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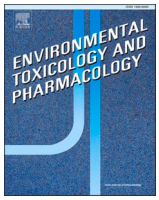

Polystyrene microplastics effects on zebrafish embryological development: Comparison of two different sizes

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ABSTRACT

Microplastics have become a great worldwide problem and it's therefore important to study their possible effects on human and environmental health. In this study, zebrafish embryos were used to compare two different sizes of polystyrene microplastics (PS-MPs), 1 μm and 3 μm respectively, at 0.01, 0.1, 1.0 and 10.0 mgL^{-1}, and were monitored up to 72 h. Toxicity tests demonstrated that neither of the PS-MPs altered the embryos' survival and the normal hatching process. Instead, higher concentrations of both sizes caused an increase of the heart rate and phenotypic changes. The PS-MPs of both sizes entered and accumulated in the larvae at the concentration of 10.0 mgL^{-1} and the same concentration caused an increase of apoptotic processes correlated to redox homeostasis changes. The reported results give a realistic view of the negative effects of exposure to PS-MPs and provide new information on their toxicity, also considering their sizes.

1. Introduction

Plastics are an immense family of unique and versatile materials and since the 1950 s, a total of 8.3 billion tons of synthetic plastics have been produced (Geyer et al., 2017). Global plastics production reached 370 million tons in 2019 (Plastics Europe, 2020) and is expected to reach 33 billion tons by 2050. These plastics reach oceans, soils, landfills, and even the atmosphere worldwide (Rhodes, 2018).

There are several categories of plastics such as polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), and polyurethane (PUR) (Letcher, 2020). Plastic pollution is mainly caused by an intentional release of small plastic debris and the deterioration of plastic products that end up in the environment (Plastics Europe, 2020; Zbyszewski et al., 2014).

These small fragments of plastic, fibers, and granules collectively defined as microplastics (MPs) are usually considered to range in size from 5 mm to 0.1 μm. MPs can be categorized as primary or secondary. Primary microplastics are intentionally produced for targeted applications, such as microbeads in personal care products (facial scrub or toothpaste) or in industrial detergent (Browne, 2015; Cole et al., 2011) while secondary microplastics are derived from the fragmentation and degradation of plastics (Galgani et al., 2013; Thompson et al., 2009).

MPs have become an emerging concern for human and environmental health because of their massive release, intentional or unintentional, into the environment, particularly into marine (Gola et al., 2021) and freshwater aquatic systems where they are deposited (Auta et al., 2017; Li et al., 2018). Indeed, significant amounts of MPs of different types, have been found in several freshwater ecosystems such as surface water and sediments (Mendoza and Balcer, 2019; Triebskorn et al., 2019).

Polystyrene is one of the most widely used types because of its corrosion resistance, ease of handling, and low cost (Chen et al., 2022a) but contains a styrene monomer that is a naturally occurring carcinogen. Many studies have recently highlighted that commercial polystyrene microplastics (PS-MPs) can accumulate in various aquatic organisms and that, starting with primary consumers, mainly represented by zooplankton, they involve all organisms in the food web chain, from invertebrates to seabirds and fish, including edible and commercial species (Cole et al., 2013; Markic et al., 2020; Setälä et al., 2014). Moreover, although it is far to understood, PS-MPs may pose a risk to human health; in fact, these particles can enter the human body through inhalation, ingestion, and skin contact and can penetrate the blood-brain barrier causing damage to brain tissue (Pitt et al., 2018). They are probably able to cross cell membranes causing oxidative stress and inflammation, respiratory diseases, or lung cancer (Vethaak et al., 2021).

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Zebrafish (*Danio rerio)* has been widely used as a bioindicator in the field of environmental toxicology, and as a model for human diseases and drug development due to its positive peculiarities, especially considering zebrafish embryos (Dai et al., 2014; Deveau et al., 2017; Zang et al., 2018). In fact, they develop rapidly and are transparent, which offers an advantage in studying the location of fluorescently or stained contaminants and allows an easy detection of alterations occurring in the early stages of development. Moreover, *Danio rerio* has genetic similarities with humans (Batel et al., 2018; Yang et al., 2009). It is a good organism model also for ecotoxicological studies and to test the adverse effects of metals such as cadmium (Monaco et al., 2016), aluminum (Bianchi et al., 2023; Capriello et al., 2021a; Capriello et al., 2022; Ferrandino et al., 2022) and copper (Pereira et al., 2016; Zhao et al., 2020) both in adult organisms and embryos (Capriello et al., 2019; Cheng et al., 2000; Monaco et al., 2017a).

In recent years, attention has been focused on the effects of microplastics and nanoplastics (NPs) in several aquatic and terrestrial organisms (Bradney et al., 2019; de Sá et al., 2018) such as mollusks (Browne et al., 2008), microalgae (Prata et al., 2019), snail (Song et al., 2019) and zebrafish (De Marco et al., 2022; Qiang and Cheng, 2019; Wang et al., 2022). In particular, it has been demonstrated that the exposure and following accumulation of MPs/NPs in zebrafish larvae caused several behavioral and morphological changes. To improve current knowledge, in this study we approached the problem of the size effect of polystyrene MPs, an aspect relatively neglected in the literature. In fact, most of the research focuses on the effects of a single type of MP on different organisms and/or tissues or on the comparative effect of microplastics and nanoplastics (Yan et al., 2021; Yin et al., 2021; Sendra et al., 2021).

For these reasons, in this work, *Danio rerio* embryos were exposed to 1 μm and 3 μm PS-MPs, and the concentrations of 0.01, 0.1, 1.0 and 10.0 mgL^{-1}, were selected to simulate microplastics pollution in the aquatic environment (Li et al., 2020). These concentrations were already used in previous research (Qiang and Cheng, 2019; Wang et al., 2022). In this paper, survival, hatching and development of zebrafish embryos were observed up to 72 h of exposure. Furthermore, to analyze in more detail the action of these particles, heart rate, oxidative stress, and cell death response were also determined. This latter is a highly conserved and regulated processes important in the morphogenesis of developing tissues and homeostasis organisms.

2. Material and methods

2.1. Preparation of PS-MPs suspension

PS-MPs of 1 μm (microParticles GmbH, Germany, density of 1.05 g/cm^3) and 3 μm (Sigma-Aldrich, Merck Life Science, Italy, density of 1.05 g/cm^3) in diameter were diluted in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2{\cdot}2\ H_2O$, 0.33 mM $MgSO_4$) to the final tested concentrations of 0.01, 0.1, 1.0 and 10.0 mgL^{-1}. Solutions were gently shaken before use.

2.2. Zebrafish maintenance and embryos collection

Adult zebrafish were bred in the facility of the Department of Biology, University of Naples Federico II, in oxygenated tanks, under a 14 h:10 h light/dark photoperiod, at a temperature of 28.0 °C, pH of 7.5. They were fed with a commercial diet (TetraMin Tropical Flake Fish®) supplemented with live *Artemia* sp. nauplii (Westerfield, 2000). All the experiments were according to the guidelines and policies dictated by European regulations on the wellness of animals employed for experimental purposes (Directive 2010/63/EU). All animal protocols used throughout the present study were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II, and the Italian Minister of Health.

Eggs were collected by siphoning the bottom of the tank in the early morning. Fertilized ones were selected under a stereomicroscope (Leica Zoom 2000) and transferred into E3 medium.

2.3. Embryos treatment with PS-MPs

Embryos at 6 h post fertilization were exposed to 10 mL of 0.01, 0.1. 1.0, 10.0 mgL^{-1} PS-MPs or to 10 mL of E3 medium (control), in 6-well plates (10 embryos per group and 2 duplicates per each group) and then were incubated at 28.0 °C, under semi-static conditions (solutions were renewed daily). All experiments were repeated in triplicate.

2.4. Survival, hatching, and morphological analyses

The survival rate was assessed at 24, 48, and 72 h of treatment, and the hatching rate at 48 h; the number of dead embryos or hatched larvae was determined over the total number of developing eggs (Malafaia et al., 2020). Phenotypical alterations were determined at 72 h of treatment, under a light microscope, after placing the larvae in a hanging drop slide. The type and percentage of alterations were determined by counting the number of malformed larvae out of the total number of observed larvae.

2.5. Cardiotoxicity

The heart rate was measured at 72 h of treatment under the microscope for a 15 s period and then calculated per minute. The data are the average of three measurements per larvae (Monaco et al., 2017a).

2.6. Nile red staining

To localize PS-MPs within embryos, Nile red (NR) staining was used on a total of 10 embryos per treatment, according to the method described by Bashirova et al. (2023). PS-MPs both of 1 and 3 μm were only used at the concentration of 10.0 mgL^{-1} because it was the one that induced the most relevant effects. Briefly, a Nile red (Sigma-Aldrich, Merck Life Science) stock solution of 250.0 mgL^{-1} was prepared in acetone (Carlo Erba, Cornaredo, Italy) 0.4%. This solution was further diluted with E3 medium or with PS-MPs suspension to obtain a final working concentration of NR 5.0 μgL^{-1}. Before use the staining solutions were shaken (200 rpm) for 24 h, at room temperature and in the dark. For each experimental group, 10 embryos were exposed to NR-MPs solution in a 6-well microplate, at 28.0 °C, for 24 h in the dark, and then at other 48 h of exposition to NR-free PS-MPs solution. Completed staining, larvae were so placed on a hanging drop slide, and the images were acquired using Axiovision 4.8 Software (Zeiss, Germany). No mortality was observed during the experiments that were repeated in triplicate.

2.7. Apoptosis evaluation

Apoptosis was investigated in 10 whole-mount larvae treated with 10.0 mgL^{-1} of PS-MPs of 1 μm and 3 μm and in the control group. At 72 h of treatment, 10 larvae for each group of treatment were rinsed three times with E3 medium and then exposed to acridine orange 5 μgmL^{-1} for 30 min, in the dark, at room temperature (Felix et al., 2017). The larvae were rinsed with E3 medium to remove excess dye and then placed individually on a hanging drop slide. Images were acquired with Axiovision 4.8 Software (Zeiss, Germany) by setting the FITC channel (emission peak at 525 nm; green spectrum) allowing apoptotic nuclei to be visualized as fluorescent spots (Capriello et al., 2021a). Semi-quantitative analyses of labeled nuclei were performed by ImageJ Software (Monaco et al., 2017b), by comparing the number of fluorescent spots. No larval mortality was registered.

2.8. Redox homeostasis

The degree of lipid peroxidation (lipid hydroperoxides - HPs) was

evaluated by following the reduction of NADPH at 340 nm in a system of coupled reaction of glutathione reductase (GR) and glutathione peroxidase (GPX) enzymes, in the presence of reduced glutathione (GSH). Briefly, 10 μg of zebrafish larval homogenate proteins (obtained from 20 larvae) were diluted in 0.1 M monobasic phosphate buffer, pH 7.4 (Heath and Tappel, 1976). HPs levels were expressed as μmol NADPH oxidized • min^{-1} • mg^{-1} protein.

In vitro, susceptibility to oxidative stress was assessed by measuring the difference between baseline hydroperoxide levels and hydroperoxide levels after stress induction (Fasciolo et al., 2023). Stress was induced by incubating the homogenate (obtained from 20 larvae) with iron and ascorbate (Fe/As), at a concentration of 100/1000 μM, for 10 min at room temperature. The reaction was stopped by adding 0.2% 2, 6-di-t-butyl-p-cresol (BHT).

Total ROS content was assessed by determining the conversion of the non-fluorescent 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) in the fluorescent dichlorofluorescein (DCF) by the samples' ROS (Fasciolo et al., 2022). Briefly, 12.5 μg of homogenate proteins (obtained from 20 larvae) were incubated for 15 min with 10 μM DCFH-DA in monobasic phosphate buffer 0.1 M, pH 7.4. To this was then added 100 μM $FeCl_3$ and incubated for 30 min to stop the reaction. DCF was assessed in a multimode microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek) (485 excitation wavelength, 530 emission wavelength).

The activity of the GPX and GR enzymes was detected in 0.01 mg of tissue homogenate proteins. Briefly, GPX activity was assessed using H_2O_2 as substrate, in the presence of GSH, following the rate of NADPH oxidation, necessary to reduce oxidized glutathione (GSSG), and catalysed by adding GR. Instead, GR activity was measured following the rate of NADPH oxidation after the addition of GSSG as substrate (Napolitano et al., 2023). For both procedures, the NADPH oxidation degree was followed at 340 nm using a multi-mode microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek), and the activity of both the enzymes were expressed as nmol NADPH oxidized • min^{-1} • mg^{-1} protein.

Superoxide dismutase (SOD) activity was measured at 25 °C assessing the decrease in the reduction rate of cytochrome c at 550 nm due to the superoxide radicals, produced by the xanthine–xanthine oxidase system. Homogenate of 20 larvae was added to a solution containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4, pH 7.8, 20 mM cytochrome c, 5 mM xanthine, and 0.01 U of xanthine oxidase. A unit of SOD activity corresponds to the enzyme concentration able to inhibit the reduction in cytochrome c by 50% (Fasciolo et al., 2023).

2.9. Statistical analyses

All tests were performed in triplicate, and data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism Software (version 8.02 for Windows, GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) method and Tukey's pairwise comparison tests were used for data analyses. Probability values were: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1. PS-MPs teratogenicity and effects on heart rate

PS-MPs effects on the early development stages of zebrafish, survival, and hatching rate were monitored up to 72 h of treatment. Results indicated that PS-MPs do not alter the survival rate or the normal hatching process of embryos. In contrast, exposure to PS-MPs caused severe morphological alterations, at the higher concentration. As can be seen in Fig. 1B-E, tail alterations, pericardial edema, and yolk sac deformities were evident compared with the control (Fig. 1A). The percentage of alteration (Fig. 1F) increased to 24.00 ± 5.66% and 25.00 ± 2.89% in the larvae exposed to 1 μm PS-MPs at 1.0 ($p < 0.05$) and 10.0 mgL^{-1} ($p < 0.05$) respectively, compared to control. In larvae exposed to 3 μm PS-MPs, a dose-dependent effect was observed: percentages in fact increased to 13.00 ± 1.41% at 1.0 mgL^{-1} ($p < 0.05$) and to 28.50 ± 2.65% at 10.0 mgL^{-1} ($p < 0.01$). No difference was found in the effects

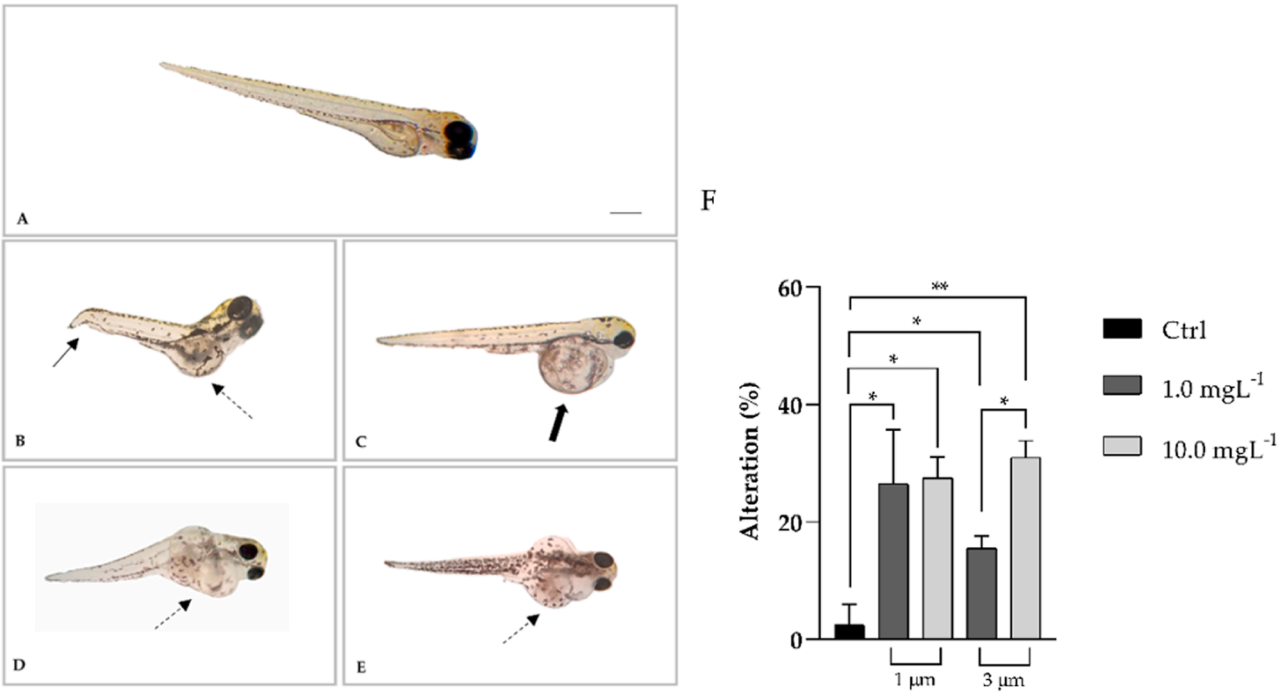

Fig. 1. Polystyrene PS-MPs induce marked dose-dependent phenotypic alterations. Control larva (A). Larvae treated with 1 μm PS-MPs at 1.0 (B) or 10.0 mgL^{-1} (C). Larvae treated with 3 μm PS-MPs at 1.0 (D) or 10.0 mgL^{-1} (E). Notice the bent tail (arrow), the cardiac edema (dotted arrow), yolk sac deformities (thick arrow). (F) Percentage of phenotypic alterations exposed to 1.0 or 10.0 mgL^{-1} of 1 or 3 μm PS-MPs. Statistical differences were determined by ANOVA followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$). Scale bar: 450 μm.

exerted by 1 μm and 3 μm PS-MPs used at the same concentration.

The heart rate assessed at 72 h of treatment showed alterations. Exposure to PS-MPs significantly increased heart rate at concentrations of 1.0 mgL^{-1} ($p < 0.05$) and even more so to 10.0 mgL^{-1} compared with control for both sizes of PS-MPs ($p < 0.01$; $p < 0.001$). This trend suggested a dose-dependent increase (Fig. 2). No significative difference was found between 1 and 3 μm PS-MPs used at the same concentration.

3.2. PS-MPs accumulation

Control larvae (Fig. 3A, D) remain completely unstained after exposure to NR. In contrast, larvae exposed to 1 μm (Figs. 3B, E) or 3 μm (Fig. 3C, F) PS-MPs showed intense, dose-dependent, fluorescence in the yolk sac and intestinal tract.

3.3. Effects of PS-MPs on apoptosis

Fluorescent spots, corresponding to apoptotic nuclei, were only occasionally seen in control larvae (Fig. 4A, D). At 10.0 mgL^{-1}, fluorescent spots significantly increased in number in both larvae exposed to 1 μm (Fig. 4B, E), or 3 μm (Fig. 4C, F) PS-MPs. Fluorescence is localized in the head, dorso-posteriorly to the eye (Fig. 4B, C) and in the tail, in correspondence to the neural tube (Fig. 4E, F). The semi-quantitative analysis demonstrated that the increase in the heads is always statistically significant ($p < 0.01$) compared with the control while the increase in the tail is significant only for larvae exposed to 1 μm PS-MPs ($p < 0.05$) (Fig. 4G).

3.4. Effects of PS-MPs on redox homeostasis

ROS content (Fig. 5A) significantly decreased in larvae exposed to 3 μm PS-MPs at 10.0 mgL^{-1} ($p < 0.001$); the other treatments did not show evident effects. HPs (Fig. 5B) increased significantly at 10.0 mgL^{-1} of 1 μm compared with the control group ($p < 0.05$) but not a 1.0 mgL^{-1}.

Coming to the enzymatic antioxidant system (Fig. 6), glutathione peroxidase activity (Fig. 6A) was unaffected by treatments, while glutathione reductase activity increases significantly in the group treated with 1 μm PS-MPs at 1.0 mgL^{-1} ($p < 0.05$) (Fig. 6B). Superoxide dismutase activity (Fig. 6C) significantly increased after PS-MPs exposure ($p < 0.0001$ vs control group), reaching the highest values at 10.0 mgL^{-1} for 3 μm PS-MPs ($p < 0.0001$).

In vitro susceptibility to oxidative stress (Fig. 6D) significantly decreased ($p < 0.01$) with the only exception of treatment with 3 μm PS-MPs at 1.0 mgL^{-1}.

4. Discussion

In this study, the first evidence emerging is that embryos exposed to the higher concentrations of both sizes PS-MPs presented phenotypic alterations such as tail malformations, pericardial edema, and yolk sac deformities. No differences were found between 1 μm and 3 μm, suggesting the same mechanism of action with these sizes of PS-MPs. Similar effects are reported with microplastics different in sizes and composition, in zebrafish (Anifowoshe et al., 2022; De Marco et al., 2022; Martínez-Álvarez et al., 2022; Zhang et al., 2023), and in other species (Bonfanti et al., 2021). In zebrafish embryos, these phenotypic alterations are common manifestations of oxidative stress (Sheu et al., 2017) and genotoxicity (Schottenfeld et al., 2007). These alterations are also found after exposure to other toxic agents such as metals (Jin et al., 2015; Xu et al., 2017), solvents (Maes et al., 2012), herbicides (Lanzarin et al., 2019) or dyes (Motta et al., 2019; Capriello et al., 2021b). The cause of these alterations can be explained by two hypotheses. In zebrafish, development occurs inside a semi-impermeable chorion presenting pores up to 500 nm that block the entrance of larger particles (Cheng et al., 2007; Duan et al., 2020). So, the first hypothesis is that MPs adhere to the chorion (Prata et al., 2022), mechanically obstructing the pores causing embryonic hypoxia and ion unbalance (Cheng et al., 2007; Wang et al., 2022), a condition at the basis of many embryonic alterations (Santos et al., 2021) also in other organisms (Jezierska et al., 2009; Mills and Barnhart, 1999). The hypoxic embryo is known to react by accelerating muscle movements and causing early hatching (Malafaia et al., 2020). Such an event was not observed in our experiments and therefore, hypoxia is an improbable cause of embryo teratogenicity. The second hypothesis is that MPs released degradation products that passed through the pores, inducing toxic responses in the developing embryos. It has been recently reported that polystyrene is not an inert material but rather releases a plethora of chemicals (Tian et al., 2019; Yousif and Haddad, 2013) which could cause changes in transcription levels in genes involved in the notochord and bone development (Xu et al., 2023).

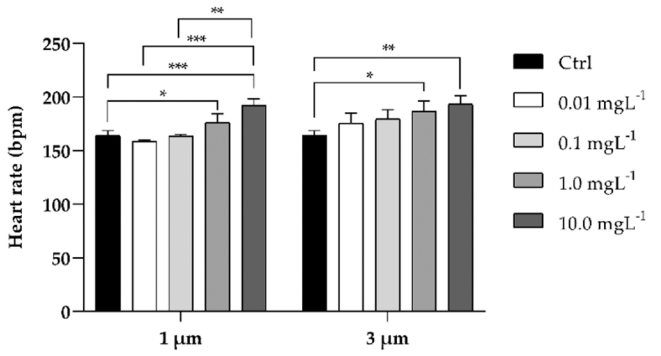

Fig. 2. PS-MPs accelerate heart rate (bpm) at 72 h of treatment. Statistical differences were determined by ANOVA followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

In this study, Nile red staining revealed that both sizes of PS-MPs used were present in the yolk sac and intestinal tract. This is a piece of significant evidence because, as far as we are aware, no reports are available on the uptake and transfer into yolk by newly hatched larva resorbing yolk residues. However, other studies have shown the entry and accumulation of MPs in the intestinal tract, stomach, mouth, and other larval districts (Bhagat et al., 2020; Malafaia et al., 2020; Qiang and Cheng, 2019). How MPs got to the yolk and intestinal tract remains to be clarified. It has been shown that chorion pores can be crossed by NPs (Bashirova et al., 2023; Ji et al., 2020; Van Pomeren et al., 2017). On the other hand, PS-NPs interact with lipid membranes (Pitt et al., 2018), and alter lipid metabolism inducing hepatic lipid accumulation in zebrafish (Lu et al., 2016). Therefore, the high lipid content of the yolk sac may be a target for polystyrene particles accumulation (Pitt et al., 2018).

Apoptotic processes were found in the head and tail after treatment with 1 μm PS-MPs and only in the head with 3 μm. The greater effect of 1 μm PS-MPs may be due to the smaller size, that facilitates tissue penetration. Apoptosis is a highly conserved and regulated process of programmed cell death that is important in tissue morphogenesis and homeostasis (Cole and Ross, 2001). It can be triggered by internal and external signals (Fadeel and Orrenius, 2005) including pollutants. Indeed, apoptosis is a biomarker for cadmium (Monaco et al., 2017b; Park et al., 2020), aluminum (Capriello et al., 2021a), copper (Zhao et al., 2020) and microplastics toxicity (Enfrin et al., 2020; Chen et al., 2022b; Suman et al., 2023; Zhang et al., 2021). In our case, polystyrene microbeads may have induced apoptosis via oxidative stress and/or by altering mitochondrial permeability (Azevedo et al., 2020; Zou et al., 2020). Recent work reports that micropolystyrene induces inflammation and pyroptosis, a process that shares several aspects with apoptosis (Hou et al., 2021). This is an intriguing aspect that should be explored further.

Heart rate significantly increased at higher PS-MPs concentrations, for both sizes used, data in line with the observed pericardial edema and other previous studies. The adhesion of microplastics on the chorion surface or presence inside the larvae can create a hypoxic condition that

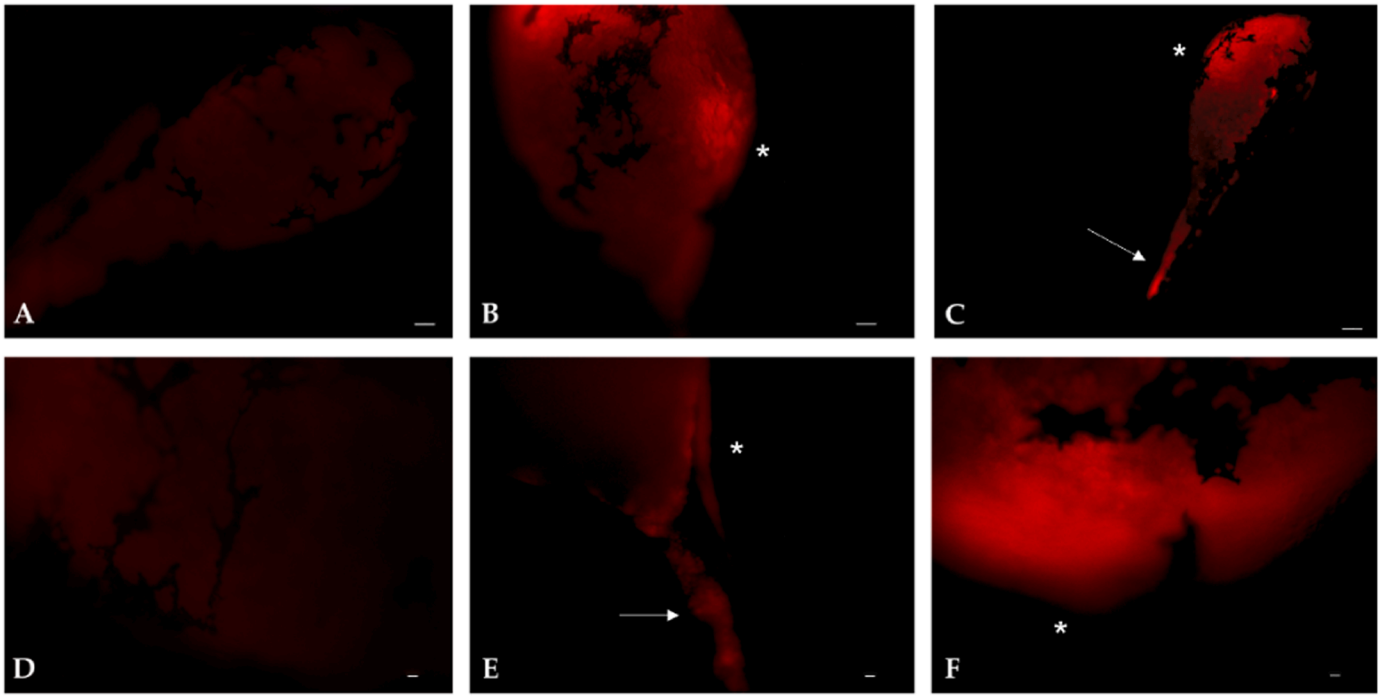

Fig. 3. Nile red staining of control larvae (A, D) or larvae exposed for 72 h to PS-MPs 1 μm (B, E) or 3 μm (C, F). (10.0 mgL^{-1}). (A, D) Unstained larvae. (B-C; E-F) Larvae with intense fluorescence in correspondence of yolk sac (*) and intestinal tract (arrows). Scale bar (A, B, C): 10 μm; scale bar (D, E; F): 5 μm.

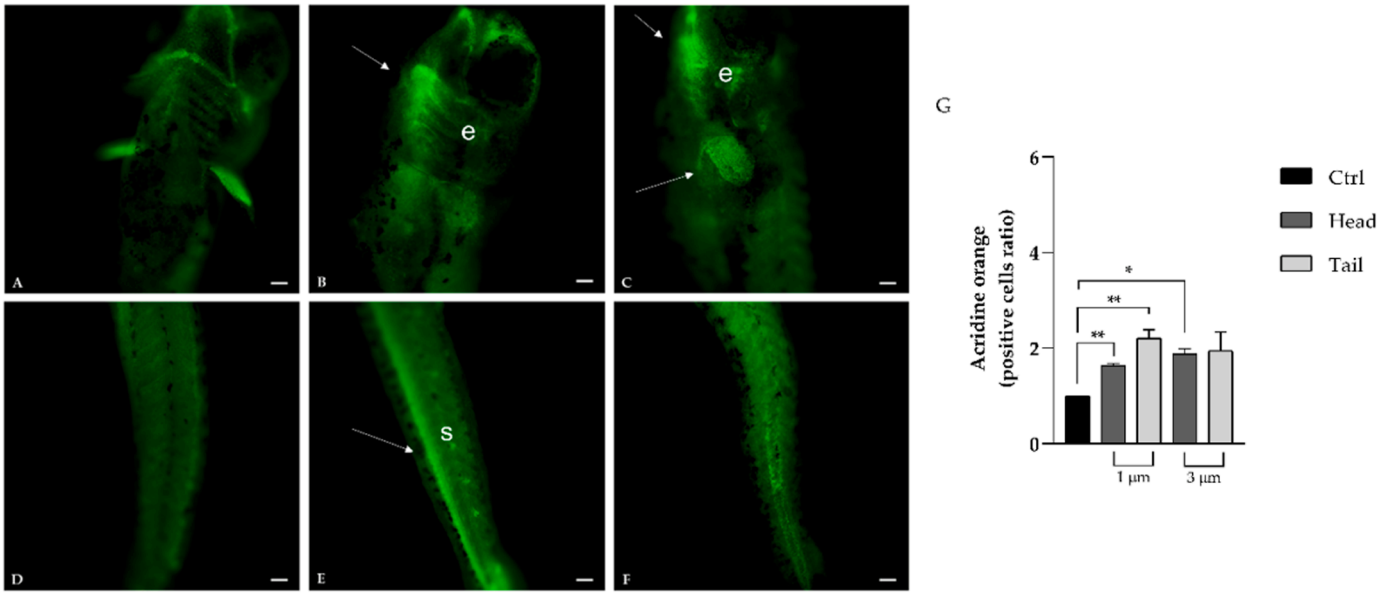

Fig. 4. Whole mount acridine orange staining of control larvae (A, D) or larvae exposed to 1 μm (B, E) or 3 μm (C, F) MPs. Upper panels: heads; lower panels, tails. Fluorescent spots (arrows); eye (e), somites (s). (G) Results of semiquantitative analyses demonstrating data significance. The graph shows the semi-quantitative analysis reported as the ratio of acridine orange positive cells. Statistical differences were determined by ANOVA followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$). Scale bar: 50 μm.

alters the blood flow consequently increasing heart rate (Duan et al., 2020; Lu et al., 2022; Zou et al., 2020). Decreased heart rate however has been reported with different MPs and NPs in sizes, composition, and exposure times. Bradycardia observed with NPs was attributed to the interaction between these particles and sarcomeres (Pitt et al., 2018; Prata et al., 2022; Zhang et al., 2020). Thus, the altered heart rate and pericardial edema could be attributable to an inflammatory process caused by exposure to these microparticles. It has been reported that altered heart rate, and cardiovascular disease are linked with oxidative stress (Doroszko et al., 2018; Ren et al., 2020). Nevertheless, in this study, there was no significant change in reactive oxygen species content, except in the 10.0 mgL^{-1} of 3 μm PS-MPs, excluding a correlation with heart rate alteration. Probably, this reduction is due to the ability of MPs and NPs to play a dual role (Agathokleous et al., 2021). Although they appear capable of inducing oxidative damage (Saborowski et al., 2022) or being vehicles for other substances (Rai et al., 2022), they can also reduce oxidative stress and inhibit ferroptosis as reported by Li et al. (2019) in a study on PS-NPs. The increased ability to remove ROS can

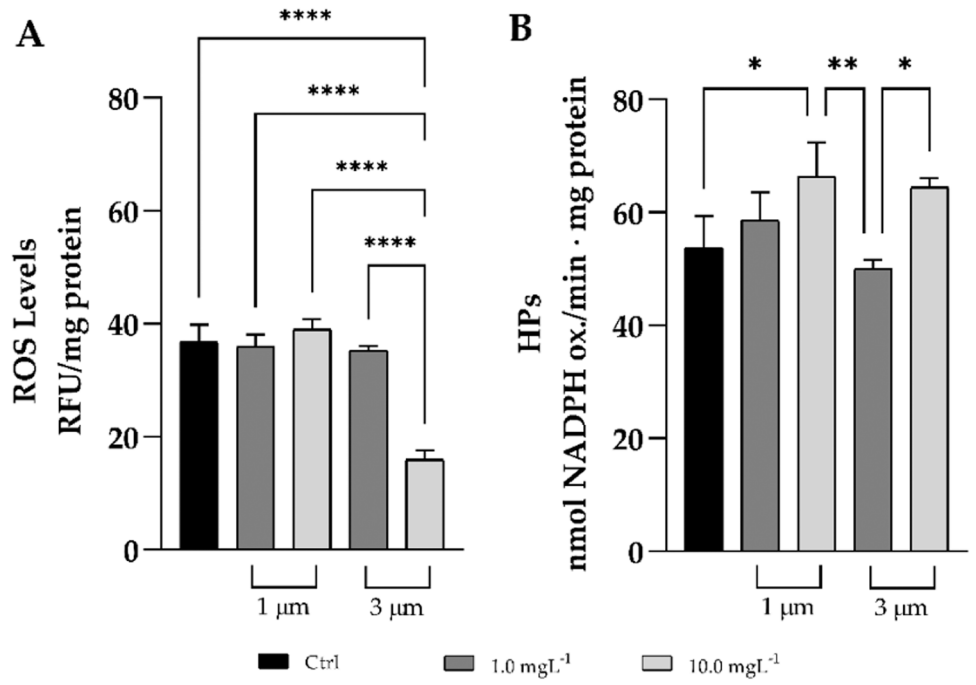

Fig. 5. Oxidative stress in control larvae or larvae exposed to 1 µm or 3 µm PS-MPs. (A) ROS content; (B) HPs, lipid hydroperoxide levels. Each determination is the mean of three repeated measures. Statistical differences were determined by ANOVA followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

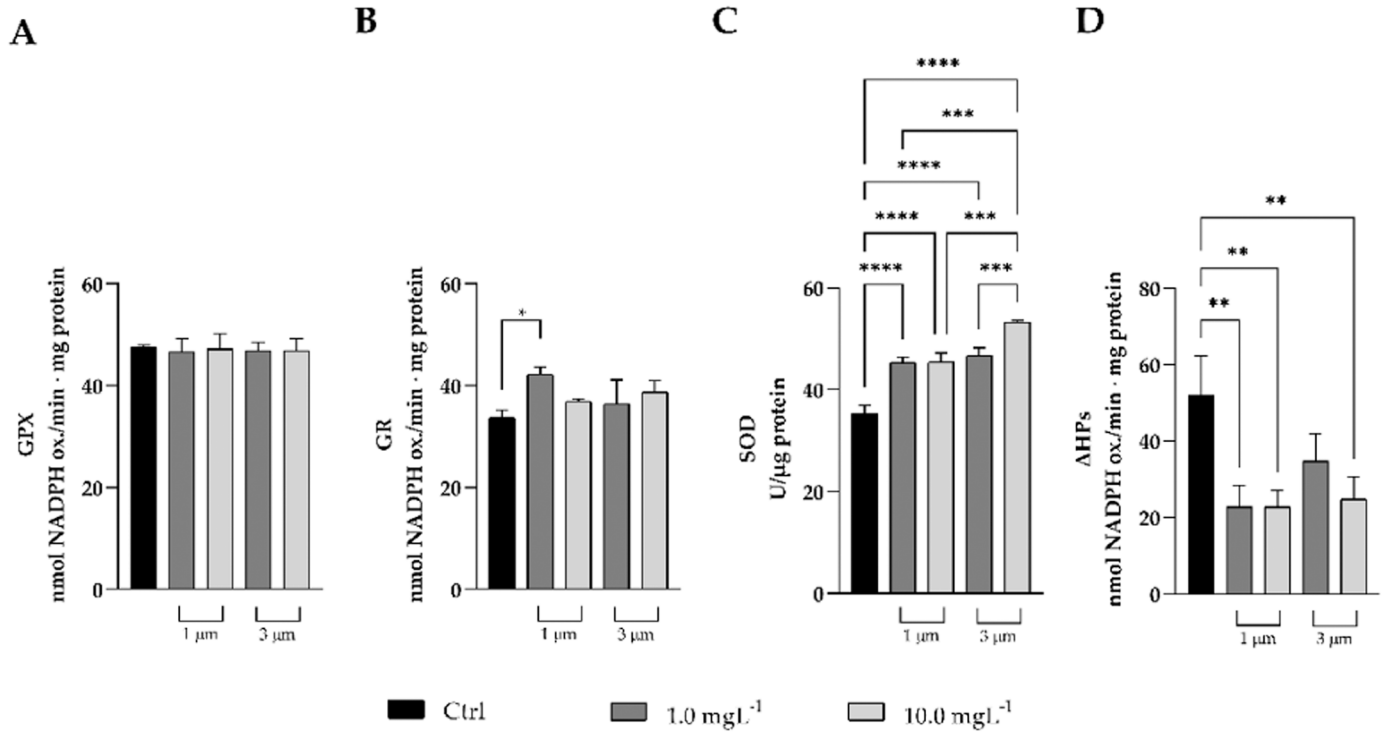

Fig. 6. Glutathione peroxidase activity (GPX, A), glutathione reductase activity (GR, B), superoxide dismutase activity (SOD, C), and *in vitro* susceptibility to oxidative stress (ΔHPs, D) in control larvae or larvae exposed to 1 µm or 3 µm PS-MPs. Each determination is the mean of three repeated measures (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

have detrimental effects on larval development. It has recently been demonstrated that a delicate balance of ROS is necessary during the early stages of development and that ROS are necessary for correct cell adhesion so that their reduction, also due to increased removal, leads to defective cell motility and progression of epibolia (Mendieta-Serrano et al., 2019).

Our data suggest an adaptation of the antioxidant system to PS-MPs exposure. ROS content does not increase after any PS-MPs treatment, and the lipid hydroperoxides increased only owing to 10.0 mgL^{-1} of 1 µm MPs treatment. Boopathi et al. (2023) reported that exposure to PS-MPs

triggers the onset of oxidative stress that, in several tissues, induces low-grade inflammation, which is associated with apoptosis. Geremia et al. (2023) reported that PS-MPs can induce oxidative stress directly, by Fenton Reaction, via the release of toxic chemicals or by generating a mitochondrial disfunction. Accordingly, we found that in the group treated with 10.0 mgL^{-1} of 1 μm PS-MPs, the HP levels increase in the larvae homogenates is associated with an increase in the apoptosis process both in the head and tail. In agreement, Gu et al. (2023) report in sea cucumber that PS-MPs exposure affects the relative expression levels of mitochondrial apoptosis-related genes reinforcing the idea that exists a relationship between the PS-MPs, low-grade inflammation, and apoptosis, a relationship found in this work. In the group treated with 10.0 mgL^{-1} of 3 μm, hydroperoxide levels were not significantly different in the larvae homogenates, notwithstanding the significant increase in apoptosis in the head. This discrepancy can depend on the higher increase in the activity of the SOD enzyme in the 10.0 mgL^{-1} of 3 μm, with respect to the other treatments. However, evidence for the lack of increased oxidative lipid damage and increased glutathione peroxidase has already been shown in zebrafish larvae (Yang et al., 2020). While glutathione reductase increases only in 1.0 mgL^{-1} of 1 μm PS-MPs treated larvae, but not in other groups, suggesting that the increase in SOD activity is a sufficient compensatory mechanism. In part, the increase in SOD enzyme activity may account for the reduction in stress susceptibility that occurs in all treated groups compared with the control group, and the decrease that verifies in the 10.0 mgL^{-1} of 3 μm PS-MPs treated group about the ROS content. As suggested by Guimarães et al. (2021) it is probable that the increased activity of the antioxidant enzyme SOD in *D. rerio* larvae, as also found for other enzymes in juvenile stages, is an initial response to exposure to MPs at the concentrations tested. In agreement, an increase in SOD activity and a lack of increase in oxidative damage upon exposure to 10.0 mgL^{-1} of PS-MPs was also demonstrated in adult zebrafish by Li et al. (2022).

5. Conclusion

This study demonstrates that 1 μm and 3 μm polystyrene particles enter and accumulate in zebrafish larvae. No mortality was observed but different size-related effects on apoptosis and redox homeostasis, suggesting different responses at the cell level. Interestingly, MPs treated larvae shown a decrease in susceptibility to stress, due to an increase in SOD activity. ROS removal was more efficient but not enough to prevent apoptosis in the group exposed to the highest concentration of 1 μm PS-MPs. These data will contribute to a more exhaustive comprehension of MPs' toxicity, attracting attention to the point that size does matter. This will prompt future studies and favor previsions on the environmental toxicity of this ubiquitous waste.

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CRediT authorship contribution statement

La Pietra Alessandra: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Motta Chiara Maria:** Data curation, Writing – review & editing. **Venditti Paola:** Data curation, Writing – review & editing. **Fasciolo Gianluca:** Formal analysis, Methodology, Writing – original draft. **Lucariello Daniela:** Formal analysis, Methodology. **Ferrandino Ida:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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.

Data Availability

Data will be made available on request.

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