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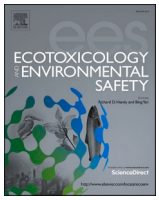

Microplastics and copper induce apoptosis, alter neurocircuits, and cause behavioral changes in zebrafish (*Danio rerio*) brain

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

[a] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, CITAB and Inov4Agro, Institute for Innovation, Capacity Building and Sustainability of Agri-food Production, Quinta de Prados, Vila Real 5000-801, Portugal
[b] University of Trás-os-Montes and Alto Douro, Quinta de Prados, Vila Real 5000-801, Portugal
[c] Centro Oceanográfico de Vigo, Instituto Español de Oceanografía, IEO-CSIC, Subida a Radio Faro 50, Vigo 36390, Spain

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ABSTRACT

The knowledge regarding the neurological and behavioral toxic effects associated with microplastics (MPs) and heavy metals exposure is still scarce. The present study aimed to evaluate the potential chronic (30 days) toxic effects of MPs (2 mg/L) and copper (Cu, 25 μg/L), alone or combined, in the zebrafish (*Danio rerio*) brain antioxidant system, cell proliferation/death, cholinergic-, serotonergic- and dopaminergic pathways and, consequently, in locomotor, anxiety, and social behaviors.

Our findings showed that MPs and Cu exposure modulated the antioxidant system of zebrafish brain, with superoxide dismutase (SOD) and glutathione reductase (GR) having higher activity in the Cu25 +MPs group, but glutathione peroxidase (GPx) being inhibited in MPs, Cu25 and Cu25 +MPs. Moreover, an increase in acetylcholinesterase (AChE) activity was observed in all exposed groups. When considering neurogenesis genes, a downregulation of proliferating cell nuclear antigen (*pcna*) was noticed in zebrafish exposed to the mixture treatment, while for dopaminergic system-related genes (*th* and *slc6a3*) an upregulation was observed in MPs, Cu25 and Cu25 +MPs groups. An increase in apoptosis-related genes expression (*casp8*, *casp9* and *casp3*) was observed in the MPs exposed group. Changes in zebrafish behavior, particularly in mean speed, total distance moved, inactivity in the aquaria, and social/shoaling behavior was also observed in the MPs and Cu exposed groups. Overall, our results highlight the multiplicity of toxic effects of MPs, alone or combined with Cu, in zebrafish brain, namely apoptosis and alterations in adult neurogenesis, neurocircuits and, consequently, behavior.

1. Introduction

The discovery of plastics and their wide applicability has progressively stimulated plastics mass production, with global production reaching 367 million tonnes in 2020 (PlasticsEurope, 2021). In turn, the high production of plastic together with ineffective waste management and disposal has caused increased pollution in ecosystems (Avio et al., 2017). In the aquatic environment, the degradation of macroplastics into smaller particles, designated microplastics (MPs, *i.e.* plastic polymer particles with a size lower than 5 mm), has become an environmental issue of global concern and a threat to aquatic organisms and human health (Avio et al., 2017). Their small size, high abundance, and widespread distribution in the aquatic environment, make MPs easily accessible for ingestion/uptake by zooplankton, bivalves or fish, increasing their potential to induce toxicological effects on aquatic organisms (Avio et al., 2017). Besides the direct impacts of MPs, there is an additional concern associated with the capacity of plastic particles to adsorb waterborne pollutants, such as heavy metals (Godoy et al., 2019), which could lead to alterations in their bioavailability and/or toxicity to aquatic organisms (Avio et al., 2017). A common heavy metal found in aquatic ecosystems is copper (Cu), which has been reported in concentrations ranging from 0.04 up to 560 μg/L in surface waters (Couto et al., 2018; Perlatti et al., 2021), and from 0.06 to 500.6 μg/g in MPs recovered from sediments and/or surface waters (Dobaradaran

* Correspondence to: Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Department of Biology and Environment, University of Trás-os-Montes and Alto Douro, Quinta de Prados, Vila Real 5000-801, Portugal.
E-mail address: dsantos@utad.pt (D. Santos).

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et al., 2018; Sarkar et al., 2021).

It has been shown that isolated MPs or Cu cause a wide range of toxic effects in aquatic organisms, such as histopathological lesions (Umamaheswari et al., 2021), oxidative stress (Barboza et al., 2018a; Santos et al., 2020, 2021a), cell death (Luzio et al., 2013; Umamaheswari et al., 2021), gut microbiota dysbiosis (Montero et al., 2022), reproductive dysfunction (Qiang and Cheng, 2021), and inflammation (Montero et al., 2022). Moreover, one of the commonly documented effects of MPs and Cu, or other heavy metals, is neurotoxicity with most of the studies reporting acetylcholinesterase inhibition (Barboza et al., 2018a; Miranda et al., 2019; Roda et al., 2020; Santos et al., 2021a). In turn, when the neurological function of the organisms is affected, behavioral phenotypes are normally associated (Fitzgerald et al., 2021), with some studies reporting impairment of olfactory-driven behavioral responses (Shi et al., 2021), swimming activity (Barboza et al., 2018b; Chen et al., 2020; Santos et al., 2021b), and predatory performance (Miranda et al., 2019) in aquatic species exposed to MPs, with or without heavy metals. Behavioral responses have been considered sensitive and ecologically relevant endpoints in ecotoxicity since they integrate the internal physiological state of organisms with their response to external stimuli at the same time (Tierney, 2011). Even sub-lethal changes in the neurological function and behavior of aquatic organisms may affect their performance and fitness, ultimately, leading to negative impacts on aquatic populations and ecosystem dynamics (Fitzgerald et al., 2021). It has been stated that behavior alterations may result from toxic effects in neuronal signaling networks (*e.g.* neurocircuits) and/or in mechanisms related to neurodevelopmental processes (*e.g.* neuronal proliferation) (Tierney, 2011). Nonetheless, research has focused mainly on the effects of isolated pollutants, with the neurological and behavioral toxic effects associated with MPs and Cu exposure remaining largely unclear. In turn, it is urgently needed to expand the knowledge about MPs and Cu neurotoxicity in aquatic organisms.

Considering the above, the present study aimed to evaluate the potential chronic toxic effects of MPs and Cu, alone or combined, in the fish brain and, consequently, in locomotor, anxiety, and social behaviors. For this, the well-established model organism zebrafish (*Danio rerio*) was used. Zebrafish is widely used in aquatic toxicity testing, including neurotoxicity screening, since it presents several advantages such as small size, easy and low-cost husbandry, characterized genome, well-conserved morphology and molecular mechanisms of the nervous system with other vertebrates, and a vast set of simple and complex behaviors (Fitzgerald et al., 2021). In the present study, to achieve the established objective, the stress response and metabolic enzymes, along with the expression of genes involved in the antioxidant/detoxification response, apoptosis, neuronal proliferation, and in the cholinergic-, serotonergic- and dopaminergic pathways, were evaluated in the zebrafish brain, after exposure for 30 days to MPs and Cu. Moreover, specific behavioral assays were performed to assess the swimming activity, social interaction, and anxiety-like behaviors of the exposed zebrafish. This study contributes to a better understanding of MPs and Cu neurotoxicity in zebrafish and warns of the potential ecological risk that mixtures of plastic particles with heavy metals may represent to the aquatic ecosystem.

2. Materials and methods

2.1. Ethical statement

All the experiment protocols with zebrafish were performed following the European Directive 2010/63/EU and in compliance with the Portuguese (Directive 113/2013) legislation for laboratory animals experimentation and welfare, and with the Guidelines of the European Union Council (86/609/EU) and the Portuguese law (Decrete law 129/92). The authors involved in fish manipulation and experimentation are accredited with FELASA category C (Federation of Laboratory Animal Science Associations) and with licenses approved by the National Competent Authority for animal research (Direção-Geral de Alimentação e Veterinária, Lisbon, Portugal).

2.2. MPs and Cu

Virgin MPs (proprietary polymer) of 1–5 μm in diameter were obtained as dry powders from Cospheric LLC (CA, USA). The polymer particles were red fluorescent-labeled (excitation and emission wavelengths of 575/607 nm, respectively), spherical, melting point of ~290 °C, and with a density of 1.3 g/cm^3. The fluorescent particles were hydrophilic, thus it was not necessary the addition of surfactants. Considering the manufacturer specifications, it was estimated a concentration of 5.44E+ 07 spheres per mg of MPs (more details in Santos et al., 2020). In the experimental procedures, 2 mg/L of MPs dry powder was used, with the particles being dispersed in the water column. This concentration of MPs (2 mg/L ~ 1.09×10^8 particles/L) was selected to simulate a high pollution scenario, as reported, for example, in playa wetlands (levels up to 5.51 mg/L, Lasee et al., 2017).

A stock solution of copper sulfate pentahydrate ($CuSO_4.5\ H_2O$, CAS 7758–99–8, Merck, Darmstadt, Germany), at 1 g Cu/L, was prepared in ultrapure water and used for the preparation of the experimental solutions at a final concentration of 25 μg/L (0.1 μM). The selected Cu concentration (25 μg/L) was chosen considering the 96 h-LC_{50} for zebrafish (94 μg/L) (Luzio et al., 2013), being one-quarter of the reported LC_{50}, and considering the range of Cu concentrations (0.2–30 μg/L) observed in unpolluted and polluted freshwater ecosystems (Couto et al., 2018; Perlatti et al., 2021).

2.3. Experimental design

Adult wild-type zebrafish (*Danio rerio*, AB strain, ~8 months) maintained in the fish facility of the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) were randomly distributed to 30 L glass-aquaria and allowed to acclimatize for 24 h. Four treatment groups, in triplicate (16 fish per aquaria, in a total of 48 fish per group), were then established as follows: control (dechlorinated water); MPs (2 mg/L); Cu (0.1 μM = 25 μg/L, Cu25); and mixture (25 μg Cu/L + 2 mg MPs/L, Cu25 +MPs). Fish were chronically exposed for 30 days and kept under a 14:10 h (light: dark) photoperiod, in a semi-closed system supplied with dechlorinated, aerated, charcoal-filtered, and UV sterilized tap water, at 28 ± 1 ºC. Water chemistry parameters were maintained under the species optimal conditions. Fish were fed twice a day with commercial fish food (TetraMin, Germany) and *Artemia* sp. nauplii.

Water/exposure solutions were partly (40 %) exchanged manually every 48 h and the aquaria were cleaned to maintain water quality and prevent plastic particles accumulation. In addition, glass aquaria were continuously aerated to guarantee the oxygen saturation (6.6 ± 0.4 mg O_2/L) and the dispersion of the MPs in the water.

During the experiment, the mortality of fish was assessed daily. Before sampling, fish were starved for 24 h. Then, after the 30-day exposure, fish behavior was assessed, followed by their euthanization with an overdose of buffered tricaine methanesulfonate (MS-222). The weight and length of each fish were measured. Whole-brain was excised, under a stereomicroscope, for the biomarkers evaluation.

2.4. Biochemical biomarkers measurements

For the biochemical biomarkers evaluation, the brain of 15 fish per group (5 from each replicate) were sampled and homogenized in an ice-cold buffer (pH 7.4, sucrose 0.32 mM, HEPES 20 mM, $MgCl_2$ 1 mM, and phenylmethylsulphonyl fluoride 0.5 mM). Samples were centrifuged (15,000 × *g*, 20 min, 4 ºC, Sigma 3K30) and the homogenate supernatant was collected. Standardized protocols, described previously by Félix et al. (2016) and Santos et al. (2020), were used to determine the activity and/or levels of the following biomarkers: reactive oxygen species (ROS), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase

(CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR), reduced (GSH) and oxidized states (GSSG) of glutathione levels, lactate dehydrogenase (LDH), acetylcholinesterase (AChE) and metallothioneins (MT). The details concerning the protocols used to evaluate each enzymatic biomarker can be found in Supplementary file 1.

The concentration of protein on each sample was determined through the method of Bradford (1976), at 595 nm. All the measurements were performed at 30 °C in a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Varian Cary Eclipse (Varian, USA) spectrofluorometer.

2.5. Gene expression analysis

For gene expression evaluation, the brain of 18 fish per group (6 from each replicate) were sampled and stored in RNA Later (Sigma, Germany), at −80 °C, until analysis. Samples were homogenized, for 2 min, in a TissueLyzer shaker (Qiagen, Germany), at 30 Hz, and the total RNA was isolated with the *Illustra RNAspin Mini Kit* (GE Healthcare, Germany) and reverse transcribed into cDNA using the *SensiFAST cDNA Synthesis Kit* (Bioline, UK).

Subsequently, the relative expression levels of 19 target genes involved in antioxidant activity (*sod1*, *cat*, *gclc*, *gstp1*), detoxification (*mt2*), apoptosis (*casp3*, *casp8*, *casp9*, *aif*), neurogenesis (*pcna*, *sox2*), cholinergic (*ache*), serotonergic (*tph1a*, *tph1b*, *tph2*, *slc6a4a*, *slc6a4b*) and dopaminergic (*th*, *slc6a3*) systems (Table S1), was determined by quantitative real-time PCR (qPCR), as described in Santos et al. (2020) and in Supplementary file 1. On each plate, a no template control was included. Each sample was run in duplicate, and real-time PCR reactions were carried out in triplicate. Only comparative threshold cycle quantification (*Cq*) values leading to a *Cq* mean with a standard deviation below 0.2 were considered.

The relative expression of each gene was calculated according to the method of Hellemans et al. (2007) and the results are expressed as normalized relative quantity (NRQ). For the statistical analysis, gene expression data were *log2* transformed (*Cq'*) to reduce the heterogeneity of variance.

2.6. Behavioral analysis

At the end of the experiment, 5 fish per group and replicate (n = 3) were randomly selected to assess behavioral parameters. The fish behavior was monitored using a video-tracking system (TheRealFishTracker) (Buske and Gerlai, 2014), and it was carried out in a temperature-controlled room (25 ± 1 °C). During recordings, disturbances (*e.g.* noise and movement) were reduced. Behavior was recorded from above with 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), at a resolution of 1920 × 1080 pixels (30 frames per second). A schematic diagram of the behavioral tests is shown in Fig. S1 (Suppl. file 1).

Animals were placed individually in glass aquaria (n = 3, with 5 fish per group and replicate) and an *open field* test was carried out, for 5 min, to evaluate the exploratory/locomotor behavior, as described by Stewart et al. (2012). The following endpoints were measured: mean speed, total distance moved, mean absolute turn angle, percentage of time active/-inactive, and mean distance to the center zone.

The light-dark test, which is used to evaluate anxiety behavior, was performed as described by Gebauer et al. (2011). Animals (n = 3, with 5 fish per group and replicate) were placed individually in a glass aquarium (10 × 20 × 4 cm) divided into two equally sized partitions (one-half of the tank was coated with a white non-reflective paper and the other area with dark non-reflective paper). Fish were placed in the aquaria and the time spent on each compartment was recorded for 5 min

To study the social behavior, 5 fish per group and replicate (n = 3) were placed in the test aquarium and, after 5 min of acclimation, their behavior was recorded for 5 min. The nearest neighbor distance (NND) and the average inter-individual distance (IID) parameters were then evaluated based on the X, Y position obtained from the tracking software.

2.7. Metal concentrations analysis

To confirm the actual Cu concentrations, 50 mL of each experimental solution was collected at the start and after 14 and 30 days of exposure. Each sample was acidified with 65 % nitric acid (HNO_3, Merck, Darmstadt, Germany), and stored at 4 °C until analysis. For the evaluation of Cu bioaccumulation in zebrafish, after the 30 days of exposure,brain samples were collected, weighed, and transferred to glass tubes. Samples digestion was then performed with HNO_3 (65 %, Merck, Germany) mixed with hydrogen peroxide (30 % H_2O_2, Merck, Germany), at room temperature, overnight. The solutions were heated until became clear, dried at 155 ℃ and cooled to room temperature. Finally, 65 % HNO_3 matrix solution was added to the digested samples and stirred. Both water and digested samples were quantified, in duplicate, by electrothermal atomic absorption spectrometry (Unicam 939 Spectrometer, GF90 furnace). The results are expressed as mean ± standard deviation (SD) and are presented in Table S2 (Suppl. file 1).

2.8. Integrated Biomarker Response (IBR) and statistical analysis

The stress index IBR was used to integrate all biomarkers, and calculated according to the method described by Beliaeff and Burgeot (2002) and later adapted by Devin et al. (2014). Data were normalized and the obtained scores were displayed in a star plot. To obtain an IBR value, all the star plot triangular areas were summed up (details in Santos et al., 2021a).

Through a redundancy analysis (RDA), a constrained ordination was applied to extract and summarize the variation in the response variables that can be explained by the explanatory variables. Variables related to enzymatic activities were standardized before all analyses to preserve the original scale. The significance of the variables (biochemistry, gene expression, and behavior) was tested (individually and all together) with 999 Monte Carlo permutations. The RDA was performed using CANOCO 5 (version 5.12) (Biometrics, Wageningen, Netherlands).

Prism 9.0 software (GraphPad Software, Inc., USA) was used to perform the rest of the statistical analysis. The experimental data were checked for assumptions of homogeneity of variance and normality with the Brown-Forsythe and Kolmogorov-Smirnov tests, respectively. A one-way analysis of variance (ANOVA) followed by Tukey's multi-comparison post-hoc test was performed to evaluate differences between groups. A two-way ANOVA followed by Tukey's multicomparison post-hoc test was also performed to assess if the presence/absence of MPs modulates Cu toxicity. When data did not fulfill a normal distribution, the non-parametric Kruskal-Wallis on Ranks test followed by the post-hoc Dunn's multi-comparison test was conducted. Statistical differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Biometric parameters of zebrafish

During the experiment, no mortality or signs of disease were observed in the fish. No significant differences were observed in body length and weight between exposed and control fish (Supp. file 2 – Fig. S1), suggesting that MPs and Cu exposure did not influence zebrafish growth. Corroborating our results, previous studies have shown that growth performance was not affected in fish exposed to low-density polyethylene MPs (Alomar et al., 2021) or combined with heavy metals (Cu, Cd, Pb and Zn) (Wen et al., 2018). Nevertheless, other authors reported a decrease in body length and weight in zebrafish and medaka (*Oryzias melastigma*), after being exposed to MPs-enriched diets

(Cormier et al., 2021). These divergences have been associated with the feeding behavior of the species, the MPs exposure route and concentration, as well as the egestion rate (Alomar et al., 2021), thus explaining the different responses of zebrafish to MPs in relation to the results reported in the literature.

3.2. Effects of MPs and Cu on the antioxidant defense system and detoxification processes of zebrafish brain

Organisms have several biological mechanisms to prevent and eliminate excess ROS levels and, therefore, minimize their negative effects on cells. Among these mechanisms, the antioxidant system, which comprises several enzymes such as SOD, CAT, and GPx, tightly regulates ROS levels to maintain cell homeostasis. The data regarding the biochemical biomarkers evaluated are presented in Table 1. In the present study, ROS and LPO levels were not significantly affected by MPs and Cu exposure, in comparison with the control group, despite the tendency of the Cu25 +MPs group to present higher levels of both oxidative stress/damage biomarkers ($p > 0.05$). Though, it was observed that the antioxidant system was modulated by MPs and Cu. The enzyme SOD showed higher activity in Cu25 ($p = 0.0026$) and Cu25 +MPs ($p < 0.0001$), while GPx was inhibited in MPs ($p = 0.0004$), Cu25 ($p = 0.0088$), and Cu25 +MPs ($p < 0.0001$) groups. CAT activity and GST, a phase II biotransformation enzyme, on the other hand, did not show significant changes in comparison to the control. Likewise, the transcription of the antioxidant gene *sod1* was upregulated in the MPs group ($p = 0.0138$), while *cat* and *gstp1* gene expression did not show significant changes in the exposed groups, in comparison with the control group (Suppl. file 2 - Table S1, and illustrated in Fig. 1).

Since ROS production is transitory, but the antioxidant system was modulated by MPs and Cu exposure, it is possible that during the 30-day experiment the treatments may have induced the generation of ROS, which subsequently activated the antioxidant defenses of zebrafish. A rise in SOD activity indicates that an excess of the free radical superoxide ($O_2^{\cdot-}$) in the cells needed to be counteracted to prevent oxidative damage. This is also supported by the non-significant induction of the CAT activity in Cu25 and Cu25 +MPs. In turn, the GPx inhibition by MPs and Cu suggests that the hydrogen peroxide (H_2O_2) produced by the SOD detoxification reaction may have surpassed the scavenging capacity of GPx. It has been stated that GPx is more effective at low H_2O_2 concentrations, whereas CAT is the predominant H_2O_2 scavenger at high concentrations (Rocha et al., 2020). Drąg-Kozak et al. (2019) suggested that inhibition of GPx activity can result directly from the damage of its functional groups and consequently its catalytic activity by the heavy metals, or indirectly from a compromised supply of NADPH. In fact, a significant increase in GR activity was observed in the MPs ($p = 0.0184$) and Cu25 +MPs ($p = 0.0030$) groups, in comparison with the control group, thus explaining a possible compromised supply of NADPH for GPx. GR activity may, in turn, be increased to counterbalance the significant increase of GSSG levels on Cu25 +MPs exposed fish ($p = 0.0330$), since GSSG is toxic to the cells, and to regenerate the GSH. Nevertheless, the *gclc* gene, that is involved in GSH synthesis, was not significantly affected in the exposed zebrafish brain (Suppl. file 2 - Table S1, and illustrated in Fig. 1). The present findings are consistent with previous studies that reported the induction of oxidative stress and/or modulation of the antioxidant system in the brain or other organs of fish exposed to MPs, alone or combined with heavy metals (Barboza et al., 2018a; Wang et al., 2021; Wen et al., 2018).

MT are cysteine-rich proteins of low molecular weight involved in the regulation of intracellular metals and the detoxification of heavy metals (Santos et al., 2021a). In the exposed zebrafish brain, no alterations in MT levels (Table 1) nor the *mt2* gene expression (Suppl. file 2 - Table S1, and illustrated in Fig. 1) were observed in any of the groups ($p > 0.05$), compared to the control. These results indicate that MPs and Cu, under these experimental conditions, were not able to induce MT synthesis in the zebrafish brain. This reflects that MT may not be

Table 1
Chronic changes in biochemical parameters observed in zebrafish (*Danio rerio*) exposed for 30 days to microplastics (MPs) and copper (Cu), alone or combined.

Enzymatic Biomarkers		Cont	2 mg/L	25 µg/L		Interaction between MPs and Cu
			MPs	Cu	Cu+MPs	
Oxidative stress and damage	**ROS** µmol DCF/mg of protein	980.7 ± 287.6	866.8 ± 264.3	994.6 ± 233.8	1165.1 ± 464.7	F- value = 3.059. *p - value = 0.085*
	LPO µmol MDA/mg of protein	909.2 ± 534.6**ab**	722.0 ± 360.3**ab**	611.1 ± 383.4**a**	1156.9 ± 679.3**b**	F- value = 8.395. *p - value = 0.005*
Antioxidant enzymes	**SOD** U /mg of protein	687.0 ± 310.3**a**	649.6 ± 289.0**a**	1197.3 ± 436.0**b**	1469.0 ± 491.4**b**	F- value = 2.500. *p - value = 0.119*
	CAT U /mg of protein	135.2 ± 31.4**ab**	108.4 ± 50.4**a**	151.8 ± 52.5**b**	171.6 ± 69.2**b**	F- value = 3.138. *p - value = 0.082*
	GPx µmol NADPH/min.mg of protein	176.2 ± 62.4**a**	64.7 ± 28.2**bc**	82.1 ± 41.8**b**	34.8 ± 21.7**c**	F- value = 9.565. *p - value = 0.003*
	GR µmol NADPH/min.mg of protein	73.1 ± 29.9**a**	123.2 ± 44.3**b**	108.7 ± 48.4**ab**	133.5 ± 56.1**b**	F- value = 1.183. *p - value = 0.281*
Detoxification	**GST** µmol CDNB/min.mg of protein	235.1 ± 86.7	170.0 ± 53.5	213.1 ± 69.1	176.2 ± 87.2	F- value = 0.563. *p - value = 0.456*
	MT µmol GSH/mg of protein	9.1 ± 3.3**ab**	7.4 ± 2.3**ab**	7.1 ± 2.3**a**	10.3 ± 3.9**b**	F- value = 9.961. *p - value = 0.003*
Glutathione levels	**GSH** µmol /mg of protein	3283.7 ± 879.4	3417.5 ± 759.7	3945.3 ± 896.0	3926.9 ± 731.7	F- value = 0.138. *p - value = 0.712*
	GSSG µmol /mg of protein	224.7 ± 59.3**a**	216.3 ± 55.7**a**	259.9 ± 57.0**ab**	294.5 ± 99.4**b**	F- value = 1.498. *p - value = 0.226*
Metabolism	**LDH** µmol NADH/min.mg of protein	741.0 ± 168.4	627.0 ± 195.7	707.2 ± 230.2	773.0 ± 256.5	F- value = 2.763. *p - value = 0.102*
Neurotoxicity	**AChE** µmol TNB/min.mg of protein	0.102 ± 0.036**a**	0.143 ± 0.026**b**	0.163 ± 0.044**bc**	0.200 ± 0.053**c**	F- value = 0.0802. *p - value = 0.778*

Data from three independent replicates (n = 3, with 5 fish per group and replicate) 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 ($p < 0.05$). The two-way ANOVA, followed by Tukey's post-test was used to assess the interaction between the microplastics and copper.

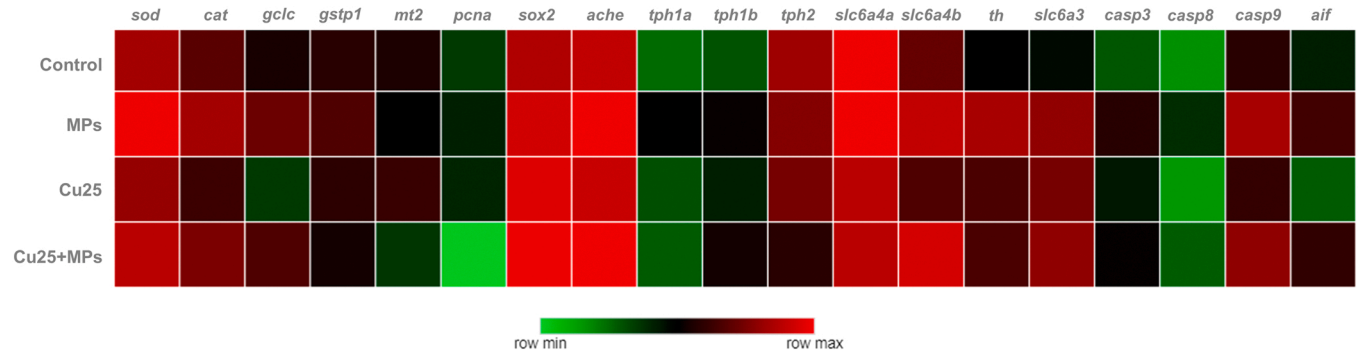

Fig. 1. Heatmap of the expression profile of the antioxidant enzymes (*sod1*, *cat*, *gclc*), detoxification (*gstp1*, *mt2*), neuronal cell proliferation (*pcna*, *sox2*), cholinergic (*ache*), serotonergic (*tph1a*, *tph1b*, *tph2*, *slc6a4a*, *slc6a4b*), dopaminergic (*th2*, *slc6a3*), and apoptosis (*casp3*, *casp8*, *casp9*, *aif*) genes in zebrafish exposed to microplastics (MPs, 2 mg/L) and copper (Cu, 25 μg/L), alone or combined, for 30 days. Data from three independent replicates (n = 3, with 6 fish per group and replicate).

determinant in MPs and Cu detoxification. Qiao et al. (2019) also found that MT levels were not affected in zebrafish exposed to MPs or Cu alone, however, in the mixture's groups, MT levels were increased in the liver and gut. Wen et al. (2018) also reported higher levels of MT in whole-bodies of discus fish (*Symphysodon aequifasciatus*) early juveniles exposed to polystyrene-MPs and cadmium. In turn, these differences between studies may be related to different responses of fish organs and species, as well as to MPs and heavy metals concentrations.

LDH is an anaerobic enzyme that catalyzes the conversion of lactate and pyruvate (Banaee et al., 2019), and is increased under oxidative stress conditions due to cell integrity disruption. In this study, LDH did not show significant changes in the brain of the exposed fish. This suggests that MPs and Cu, alone or combined, did not interfere with the anaerobic metabolic energy supply in the zebrafish brain.

Considering our results, zebrafish brain showed different antioxidant responses to counteract the toxic effects of MPs and Cu, alone or combined, thus avoiding oxidative damage. Nevertheless, apart from this, the inhibition of GPx, considered one of the major antioxidant enzymes in the nervous system (Franco and Cidlowski, 2009), renders neuronal cells susceptible to other alterations, such as apoptosis.

3.3. Effects of MPs and Cu in zebrafish neurogenesis

Proliferating cell nuclear antigen (*pcna*) is a cyclin essential for cell cycle control, DNA replication, and excision repair mechanisms (Schmidt et al., 2013). PCNA is expressed throughout the cell cycle of actively growing cells, with the highest expression being observed in the *S* phase, being, therefore, commonly used as a marker of cellular proliferation (Schmidt et al., 2013). *Sex-determining region Y (SRY)-related HMG box 2* (*sox2*) encodes a transcription factor that plays a crucial role in the maintenance of neural stem/progenitor cell pluripotency, being used as a neural stem cell marker (Schmidt et al., 2013). In the present study, for the two proliferation/neural stem cell markers, *i.e.*, *pcna* and *sox2* (Suppl. file 2 - Table S1, and illustrated in Fig. 1), no significant effects were observed in *sox2* gene expression ($p > 0.05$); however, downregulation of *pcna* gene was observed in Cu25 +MPs exposed zebrafish ($p = 0.0017$), in comparison to the control group. Changes in *pcna* expression reflect an alteration in neuronal proliferation activity. Thus, the present results suggest that MPs combined with Cu can impair the cellular proliferation process in the zebrafish brain. In teleost species, such as zebrafish, the adult brain has small niches, called "proliferation zones", that are responsible to generate new neurons from neural stem cells (Schmidt et al., 2013). Contrarily to mammals, teleost fish have a high neurogenic activity throughout life (Schmidt et al., 2013), allowing them to respond to environmental changes. Taking this into consideration, interference of MPs and Cu with adult neuronal proliferation can therefore affect brain plasticity and specific neurocircuits, with possible negative consequences in fish cognitive function and behavior.

3.4. Effects of MPs and Cu on the cholinergic, serotonergic, and dopaminergic systems

AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine in the cholinergic neurons postsynaptic membrane and has been used as a marker of neurotoxicity (Roda et al., 2020). Most of the studies have reported inhibition of AChE activity in fish (Barboza et al., 2018a; Roda et al., 2020) exposed to MPs alone, or combined with other pollutants. Surprisingly, in the present study, AChE activity increased significantly in MPs ($p = 0.049$), Cu25 ($p = 0.0009$), and Cu25 +MPs ($p < 0.0001$) groups, in comparison with the control group (Table 1). However, the *ache* transcript did not show significant changes in the exposed groups, in comparison with the control group (Suppl. file 2 - Table S1, and illustrated in Fig. 1). Similarly, an increase of AChE activity was observed in zebrafish juveniles exposed to polystyrene MPs (4×10^4 and 4×10^6 particles/m^3), for five days (Guimarães et al., 2021). Additionally, increased AChE activity was shown in discus fish (Huang et al., 2022) exposed to MPs. In this study, the AChE increase in the zebrafish brain may be related to the observed apoptosis induction and/or the tendency to higher ROS and LPO levels, and consequent cell damage. Some authors have shown that elevated AChE activity is associated to oxidative stress (Melo et al., 2003). Actually, it has been suggested that such processes can lead to the rupture of the presynaptic vesicle membranes containing acetylcholine, resulting in this neurotransmitter release and an over-stimulation of the post-synaptic receptors (Guimarães et al., 2021). The opposing results between studies, including ours, could be explained by differences in the experimental context (*e.g.* route and time of exposure), species, and type/-concentrations of the MPs (Guimarães et al., 2021). Nevertheless, regardless of the signaling pathway involved, our findings reinforce the neurotoxicity potential of MPs and Cu in aquatic organisms.

The monoamine serotonin (5-hydroxytryptamine, 5-HT) and the catecholamine dopamine are two important neurotransmitters that regulate neural activity and mediate a broad range of behaviors, including locomotor, aggressiveness, cognition, and anxiety-like behaviors (Herculano and Maximino, 2014). 5-HT is synthesized from tryptophan by tryptophan hydroxylase (TPH) enzyme, with zebrafish having three paralogs genes of the TPH, namely the *tryptophan hydroxylase 1a* (*tph1a*), the *tryptophan hydroxylase 1b* (*tph1b*), and the *tryptophan hydroxylase 2* (*tph2*) (Herculano and Maximino, 2014). In turn, after 5-HT is being released into the synaptic cleft, the 5-HT transporter (SERT), encoded by the two *solute carrier family 6 a, member 4a* and *4b* (*slc6a4a* and *slc6a4b*), removes it and terminates synaptic activity (Herculano and Maximino, 2014). In the present study, the evaluated transcripts of the serotonergic pathway were not affected by MPs and Cu in zebrafish brain ($p > 0.05$), in comparison with the control group

(Suppl. file 2 - Table S1, and illustrated in Fig. 1). Considering the dopaminergic pathway, the gene expression of the rate-limiting enzyme *tyrosine hydroxylase* (*th*), involved in dopamine synthesis, and the *dopamine transporter* (*slc6a3/dat*), which terminates the dopamine action in the synaptic cleft (Holzschuh et al., 2001), was evaluated. In our study, *th* gene expression was upregulated in the zebrafish brain exposed to MPs ($p = 0.0084$), while the *slc6a3* transcript was upregulated in the MPs ($p = 0.0078$), Cu25 ($p = 0.0403$), and Cu25 +MPs ($p = 0.0113$) groups, comparatively to the control group (Suppl. file 2 - Table S1, and illustrated in Fig. 1).

The above results indicate that, at least in the present experimental conditions, MPs and Cu do not interfere with the 5-HT synthesis, transport, and uptake, of the serotonergic system in the zebrafish brain. However, MPs and Cu, alone or combined, modulated the dopamine synthesis and transport in the exposed zebrafish brain, highlighting the neurotoxic potential of plastic particles and that different signaling pathways, other than AChE, may be involved in MPs action. Supporting these findings, previous studies have found that MPs, alone or combined with other pollutants, can modulate neurotransmitters levels and/or transcripts in several species, including fish (Huang et al., 2022; Shi et al., 2021) and bivalves (Shi et al., 2020).

3.5. Effects of MPs and Cu in the apoptotic pathways of zebrafish brain

Apoptosis, also referred as programmed cell death, is a complex process tightly regulated by specific death-signaling pathways, and that occurs to maintain cell populations' homeostasis or as a defense mechanism against cellular stress or pathological stimuli (AnvariFar et al., 2018). Apoptosis can be triggered by two main pathways - the mitochondrial or intrinsic pathway, which involves cell death signals from the mitochondria (*e.g.* cytochrome *c*); and the death receptor or extrinsic pathway, which is activated by transmembrane receptor-mediated interactions (AnvariFar et al., 2018). In turn, these pathways involve the activation of a cascade of cysteinyl aspartic acid-specific proteases called caspases (AnvariFar et al., 2018). The present study evaluated the expression of the initiating caspases, namely, *caspase-8* (*casp8*) and *caspase-9* (*casp9*), that are part of the extrinsic and intrinsic pathways, respectively, and the executioner *caspase-3* (*casp3*). Additionally, the *apoptosis-inducing factor* (*aif*) gene, a specific mitochondrial factor that is part of the apoptotic caspase-independent pathway (Luzio et al., 2013), was evaluated. The data regarding the apoptosis genes expression is presented in Supplementary file 2 - Table S1, and illustrated in Fig. 1. An increase of *casp8, casp9* and *casp3* gene expression was observed in the brain of MPs exposed zebrafish ($p < 0.05$), compared to the control group. Despite the lack of statistical significance, in Cu25 and Cu25 +MPs groups, a trend to higher expression levels of caspases ($p > 0.05$) was also observed. For *aif* gene, no significant effects were observed in the brain of the exposed groups. In turn, these results suggest that MPs induced apoptosis in the zebrafish brain, through both intrinsic and extrinsic pathways. Data regarding the effects of MPs on apoptosis pathways in the fish brain are still lacking. However, it was recently reported that mixed neuronal cells isolated from mouse embryonic cortex underwent apoptosis, by showing an increased expression of the apoptotic marker cleaved caspase-3, upon exposure to polystyrene nanoplastics (100 nm) (Jung et al., 2020). Likewise, an upregulation of apoptosis-related genes after exposure to MPs was previously shown in the gills (Umamaheswari et al., 2021) and male gonad (Qiang and Cheng, 2021) of zebrafish and in sheepshead minnow (*Cyprinodon variegatus*) larvae (Choi et al., 2018). An increase of the caspase-3 activity was also observed in the blood clam (*Tegillarca granosa*) upon exposure to polystyrene MPs and sertraline, for 14 days (Shi et al., 2020).

Is not clear how MPs alone induced apoptosis in the zebrafish brain. It has been reported that GPx, an antioxidant enzyme, has antiapoptotic properties, by preventing *cytochrome c* release from the mitochondria and by the activation of caspase-3 (Franco and Cidlowski, 2009). Therefore, a possible explanation for the activation of the apoptotic pathway in the zebrafish brain may be associated with the GPx activity depletion also observed in the MPs exposed group. Another possible mechanism by which MPs induced apoptosis could be related to the alterations observed in the dopaminergic pathway, particularly the potential increase of dopamine synthesis and transport to the cells, as a consequence of the upregulation of the *th2* and *slc6a3* genes. In support of this, accumulating evidence has shown that dopamine itself can be neurotoxic and that high levels of dopamine, which when oxidized generates ROS, free radicals, and quinones, can trigger caspases activation and subsequent apoptosis, *via* the p38 kinase phosphorylation/activation and/or the c-Jun N-terminal kinase (JNK) pathway (Junn and Mouradian, 2001; Luo et al., 1998). Overall, our findings suggest that oxidative stress, GPx, and dopamine may play a role in MPs-induced apoptosis in the fish brain, and, consequently, neurotoxicity.

3.6. Effects of MPs and Cu in zebrafish behavior

The evaluation of fish behavior has been considered an important and sensitive endpoint in ecotoxicological studies since behavior output provides an early warning sign of the pollutants toxicity and integrates multiple levels of biological effects (Fitzgerald et al., 2021). In this study, exposure to MPs or Cu25 decreased the mean speed ($p < 0.01$, 584.1 ± 33.5 and 584.6 ± 2.2 cm/min, respectively), while exposure to Cu25 +MPs (812.3 ± 30.1 cm/min) increased the mean speed of zebrafish ($p = 0.0028$), in comparison to the control group (695.9 ± 27.1 cm/min) (Fig. 2A). The total distance moved by fish was also decreased in the MPs exposed group ($p = 0.0395$, 2863.4 ± 1.8 cm), comparatively to the control fish (3445.8 ± 234.4 cm) (Fig. 2B). The absolute turn angle was increased in the Cu25 +MPs group ($p = 0.0061$, 31.0 ± 2.1°), in comparison with the control group (24.9 ± 1.1°) (Fig. 2 C). The time that fish remained inactive in the aquaria was increased in MPs ($p < 0.0001$, 10.4 ± 0.3 %) and Cu25 +MPs ($p = 0.0002$, 9.8 ± 1.9 %), in relation to the control group (3.0 ± 0.3 %) (Fig. 2D). Regarding the time that animals spent in the central zone of the aquaria, it was observed a decrease in the Cu25 group ($p = 0.0055$, 15.2 ± 0.03 %), but an increase in the Cu25 +MPs exposed fish ($p < 0.0001$, 66.0 ± 7.2 cm), when compared to the control group (31.1 ± 0.9 %) (Fig. 2E). Overall, the results show that MPs and Cu25 alone caused hypoactivity in zebrafish, while the exposure to the mixture Cu25 +MPs resulted in a state of hyperactivity. A state of hypoactivity or hyperactivity implicates negative consequences for fish performance, making them, for example, more susceptible to predation or affecting their hunting behavior. The increased AChE activity observed in the exposed fish may have contributed to the swimming pattern changes. It has been stated that, in some cases, the persistent stimulation of the cholinergic system could result in excitotoxicity and consequent neuronal apoptosis (Tierney, 2011), which in our case may explain the observed hypoactivity behavior and the induction of apoptosis in MPs and Cu groups. Additionally, the observed locomotor hypoactivity could be related to muscle morphological changes. Although in this study we did not determine such changes, previous studies have shown an impairment of the integrity and function of muscle fibers (*e.g.* thickness reduction of muscularis layer) in fish exposed to MPs (Chen et al., 2020) or heavy metals (Avallone et al., 2015), with a consequent alteration of swimming activity. Besides, interference with glucose and lipid metabolism, which can affect energy homeostasis and consequently the support of vital processes such as the locomotor behavior and the development of brain, is another hypothesis for the observed effects of MPs and Cu in this study. Chen et al. (2020), for example, showed that polystyrene MPs (5 μm) exposure. at a wide range of concentrations (0.001–20 mg/L) affected zebrafish energy-supplying substances, such as the glycolytic pathway. Considering the Cu25 +MPs group, the interaction of both pollutants seems to exert neurotoxicity, and a consequent change in swimming behavior,

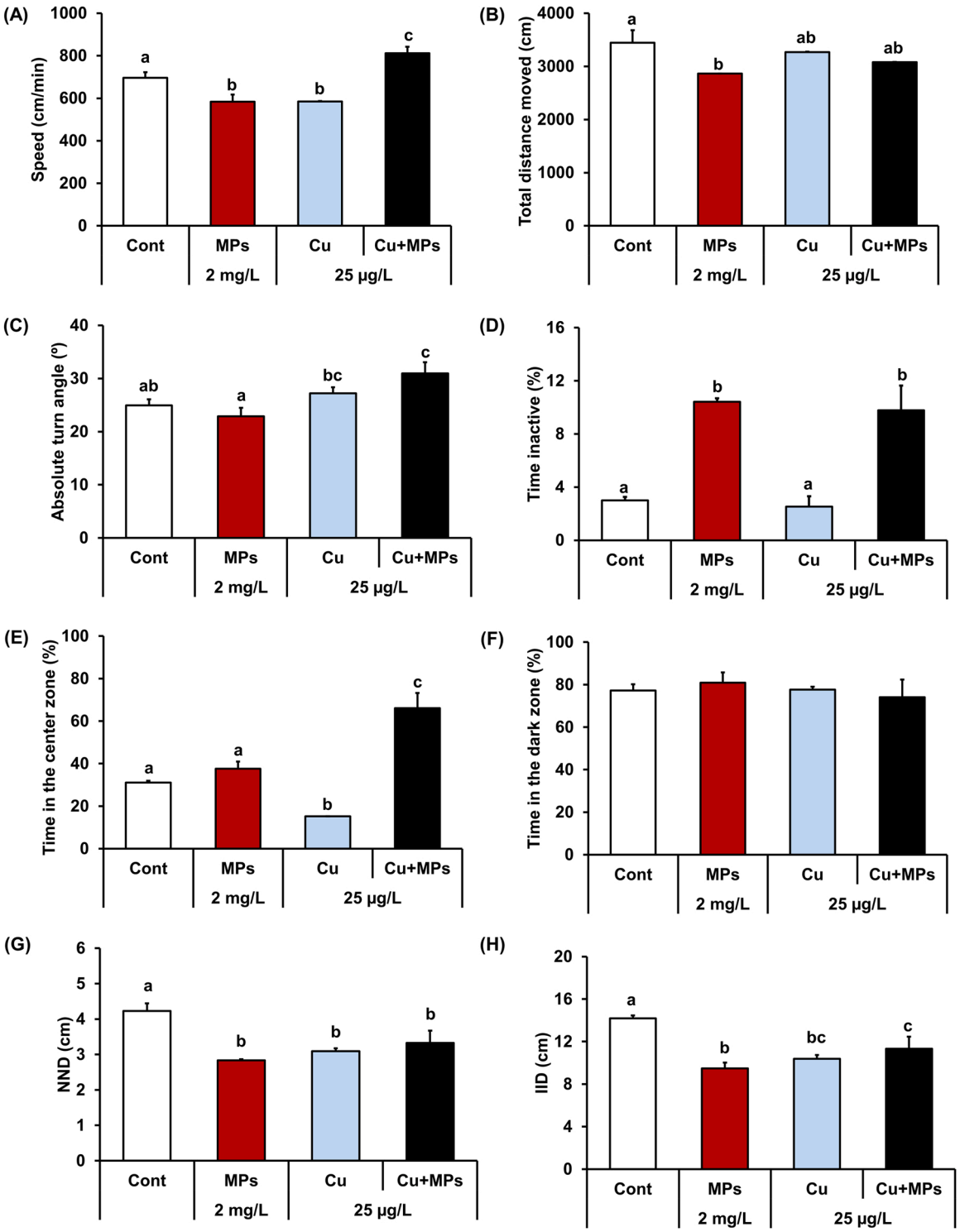

Fig. 2. Locomotor (A-E), anxiety-like (F), and social (G-H) behavior of zebrafish exposed for 30 days to microplastics (MPs, 2 mg/L) and copper (Cu, 25 µg/L), alone or combined. (A) Mean speed, (B) total distance moved by fish, (C) absolute turn angle, (D) percentage of time that fish were inactive, (E) percentage of time that fish remained in the center zone, (F) percentage of time that fish remained in the dark zone, (G) NND, nearest neighbor distance and (H) IID, inter-individual distance. Data from three independent replicates (n = 3, with 5 fish per group and replicate) and presented 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 ($p < 0.05$).

differently than the pollutants alone. Possibly, the induction of a hyperactivity state in Cu25 +MPs exposed zebrafish may be related, not only with the increase in AChE activity and the consequent transient state of adaptation of fish to such excitatory stimulation, but also with dopamine synthesis/transport (*th* and *slc6a3*) increase. In fact, previous research established that the dopaminergic system contributes to the control of locomotor behavior, and with an increase in dopamine being associated with a stimulating effect in swimming activity (Puttonen et al., 2013). Supporting our results, it was reported that exposure to MPs, as a single pollutant or combined with other contaminants, interferes with the swimming activity of fish (Barboza et al., 2018b; Chen et al., 2020; Choi et al., 2018; Qiang and Cheng, 2019; Santos et al.,

2021b).

The anxiety-like behavior is elicited by dangerous stimuli, being important for antipredator defense, foraging, and resources finding (Fitzgerald et al., 2021). The swimming activity response of zebrafish to white and dark areas was not affected ($p > 0.05$, Fig. 2F), suggesting that anxiety behavior was not modulated by MPs and Cu, alone or combined, at least in the present experimental conditions.

Fish social behavior which includes, for example, shoaling, is essential for their survival, foraging, and reproduction (Geng and Peterson, 2019). This behavior, more precisely, the tightness of a shoal, is usually evaluated by quantifying the NND and IID parameters (Geng and Peterson, 2019). In this study, a decrease of these parameters was observed in the MPs ($p < 0.001$), Cu25 ($p < 0.001$), and Cu25 +MPs ($p = 0.0034$) exposed fish, in comparison with the control group (Fig. 2G-H), implying a modulation of social/shoaling behavior by MPs and Cu in zebrafish. Social behavior is dependent upon a wide array of molecular and cellular mechanisms, including the dopaminergic system (Oliveira, 2013; Saif et al., 2013). In zebrafish it has been shown that after social stimuli (*e.g.* conspecific images) there is an increase of dopamine levels in the brain (Saif et al., 2013). Nevertheless, in the present study, the upregulation of *th* and *slc6a3* genes implies a possible increase of dopamine levels, while the social behavior decreased, which suggests that the disruption of this behavior in the exposed zebrafish may have occurred through other cellular/molecular mechanisms. A possible explanation may be related to alterations in visual and/or olfactory senses since both are essential in social recognition (Oliveira, 2013). In fact, visual impairment induced by MPs and/or heavy metals exposure has been reported in previous studies (Chen et al., 2017; LeFauve and Connaughton, 2017). Additionally, the impairment of the locomotor activity observed in the exposed zebrafish may have contributed to the alteration of social behavior.

3.7. Interactive effects of MPs on Cu toxicity

When combined, MPs and heavy metals can exhibit interaction effects, leading to alterations in the toxicokinetics of metals in fish (Yuan et al., 2020). In our study, significant interactions were found between MPs and Cu, indicating that the presence of plastic particles may have influenced the Cu induced-toxicity in biochemical (LPO, GPx, MT) and genetic (*mt2*, *pcna*, *slc6a4a*, *th2*, *slc6a3*) parameters of the brain, and in the behavior (speed, distance moved, abs. turn angle, time in the center zone, NND and IID) of the zebrafish (Table 1 and Suppl. file 2 - Tables S1 and S2). Among the biochemical parameters, it was noticed that LPO and MT showed significantly higher levels in Cu25 +MPs ($p < 0.01$), compared to the individual MPs and Cu groups, thus suggesting a possible synergistic effect of the combination. Similarly, for the parameters mean speed and time spent in the center zone, a potential synergistic effect of the combination was found, with fish exposed to Cu25 +MPs showing higher values ($p < 0.0001$) in comparison with fish exposed to MPs or Cu alone. On the other hand, in molecular biomarkers, a lower expression for *pcna* gene was observed in the Cu25 +MPs group ($p < 0.0001$), in relation to MPs and Cu alone groups, suggesting a possible antagonistic effect. Corroborating our results, previous reports have shown the modulation of heavy metals by MPs in different aquatic species, with some authors reporting synergistic effects and, therefore, an increase of the metal toxicity in the MPs presence (Banaee et al., 2019; Qiao et al., 2019; Santos et al., 2021a) and other authors reporting antagonistic effects, in which MPs alleviate the heavy metals toxicity (Miranda et al., 2019; Santos et al., 2020; Wen et al., 2018). As in our study, different interactions between MPs and heavy metals have also been observed for different endpoints in the same study (Wang et al., 2021). In turn, the discrepancy and variability between studies regarding the interactions between MPs and heavy metals have been associated with the physicochemical features of MPs (*e.g.* type of plastic), the chemical bond strength between the heavy metal and MPs, the experimental conditions (*e.g.* concentrations, exposure route) and/or the species (Yuan et al., 2020). Overall, our data suggest that MPs can modulate the toxicity of Cu in zebrafish, emphasizing the importance to evaluate the interaction between plastic particles and waterborne pollutants, and which may help to unravel the real ecological impact of MPs in aquatic ecosystems.

3.8. Integrated biomarker response (IBR) and RDA analysis

The IBR index is considered an effective tool to evaluate in an integrated manner the most sensitive endpoints/biomarkers of organisms in response to pollutants (Santos et al., 2021a). The IBR index and the corresponding star plots obtained in the present study are shown in Fig. 3. The IBR index applied to biochemical and genetic biomarkers results showed that the treatments with plastic particles presented the highest index values (2.033 and 2.097 for MPs and Cu25 +MPs, respectively), thus reflecting higher stress (Fig. 3A). In the MPs exposed fish, the most discriminant factors were the genes *sod1*, *cat*, *mt2* and *th2*, while in the Cu25 +MPs group they were the biomarkers SOD, LPO, LDH, *slc6a3*, *casp9* and *aif* (Fig. 3B). Overall, these results suggest that the treatments with plastic particles were more toxic than Cu alone in the zebrafish brain, highlighting the individual neurotoxicity of MPs reported in the literature (Banaee et al., 2019; Barboza et al., 2018a). Moreover, these findings suggest that MPs effects may have overlapped Cu toxicity.

The RDA results showed which variables related to zebrafish brain enzymatic activities, gene expression, and behavior contributed more to the explained variance in response to MPs, Cu25, and Cu25 +MPs (Fig. 4). The first two axes explained 91.75 %, 92.56 %, and 88.57 %, of the overall variation of the biochemical, gene expression and behavioral responses, respectively (Fig. 4 and Table 2). The enzymatic variables accounted for 23.1 % of the variance explained, in response to MPs and Cu, alone or combined. The most correlated variables with axis 1 were GPx (negatively), SOD, GR, and AChE (positively), with these last three

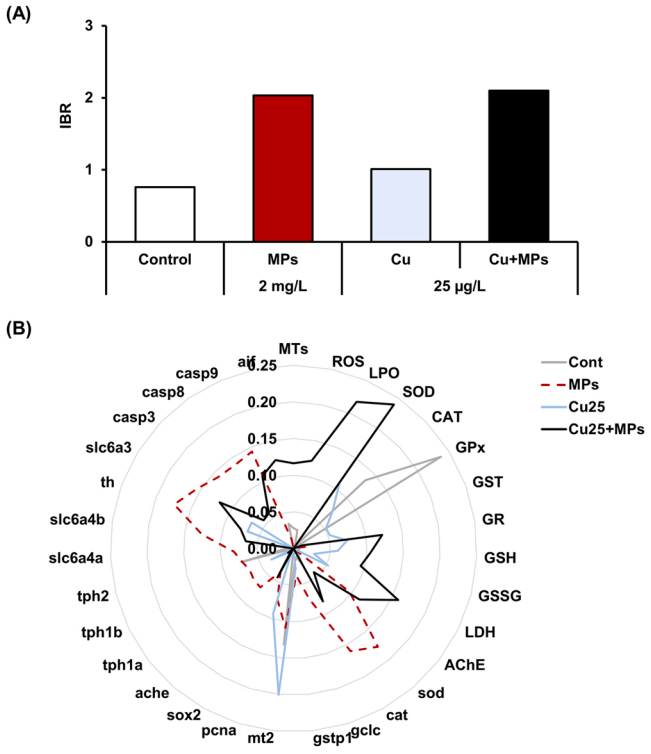

Fig. 3. Integrated biomarker response index (IBR) values (A) and star plot (B) of the biochemical and molecular biomarkers evaluated in zebrafish exposed to microplastics (MPs, 2 mg/L) and copper (Cu, 25 µg/L), alone or combined, for 30 days. Data from three independent replicates.

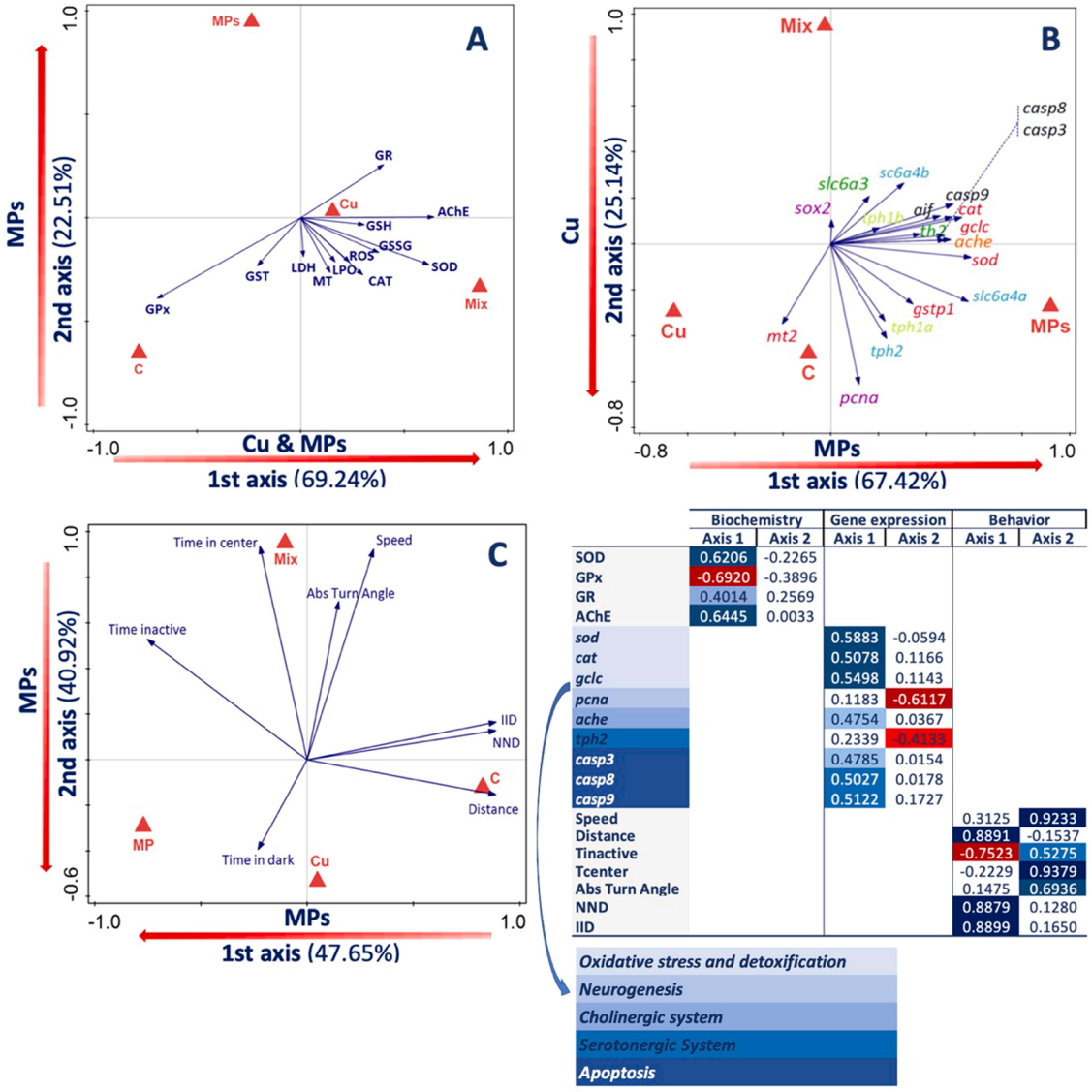

Fig. 4. Redundancy analysis (RDA) showing the total variance explained, for the first two axes, for each of the studied variables in the different groups [Control (C), microplastics (MPs), copper (Cu25), and the mixture of both (Cu25 +MPs (Mix))]: A) Biochemistry (23.1 %), B) gene expression (23.4 %), and C) behavior (82.9 %) parameters. The arrows show the gradient presented in the different plots and axis. The table shows the variables most correlated with the first axis (>0.4000), in blue positively, and in red negatively. Data from three independent replicates.

Table 2
Summary of the all redundancy analysis (RDA) results in zebrafish (*Danio rerio*) exposed for 30 days to microplastics (MPs) and copper (Cu), alone or combined.

	Biochemistry		Gene expression		Behavior		All variables	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Total variation	768.00000		722.00000		96.00000		1178.000	
Explanatory variables account for	23.07 %		23.44 %		82.88 %		23.68 %	
Explained fitted variation (cumulative)	69.24 %	91.75 %	67.42 %	92.56 %	47.65 %	88.57 %	52.88 %	85.63 %
Permutation Test Results								
On First Axis	F= 3.8, $p = 0.001$		F= 2.1, $p = 0.001$		F= 1.7, $p = 0.001$		F= 1.6, $p = 0.001$	
On All Axes	F= 6.0, $p = 0.001$		F= 3.5, $p = 0.001$		F= 12.9, $p = 0.001$		F= 3.5, $p = 0.001$	

Data from three independent replicates.

enzymes being positively correlated with the Cu25 +MPs, but negatively correlated with MPs. The gene expression variables accounted for 23.4 % of the variance explained, with the genes related to the oxidative stress (*sod* and *gstp1*), neurogenesis (*pcna*), cholinergic (*ache*) and serotonergic (*tph2* and *slc6a4a*) systems being positively correlated with axis 1 and with the presence of MPs. On the other hand, the *cat*, *gclc*, *slc6a4b*, *casp3*, *casp8* and *casp9* were negatively correlated with Cu. The variables related to behavioural responses accounted for 82.9 % of the variance. The time that animals spent in the central zone, the mean speed, and the absolute turn angle parameters were positively correlated with the Cu25 +MPs, but negatively correlated with Cu.

When considering all the variables (Fig. 5), the total variation

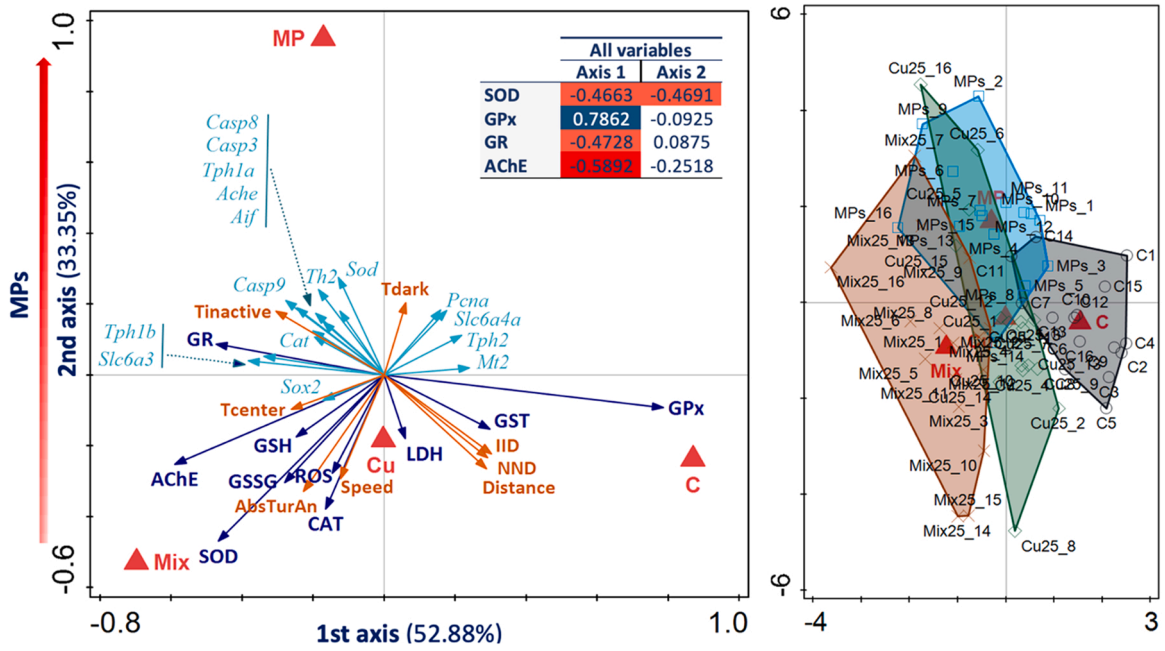

Fig. 5. Redundancy analysis (RDA) results show the total variance explained by all the studied variables in the zebrafish brain exposed for 30 days to the different groups [Control (C), microplastics (MPs), copper (Cu25), and the mixture of both (Cu25 +MPs (Mix))]. Dark blue – biochemistry, light blue – gene expression, and orange – behavior parameters. The arrows show the gradient presented in the different plots and axis. Data from three independent replicates.

explained was 1178.000, with the explanatory variables accounting for around 23.7 % (Table 2). Supporting the data presented in the above sections, the enzymatic activities of AChE, SOD, and CAT, and the behavior variables mean speed, time in the center, and the absolute turn angle were positively correlated with the Cu25 +MPs group. Positively correlated with the MPs group were the GR enzyme and the genes mostly related to apoptosis, cholinergic, serotonergic, and dopaminergic systems. Contrarily, the enzymatic activities of GPx, GST, and LDH, the variables related with social behavior, and the total distance traveled by fish were negatively correlated with the MPs presence in the medium. Overall, based on redundancy analysis, our results suggest that the disruption of AChE activity may have contributed to the alterations in the zebrafish locomotor behavior, while the disturbance of the social behavior may be related to changes in the dopaminergic system and the induction of apoptosis. Moreover, the negative correlation between the enzyme GPx and the apoptosis-related genes suggests that the observed inhibition of this enzyme, which is known to have antiapoptotic properties, may have contributed to the induction of apoptosis in the brain of zebrafish exposed to MPs. Nonetheless, more studies are still needed to further increase our knowledge about the role of MPs, and associated heavy metals, in the induction of apoptosis, and alterations in the neurocircuits and behavior in fish.

4. Conclusions

The results of this study demonstrated that the zebrafish brain is affected by MPs, alone or combined with Cu, at both molecular, cellular, and physiological levels. Overall, it was observed a modulation of the antioxidant system, which could counteract the induced oxidative stress and prevent oxidative damage. Furthermore, it was observed the induction of apoptosis in the zebrafish brain, accompanied by a decrease in neuronal proliferation, an AChE activity increase, and alterations in the dopaminergic system. In turn, these toxicological changes resulted in behavioral alterations, namely the disruption of the swimming activity and social behavior. The present study highlights the neurotoxic effect of MPs and provides new findings regarding the potential molecular and/or cellular mechanisms involved. Nevertheless, considering the negative ecological implications of the neurotoxic effects of MPs, alone or combined with heavy metals, future studies should pay more attention to the plastic particles' effects in fish brain.

CRediT authorship contribution statement

Dércia Santos: Investigation, Validation, Writing – original draft, Writing – review & editing. **Ana Luzio:** Validation, Writing – review & editing. **Luís Félix:** Validation, Writing – review & editing. **Edna Cabecinha:** PCA analysis, data interpretation. **Sandra M. Monteiro and Juan Bellas**: Conceptualization, Resources, Writing – review & 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113926.

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