groups, despite the slight decrease of its expression in all treatments with respect to control ($p > 0.05$).

3.6. Effects of MPs, Cu and their mixture on behavior parameters

The effects of MPs, Cu and their mixture on behavior of 96 hpf larvae are shown in Fig. S3 and Fig. 6. According to the results, for mean speed (Fig. S3A) and distance to center (Fig. S3B) parameters, no significant effects were observed in the exposed larvae ($p > 0.05$). However, exposure to Cu125 induced a significant increase of the total distance traveled (353.6 ± 55.2 cm), compared to control group (229.6 ± 45.2 cm) ($p < 0.05$) (Fig. 6A). Although no

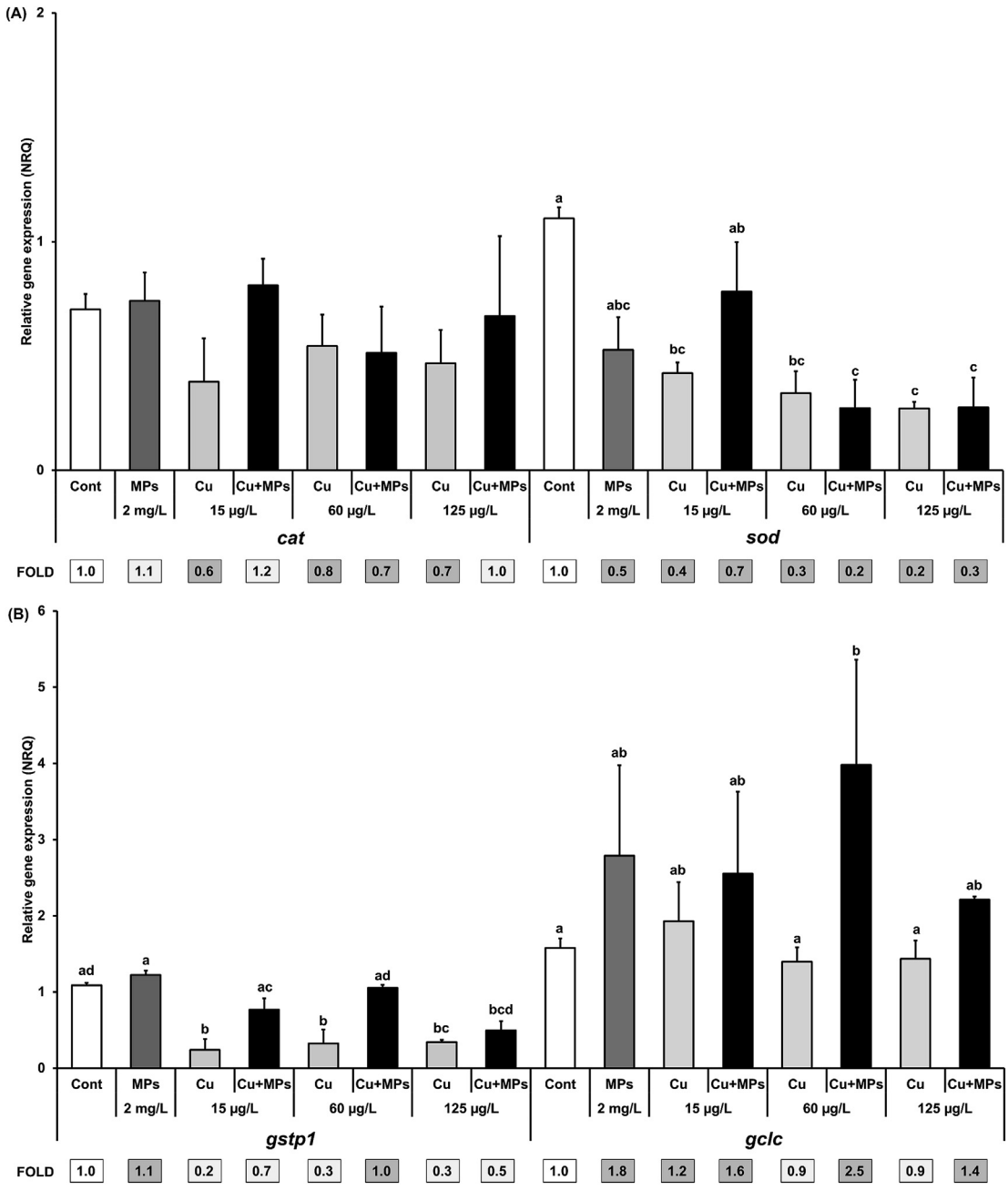

Fig. 4. Relative gene expression levels of (A) *catalase* (*cat*) and *superoxide dismutase 1* (*sod1*), and (B) *glutathione S-transferase pi 1* (*gstp1*) and *glutamate-cysteine ligase, catalytic subunit* (*gclc*), in 96 hpf zebrafish larvae following exposure to microplastics (MPs) and copper (Cu), alone or combined. Data shown as mean ± S.D., normalized to the *β-actin* (*actb2*) and *tubulin* (*tuba1b*) reference genes. Fold-changes are presented in boxes under each graphic, with dark grey box representing an upregulation and light grey a downregulation, comparatively to the control group (white box). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

significant, a slight increase of total distance traveled was also observed in the Cu125+MPs (319.7 ± 32.8 cm) ($p > 0.05$). Regarding the absolute turn angle (Fig. S3C) and the percentage of time active (Fig. S3D), no interference was observed in these parameters in the exposed larvae in relation to the control group ($p > 0.05$). No significant differences between the groups with Cu and the respective mixtures were observed in the exploratory behavior parameters ($p > 0.05$).

The avoidance behavior (Fig. 6B) was assessed through the presence of a visual stimulus. For most of the groups, in the absence of the aversive stimulus, zebrafish larvae presented neither a preference for the upper nor for the bottom area of the well. On the other hand, in the presence of the aversive stimulus, under the form of a red bouncing ball, there was a significant increase in the time spent in the non-stimulus area for most of the groups. However, in the groups exposed to Cu60, Cu125 and Cu125+MPs, no significant differences were observed between the time spent in the stimulus and in the non-stimulus area ($p > 0.05$), suggesting that these exposed larvae did not perceive the aversive stimuli.

The social behavior was assessed by quantifying the nearest neighbor distance (NND) and the inter-individual distance (IID) (Fig. 6C and D, respectively). A significant decrease of both NND

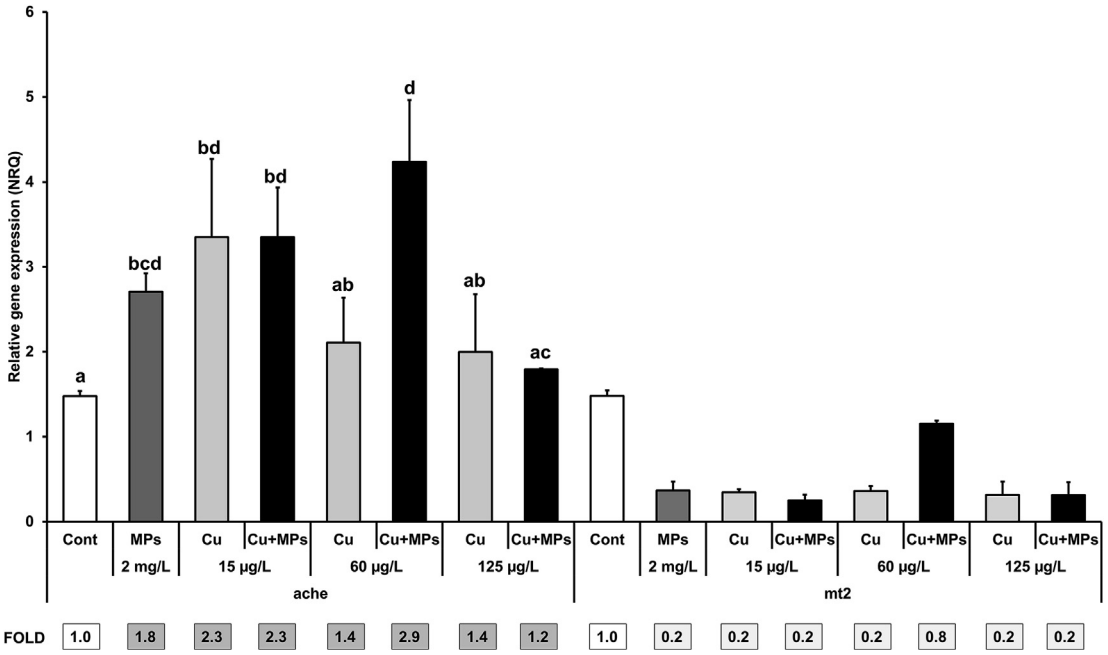

Fig. 5. Relative gene expression levels of *acetylcholinesterase* (*ache*) and *metallothionein 2* (*mt2*), in 96 hpf zebrafish larvae following exposure to microplastics (MPs) and copper (Cu), alone or combined. Data shown as mean ± S.D., normalized to the *β-actin* (*actb2*) and *tubulin* (*tuba1b*) reference genes. Fold-changes are presented in boxes under each graphic, with dark grey box representing an upregulation and light grey a downregulation, comparatively to the control group (white box). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

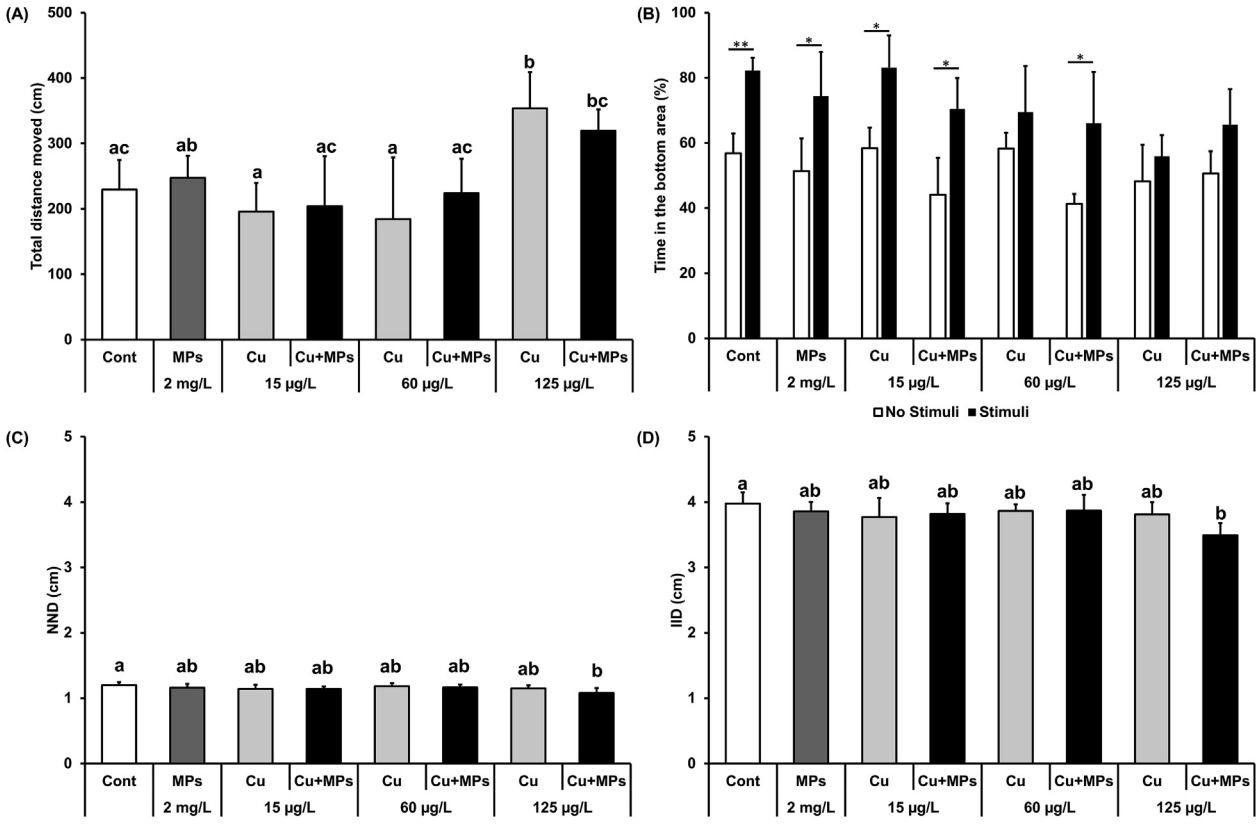

Fig. 6. Exploratory (A), avoidance (B) and social (C–D) behavior of 96 hpf zebrafish larvae following exposure to microplastics (MPs) and copper (Cu), alone or combined. (A) Total distance moved by larvae; (B) Percentage of time that larvae spent in the upper area of the well without (white bars) or with (black bars) the presentation of an aversive stimulus (bouncing ball) in the bottom area of the well; (C) NND, nearest neighbor distance and (D) IID, inter-individual distance. Data presented as mean ± S.D. Statistical analysis of exploratory and social behavior was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$). For avoidance behavior, a paired *t*-test was performed and the asterisks indicate significant differences between groups before and after the stimulus (*$p < 0.05$ and **$p < 0.01$).

($p < 0.05$) and IID ($p < 0.001$) was observed in the Cu125+MPs group, indicating a disruption on the social cohesion of these exposed larvae. No interference in these parameters was observed in the remaining groups ($p > 0.05$), and no significant differences between the groups with Cu and Cu + MPs were observed ($p > 0.05$).

The locomotor response to light and dark transitions is shown in Fig. S4. As expected, zebrafish larvae exhibit weak movements in light, but when switched from light to dark larvae increase their swimming activity. In the periods of light, no significant differences were observed between the exposed larvae and the control ($p > 0.05$). However, the larvae exposed to Cu125+MPs presented a significant increase of the distance moved in the dark periods, in comparison to the control group ($p < 0.001$), indicating an augmented reaction to the darkness stimulus. In the second dark period, the Cu125+MPs group was significantly different from the respective Cu group ($p < 0.001$), suggesting a modulation of Cu toxicity by MPs.

3.7. Principal component analysis (PCA)

PCA was applied to reduce the dimensionality of data and extract the dominant factors. The results of the PCA (Fig. 7) showed that the studied variables related to mortality, hatching, biochemistry, gene expression, metallothionein' and behavior of the larvae were sensitive to the concentration of Cu. The first principal component explained 28.94%, while the second principal component explained 13.76% of the overall variance. Therefore, the first two principal components explained 42.7% of the total variance and were considered enough in reducing dimension and avoiding multicollinearity. For all the variables, the highest contributions of the individual responses to the ordination's axis were CAT (0.96), LDH (0.92), mortality (0.73) and total distance (0.74), and the contributions of the other environmental variables were more similar. PC1 links the Cu125 and Cu125+MPs groups with mortality and CAT activity, as well with GPx, GST and the behavior variables, speed, total distance and absolute turn angle. The genetic expression *cat* and *gstp1* had opposite responses of CAT and GST, respectively. LDH activity and GSSG levels were strongly associated with each other, with negative contribution to PC1. PC2 was also defined by the gradient of Cu concentration, with the Cu60+MPs group being positively correlated with this axis. Consequently, this group was related with higher expression of *ache* and *gclc* and the behavioral variables, distance to center and percentage of inactivity of larvae, that were negatively associated to the percentage of time active. The gene expression of *ache* had also an opposite response of AChE.

4. Discussion

The knowledge about the toxicological effects of MPs particles and their mixture with heavy metals on fish early life stages is still limited, particularly on freshwater species.

In this study, several developmental parameters were evaluated in the exposed zebrafish. The MPs used in the present study are in the range of 1–5 μm; therefore, the smallest particles have a size within the range of the zebrafish chorionic pores (300 nm–1 μm, Lin et al., 2013). In fact, the evaluation of 24 hpf zebrafish under fluorescence microscopy, suggested that the smallest MPs were able to cross the embryos chorion. Moreover, in later stages, at 96 hpf, MPs particles were observed in the interior of the head area and in the retina of some larvae. Since zebrafish were exposed to MPs, alone or in combination with Cu, shortly after fertilization (~2 hpf), it can be speculated that the diffusion of MPs through the chorion may have led to the accumulation of these particles on the embryo yolk sac or skin epithelium and to the possible uptake and biodistribution over the embryo organogenesis. Supporting this hypothesis, it was recently reported that polystyrene nanoparticles are able to penetrate fish chorion and accumulate in the blood stream, yolk sac, heart region, head and in organs such pancreas and liver (Pitt et al., 2018; Veneman et al., 2017). More recently, Qiang and Cheng (2019) reported that 1 μm polystyrene MPs only adhered to the chorion of 4 hpf zebrafish embryos. This disparity between studies, however, may be attributed to the concentration of MPs used (2 mg/L vs. 0.1 and 1 mg/L), type of MPs, initiation of the experiment (~2 hpf vs. 4 hpf) and time and conditions of exposure. Regardless of these findings, there is a lack of information about the uptake and biodistribution of MPs by fish embryos, and

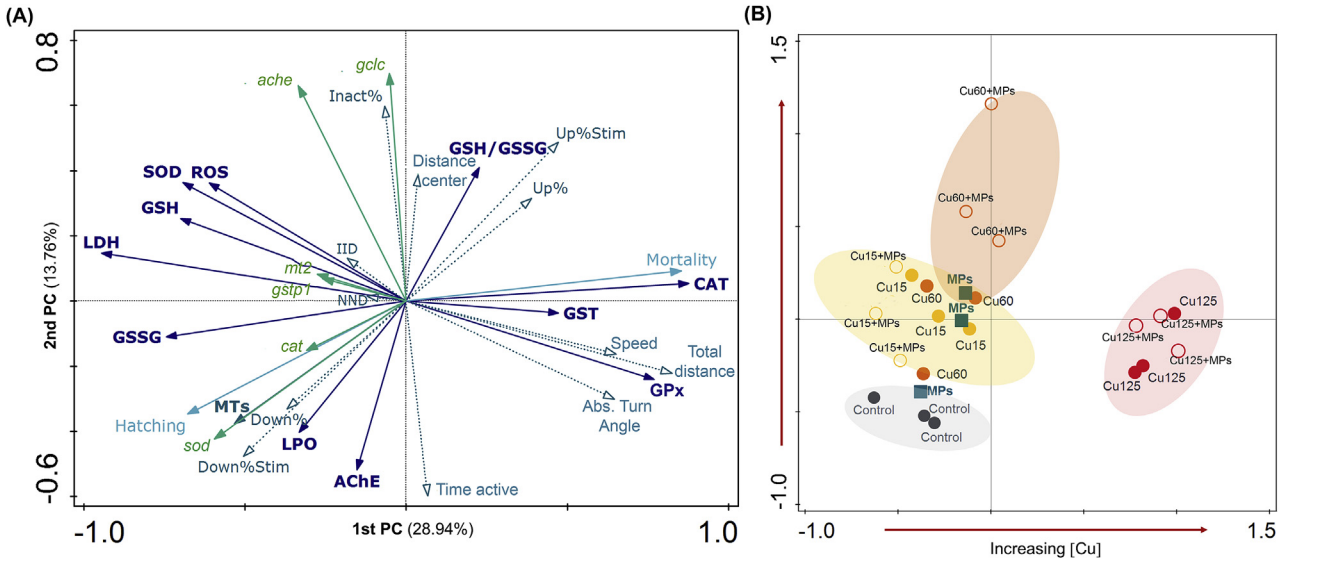

Fig. 7. Principal component analysis (A) of the mortality, hatching, metallothionein levels and behavioral, biochemical and molecular variables analyzed in zebrafish larvae exposed to microplastics (MPs) and copper (Cu), alone or combined. The first two axis explained 42.7% of the total variance. (B) The colors represent the different treatments, with red corresponding to high concentrations; orange to moderate; yellow to low concentrations and grey corresponding to the control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

future studies should be carried out.

Notwithstanding, minor impacts on survival of the embryos and larvae exposed to MPs alone were found. This exposure had no effects on the mortality and hatching rates or in the body length and heart rate overall the experiment. Our data are in agreement with previous studies, reporting that exposure to MPs alone did not produce significant effects on lethal and sub-lethal morphological parameters on fish embryos and larvae (Chen et al., 2017; LeMoine et al., 2018; Pannetier et al., 2020, 2019; Qiang and Cheng, 2019). On the other hand, exposure to Cu alone influenced the development of zebrafish embryos and larvae, by increasing the mortality on the first 24 h and mostly by inhibiting the hatching of the embryos. An increase of malformations rate was also observed. It is known that even at low concentrations, Cu exerts toxic effects on embryos, by increasing mortality, delaying embryo hatching and causing malformations (Dorts et al., 2016; Sonnack et al., 2015). Egg hatching is a crucial period during embryogenesis, being a common and sensitive parameter to evaluate embryotoxicity. The mechanisms involved in the hatching delay caused by exposure to pollutants are not clear, though it has been proposed that the decrease in embryo motility, changes in the levels of hatching enzymes or alteration of oxygen uptake by the embryo can contribute to hatching inhibition (Dorts et al., 2016; Zhang et al., 2018). Considering this, the observed inhibition may have resulted from the effects of Cu on more than one of these mechanisms. In the MPs + Cu groups, reduced survival and hatching rates, and changes in body length and heart rate were also observed. However, given that differences between the groups with Cu alone and the respective mixtures were not significant, then it suggests that MPs did not influence the Cu-induced toxicity in these lethal and sub-lethal endpoints in zebrafish.

Fish early life stages are extremely susceptible to oxidative stress (Félix et al., 2016), which results from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses. In the present study, increased ROS levels were observed in larvae exposed to Cu15, Cu60 and respective Cu + MPs, suggesting that an induction of oxidative stress occurred. Concomitantly, alterations in the antioxidant enzymes and glutathione levels were further observed.

The SOD and CAT enzymes are considered the primary line of defense towards the production of ROS under environmental stress (Lushchak, 2016). Our results show that SOD activity decreased in larvae exposed to Cu125 and Cu125+MPs, with MPs having no influence in Cu action. Being a metalloenzyme, SOD inhibition may have resulted from the direct binding of Cu ions to the active site of the enzyme, which results in changes of SOD conformation and therefore improper folding or misfolding. In fact, it was proposed that Cu-related toxicity may be due to its indiscriminate binding to proteins rather than the generally accepted ROS-generation hypothesis (Letelier et al., 2005). In agreement with our findings, in goldfish (*Carassius auratus*) higher Cu concentrations (>0.01 mg/L) inhibited this enzyme (Liu et al., 2006). The levels of *sod* gene expression were also downregulated in Cu and Cu + MPs exposed larvae. The basic region leucine-zipper transcription factor *Nrf2* (*nuclear factor erythroid 2-related factor 2*), which after nuclear translocation binds to the antioxidant response element (ARE) within gene promoters, plays a key role in the activation of genes regulating the cellular antioxidant response in fish (Ma, 2013). In Jian carp (*Cyprinus carpio* var. Jian) exposed to Cu, for 4 days, there was a decrease in nuclear Nrf2 levels and in ARE binding ability, as well as a downregulation of antioxidant related genes. Taking this into consideration, although *Nrf2* was not evaluated in the present study, the observed downregulation of *sod* gene may also have resulted from disruption of Nrf2/ARE signaling. In this study, a dose-dependent enhanced CAT activity was also observed in all Cu and Cu + MPs groups, indicating an antioxidant response to overcome metal and MPs stress. Moreover, the higher increase of CAT in Cu125 and Cu125+MPs suggests an adaptive mechanism to compensate SOD inhibition observed in these groups and maintain the cellular redox homeostasis. Still, the expression of *cat* gene did not suffer alterations in response to Cu and Cu + MPs exposure, showing that mRNA levels do not always correlate with the respective protein levels (Lushchak, 2016). GPx which detoxifies H_2O_2 and hydroperoxides to hydroxy molecules with GSH as cofactor (Lushchak, 2016), also showed a significant increase in zebrafish exposed to Cu125 and Cu125+MPs. In agreement with our results, induced oxidative stress and SOD, CAT and GPx alterations have been reported in fish species exposed to heavy metals or to mixtures of MPs and metals (Jiang et al., 2014; Liu et al., 2006; Lu et al., 2018; Wen et al., 2018). Additionally, our results showed that GST activity, a phase II biotransformation enzyme, was induced in the Cu125 and Cu125+MPs groups, demonstrating the activation of detoxification mechanisms to overcome metal and MPs stress. GST may have played an important role in controlling oxidative stress in these two groups, compensating the SOD inhibition. Alterations in the GST enzyme in response to heavy metals (Liu et al., 2006) or mixtures of MPs and pollutants (Wang et al., 2019) have also been reported. The positive correlation observed between CAT, GPx and GST suggest that these enzymes may have played an important role in the elimination and homeostasis of ROS in zebrafish larvae exposed to Cu and Cu + MPs.

In the present study, GSH, known to play an important role as ROS scavenger or by serving as a cofactor for ROS-detoxifying enzymes such as GPx or GST (Lushchak, 2016), progressively decreased in Cu groups, becoming significantly different from control in the Cu125 group. Likewise, GSSG levels also decreased. This depletion may be attributed either to the use of GSH to scavenge the free radicals or ROS generated by metal exposure. Supporting this, a positive correlation between GSH and ROS levels was noticed. Conjugation of GSH with metal ions catalyzed by GST may have contributed to its decrease, since an increase of GST was also observed in the Cu125 group. In fact, it has been proposed that GSH is capable of complexing and detoxifying heavy metal cations, resulting therefore in a reduced availability of GSH (Canesi et al., 1999). Moreover, GPx, which uses GSH as a cofactor, was shown to be increased in Cu125 and Cu125+MPs, therefore explaining in part the depletion of GSH in these exposed groups. Although not measured in the present work, an inhibitory effect on GR enzyme can also contribute to this depletion, by interfering with the supply of NADPH in GSH recycling. GSH levels reduction was also reported in fish exposed to heavy metals (Jiang et al., 2014; Lu et al., 2018; Wen et al., 2018). These findings also emphasized the antagonistic action of MPs in relation to Cu, with Cu125+MPs showing higher levels of GSH than Cu125 alone, and therefore, evidencing an alleviation of the effects of Cu and restoring of GSH levels. Oppositely, in the lowest mixture groups, GSH levels tended to increase, becoming significantly different from the control in the Cu60+MPs group. Besides, these groups differed significantly from the corresponding group exposed to Cu alone, suggesting an antagonistic interaction between MPs and Cu. Similarly, other studies have reported an antagonistic interaction between MPs and pollutants (Chen et al., 2017; Miranda et al., 2019; Pacheco et al., 2018), with MPs alleviating the chemical pollutants toxicity. An upregulation of *gclc* gene expression, which is involved in GSH synthesis, was also observed in these groups, being significantly different from control in Cu60+MPs group and differing from the groups exposed to Cu alone. Again, an antagonistic response was observed between MPs and Cu for this parameter. Overexpression of glutamate cysteine ligase (GCL) subunits genes, such as *gclc*, under stressful conditions

have been correlated with changes in cellular GSH, therefore increasing cell potential for *de novo* GSH synthesis (Krzywanski et al., 2004). Thus, a stimulated GSH synthesis through an enhanced *gclc* expression contributed to the increase of GSH levels observed in the two lowest Cu + MPs exposed larvae. The elevated GSH levels observed may be resultant from the presence of MPs and as an adaptive mechanism to prevent Cu-induced toxicity. Overall, the above findings suggest that under low and middle stress, Cu and Cu + MPs induced a cellular response to the increased ROS levels, by increasing CAT activity and GSH levels to overcome the oxidative stress and prevent oxidative damage. Whereas under severe stress, GSH levels decreased probably due to its increased use as cofactor of the antioxidant enzymes and, consequently, the reestablishment of the cellular ion and redox homeostasis. Notwithstanding, GSH levels findings also emphasized the antagonistic action of MPs in relation to Cu.

Generally, high levels of ROS and induction of oxidative stress lead to increased lipid peroxidation. For most of the exposed groups, LPO levels did not suffer significant alterations, indicating that no significant lipid oxidative damage occurred, and that the response of the antioxidant defense system overcame the metal and MPs stress. Surprisingly, lower LPO levels were noticed in the Cu125 and Cu125+MPs groups. This may be related with the increased activity of GPx and GST, which are efficient protective enzymes against lipid peroxidation (Lushchak, 2016). Indeed, a negative correlation between LPO levels and these two enzymes was observed. Although a slight increment of ROS levels in zebrafish larvae exposed to MPs alone was observed, no significant alterations of the response of both enzymatic and non-enzymatic antioxidants were detected, nor lipid oxidative damage. Chen et al. (2017) also observed that exposure of zebrafish to MPs alone do not elicit significant changes in such enzymes, corroborating our results. The effects of MPs alone seem to be a controversial issue, since a few studies have documented the induction of oxidative stress, increase of LPO levels, as well as modulation of SOD and CAT activity in fish exposed to MPs (Barboza et al., 2018a; Lu et al., 2016; Qiao et al., 2019b; Wan et al., 2019). However, no direct comparisons are possible, since most of these studies were performed in adult fishes. Besides, differences in species sensitivity and exposure conditions, such as type and concentration of MPs, route and time of exposure may be responsible for such opposite findings. Overall, the results of the present study indicate that Cu and Cu + MPs induced oxidative stress, which was compensated with an increase of the antioxidant system enzymes, therefore, protecting zebrafish against lipid oxidative damage. Hence, this response may have prevented severe malformations on larvae.

Metabolic regulation plays a crucial role in stress tolerance. LDH is an anaerobic glycolysis enzyme used as a biomarker of cellular energy production and tissue damage (Shi et al., 2019). Our findings show that LDH activity decreased in larvae exposed to Cu125 and Cu125+MPs, which in turn suggests a possible inhibition of the anaerobic metabolism in the larvae, raising potential negative consequences to the energetic balance when dealing with metal and MPs stress. In agreement with our findings, Teodorescu et al. (2012) reported an inhibition of LDH activity in goldfish (*Carassius auratus gibelio*) exposed to 100 and 250 μg/L of Cu, for 72 h. These authors proposed that Cu may interfere with Na+/K + ATPase, which in turn generates acidosis and inhibition of phosphofructokinase, the rate limiting enzyme of glycolysis pathway. Direct binding of the metal to the active site thiol group of LDH and formation of an enzyme-inhibitor complex or changes in the activity of mitochondrial membrane function were also proposed as mechanisms responsible for LDH inhibition (Sastry and Gupta, 1980; Teodorescu et al., 2012). Modulation of LDH activity has also been observed in other aquatic biota exposed to heavy metals (Banaee et al., 2019; Shi et al., 2019) and mixtures of MPs and pollutants (Banaee et al., 2019).

MT are a family of proteins involved in heavy metal detoxification and protection of tissues against oxidative damage (Lindeque et al., 2010). In general, it has been shown that MT are induced in response to heavy metals (Lindeque et al., 2010; Lushchak, 2016). In the present study, contrary to the expected, MT protein levels decreased in the Cu60, Cu125 and respective Cu + MPs groups. A possible reason for this decrease may be related with the inhibition of MT synthesis.

Acetylcholinesterase (AChE) is a key enzyme of the nervous system, having an essential role in neurotransmission. The results of the present study showed that exposure to Cu alone and Cu + MPs induced AChE inhibition. In PCA analysis, a positive correlation between AChE and hatching was observed, which suggests that a decrease in embryo motility derived from AChE inhibition may have contributed to the lower hatching observed in the exposed embryos. Previous studies have reported inhibition of AChE activity in fish exposed to Cu (Haverroth et al., 2015; Roda et al., 2020; Tilton et al., 2011). In fish exposed to the MPs alone, no significant differences were perceived in this parameter, although a slight decrease of AChE activity was noticed. Similarly, a slight decrease of AChE activity was previously reported in zebrafish larvae exposed to 1 mg/L of polystyrene MPs (Chen et al., 2017) and in common goby (*Pomatoschistus microps*) juveniles exposed to 0.184 mg/L of MPs (Fonte et al., 2016). Moreover, significant inhibition of AChE activity by MPs has been reported previously in fish (Banaee et al., 2019; Barboza et al., 2018a; Roda et al., 2020). Reacting to the neurotoxicity induction, an adaptive response was observed in zebrafish larvae, rising the *ache* expression in the MPs, Cu15, Cu60 and respective mixtures. Therefore, this answer may have alleviated the neurotoxicity induced by some treatments, such as MPs alone. Notwithstanding, the present results and the mentioned studies suggest that MPs and heavy metals may lead to adverse effects in the cholinergic neurocircuit, causing neurotoxicity. Supporting the MPs and Cu induced neurotoxicity, in the present study, the histological analysis also revealed changes in the morphology of brain and retina in the MPs and Cu exposed zebrafish larvae, with epithelial detachment being the histopathological change with higher prevalence in exposed animals. The fish nervous system is involved in a wide range of physiological and regulatory functions that are crucial for the survival and performance of organisms. Therefore, brain lesions induced by pollutants can compromise the individual and, ultimately the populations' fitness. Although the incidence of histological changes observed in the exposed larvae was low, the analysis evidences that brain and retina may be sensitive organs, not only to Cu, but also to MPs. Histopathological changes have also been reported in fish after exposure to heavy metals, such as disruption of the boundaries within the brain and decrease of differentiated neurons and glia numbers (Al-sawafi et al., 2017; Chow et al., 2008), and to MPs, like epithelial detachment and mucous hypersecretion on intestinal mucosa and gills (Limonta et al., 2019; Qiao et al., 2019b).

Both predator avoidance and social behaviors are crucial to a successful and adapted life strategy, being dependent of a healthy and functional nervous system. However, exposure to aquatic pollutants can alter these behavioral patterns, compromising the adaptive performance and survival of fish populations (Acosta et al., 2016). In the present study, most of the exploratory behaviors were not altered significantly. However, exposure to Cu125 and Cu125+MPs increased the total distance traveled by larvae. Besides, when subjected to alternating light and dark periods, the larvae exposed to Cu125+MPs also presented a significant increase of the distance moved. These findings illustrate hyperactivity and disturbances in the modulation of locomotion, which, consequently,

can have detrimental effects on fish performance due to increased energy expenditure. In fact, an opposed tendency was revealed by PCA analysis between LDH activity, involved in the metabolic rate, and the total distance moved by larvae, emphasizing that assessing behavioral alterations may provide a linkage between physiological processes and interaction with the environment (Sandoval-Herrera et al., 2019). Similarly, locomotor activity disturbances have been reported in fish exposed to Cu or to MPs, alone or combined (Acosta et al., 2016; Chen et al., 2017; Haverroth et al., 2015; Qiang and Cheng, 2019). The locomotor response involves several neurochemical systems (Sharma, 2019), which when disrupted by exposure to xenobiotics can be translated in behavior impairment. Along with the histopathological lesions observed in some individuals, on the mechanism underlying the behavioral response of larvae after Cu and Cu + MPs exposure, the AChE inhibition observed in the present study seems to be the primary candidate to explain the altered locomotor response. In fact, a negative correlation between the total distance traveled by larvae and AChE was observed in this study, implying that an inhibition of AChE may have caused an increase of the distance traveled. Indeed, inhibition of AChE leads to accumulation of acetylcholine in cholinergic synapses, keeping the acetylcholine-gated sodium channels open for more ions to flow into the cell. Ultimately it leads to an action potential and hyperstimulation (Ogungbemi et al., 2019). Accordingly, locomotion alterations associated with inhibition of AChE have been previously reported in zebrafish (Chen et al., 2017; Haverroth et al., 2015).

Zebrafish larvae display both sensory and motor systems that allow them to detect and avoid threatening environmental stimuli (Colwill and Creton, 2011). In fact, in the presence of an aversive stimulus, which in this study was a bouncing ball (mimicking a predator or the shadow of a predator), zebrafish react with an adaptive escape reaction (Colwill and Creton, 2011). Our findings showed that Cu and Cu + MPs exposure disrupted avoidance behavior of zebrafish larvae since they did not perceive the aversive stimuli. Besides, social behavior was also impaired in the Cu125+MPs group, implying a disruption of the social cohesion of these exposed larvae. Given that social behavior of zebrafish during early stages is crucial for the development of avoidance reactions (Groneberg et al., 2020), its disruption may be linked with the negative effects on avoidance behavior of the exposed larvae. The avoidance behavior alterations induced by Cu and Cu + MPs may be also related to alterations on the cholinergic system, given that a close association between the parameters of avoidance behavior and AChE inhibition was observed in PCA analysis. Supporting our results, alterations of avoidance behavior have also been linked to AChE inhibitors (Carlson et al., 1998; Sandoval-Herrera et al., 2019). Zebrafish rely on sensory, olfactory and vision systems to avoid predators, thus, impairment of these systems may also be involved in avoidance behavior changes. It has been demonstrated that Cu exposure disrupts the mechanosensory lateral line system, inducing neuromast hair cells death (Olivari et al., 2008; Sonnack et al., 2015), and the olfactory sensor cells and olfactory transduction signaling (Lazzari et al., 2017). Visually-guided behaviors and visual impairment has also been reported in zebrafish exposed to heavy metals (Chow et al., 2009; LeFauve and Connaughton, 2017) and to MPs, alone or co-exposed with other pollutants (Chen et al., 2017). Data of the present study provide an initial contribution about the effects of MPs and Cu on locomotor response, avoidance and social behaviors of early life stages of fish. Considering our findings, the long-term behavioral alterations in response to heavy metals and MPs, and its implications in fish population fitness still need to be clarified.

5. Conclusions

Overall, the present study emphasizes that a 96 h exposure to MPs had no relevant effects on early life stages of zebrafish. However, it is necessary to consider that the interaction of MPs with fish early life stages may depend of the exposure conditions such as time of exposure. On other hand, environmentally relevant concentrations of Cu, alone or co-exposed with MPs, increase mortality of embryos, inhibited hatching rate and acted through different cellular mechanisms, leading to oxidative stress, AChE inhibition and, ultimately, behavioral alterations in early life stages of zebrafish. For some parameters, namely, glutathione levels and *gstp1* and *gclc* genes, the effects induced by the mixture of Cu and MPs were lower than the effects of Cu alone, evidencing an antagonistic response of MPs in relation to Cu.

Further research on the effects of heavy metals and MPs co-exposure in fish development needs to be investigated in more detail, particularly regarding to neurotoxic effects. This work also alerts to the need for biomonitoring areas potentially polluted with heavy metals and MPs.

Credit author statement

Dércia Santos: Investigation, validation, writing – original draft, review and editing. **Luís Félix:** behavior analysis, methodology, results validation, writing – review and editing. **Ana Luzio:** results validation, writing – review and editing. **Susana Parra:** methodology in biochemical determinations performed. **Edna Cabecinha:** PCA analysis, data interpretation. **Juan Bellas and Sandra M. Monteiro**: conceptualization, resources, writing – review and editing, supervision.

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.

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Appendix A. Supplementary data

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