



Morphological Alterations in the Early Developmental Stages of Zebrafish (*Danio rerio*; Hamilton 1822) Induced by Exposure to Polystyrene Microparticles

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Abstract

Microplastics (MPs) are emerging pollutants of widespread concern in aquatic environments. The aim of this study was to evaluate the negative impact of pristine MPs of polystyrene of 100 µm on embryo and larvae of *Danio rerio* exposed to three environmentally relevant concentrations of polystyrene (3.84×10^{-6} , 3.84×10^{-7} , and 3.84×10^{-8} g/mL). The exposure effect was evaluated through the general morphology score, biometrics, and integrated biomarker response version 2 index. No mortality was observed but the anatomical structure of fishes was affected showing pigmentation deficiency and alterations in the head region as the main affected endpoints. The general morphology score and the integrated biomarker response values were highly sensitive to address the effect of the three concentrations of MPs used here. Our results provide solid evidence of the negative impact of 100 µm pristine polystyrene MPs exposure on early stages of zebrafish.

Keywords Larvae · Zebrafish · Microplastics · Bioassay · Morphological development

Introduction

The negative effects of plastic contamination on biodiversity and human health are topics of great concern worldwide

due to their long-term persistence and ubiquity in the environment. Microplastics (MPs) are defined as small pieces of plastics with varied sizes ranging between 1 µm and 5 mm (Barnes et al. 2003; Thompson 2015). Today, MPs are found in almost all marine environments, including remote marine habitats like the deep-sea, unreachable islands and polar ice caps (Andrady 2011; Woodall et al. 2014; Obbard et al. 2014; Lusher et al. 2015).

In aquatic environments, MPs are difficult to recover, and their presence is becoming a menace to wildlife and ecology because their ingestion is considered a major threat to a wide variety of taxa (Cole et al. 2013; Watts et al. 2015; Green et al. 2017; Huang et al. 2020; Yan et al. 2020; Trotter et al. 2021). Polyethylene (PE), polypropylene (PP), and polystyrene (PS) particles are found more frequently in the aquatic environments because of their wide use in daily products (Andrady, 2011; Al-Thawadi et al. 2020). In fish larvae, they have produced neurotoxicity, oxidative stress, dysbiosis, intestine occlusion, accumulation through the food web and even mortality (Galloway et al. 2017; Green et al. 2017; Al-Thawadi, 2020).

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Exposure bioassays are effective to evaluate the impact of many stressors including MPs and the use of model organisms is relevant because causative relationships are simpler to discern than using field-based evaluations. Zebrafish *Danio rerio* has been very valuable because it is easy to maintain in the laboratory, its life cycle is well described, and its embryonic development is easy to follow inside the eggs (Kimmel et al. 1995).

To date, there is still controversy about the impacts of microplastics ingestion by aquatic organisms using virgin plastic particles because some authors attest that they do not reflect accurately the conditions from the environment, while others have reported detrimental effects in fish (Cormier et al. 2019; Bhagat et al. 2020; Tarasco et al. 2022). The effects have been evaluated widely with MPs of 1 to 50 μm in size, based on the reported number of particles per mL found in aquatic environments (Eriksen et al. 2014; Koelmans et al. 2019). For example, in zebrafish embryos exposed to PS of 1 to 50 μm in size, physical damages, ingestion with occlusion and non-occlusion, dysbiosis, metabolic disorders, growth inhibition, oxidative stress, and genotoxicity were observed (Eriksen et al. 2014; Sadri and Thompson 2014; Horton et al. 2017; Koelmans et al. 2019). Also, exposure to PE of 40 μm in size produced a reduction of embryo's hatching and reduction of larvae survival rates with evident teratogenic and morphological effects (Malafaia et al. 2020). PS-MPs of 1 μm in size were found adhered to the chorion's shell of zebrafish and were able to enter into the digestive tract of larvae during the transition from eggs to larvae, causing dysbiosis and affecting their natatory skills (Qiang and Cheng 2019). The same was observed in embryos exposed to low-density PE of 5 to 18 μm in size (Karami et al. 2017). Furthermore, PE of 11 to 13 μm in size co-exposed with oxybenzone, and benzo[*a*]pyrene induced the increase in the ethoxyresorufin-*O*-deethylase (EROD) activity and the CYP1A transcription, proposed as biomarkers of chemical exposure activity (Cormier et al. 2019).

In adults, the sub-lethal effect of MPs includes changes in social behavior, reduction of physical activity and feeding rates, a decrease of egg hatching and morphological alterations (Tarasco et al. 2022).

Therefore, in order to partially fill this gap of knowledge on the impact of MPs of 100 μm in freshwater organisms, we performed a static-exposure bioassay of 120 h using three increasing concentrations of 100 μm PS-MPs to test two hypotheses: (1) the external exposure to 100 μm PS-MPs microparticles triggers development and morphology related alterations to embryo and larvae of *D. rerio*, and (2) the biological effect of 100 μm PS-MPs exposure is directly proportional the exposure concentrations.

Methods and Materials

Adults *D. rerio* wild type were sexed and acclimated for a week in controlled conditions in the Immunology and Molecular Biology laboratory at the Center for Research and Advances Studies of the National Polytechnic Institute - Merida Unit (CINVESTAV-IPN Unidad Mérida). Fish were maintained in 5 L glass tanks of dechlorinated water (26 ± 1 °C) with replacement every second day with 20% of its capacity with 14:10 light/dark photoperiodicity, and fed three times a day with a commercial flake diet (Feeder[®]). For reproduction and spawning, adults were placed overnight in an aquarium at a ratio of 2:1 (females/males). Eggs were collected in a Petri dish and washed three times with bottled water. Fertilized eggs were selected carefully using a stereomicroscope (Stemi 305, ZEISS) and photo-documented with a camera (AxioCam 208 color, ZEISS) coupled to ZEN 3.4 (Blue edition) software (equipment used for each of the subsequent analyzes). The non-viable eggs observed with a flocculation inside, no visible heartbeat and lack of spontaneous movements were discarded (Lammer et al. 2009).

Virgin polystyrene microplastics [PS-MPs (5% of solids in an aqueous suspension (AS))] were purchased from Sigma-Aldrich[®] (# 56,969). PS-MPs were dark red, spherical beads of 100 ± 1.06 μm in diameter and a density of 1.05 g/cm^3 . For the purposes of this study, the PS-MPs and AV were separated by centrifugation at 10, 400 *g* and analyzed individually. The material of the MPs was identified through Energy-dispersive X-ray using a M-max 50 mm² Oxford detector and Infrared Spectroscopy (IR), recorded on an Agilent Cary 630 Fourier Transform Infrared Spectroscopy (FTIR) spectrometer (range: 4000 – 600 cm^{-1}) using an ATR interface. While the AS was analyzed by IR and nuclear magnetic resonance (¹H NMR), recorded in deuterated chloroform (CDCl₃) and deuterated methanol (CD₃OD) using an Agilent DD2 600 spectrometers at 600 MHz. The chemical shifts are reported in ppm relative to residual solvents. To process the spectra we used the MestReNova software (Fulmer et al. 2010). The PS-MPs were characterized by Scanning Electron Microscope (SEM), at 15 kV in STEM mode, briefly, the MPs were placed in a lead-base, for examination under a scanning electron microscope (model JEOL JSM-7600 F).

Sampling and the experimental design were reviewed and approved by the Institutional Animal Care and Use Committee of the CINVESTAV (approval number 2875-1), in accordance with the Mexican Official Norm (NOM-062-ZOO-1999).

The experiment was performed in a biostatic assay with no replace with eggs of 12 h post-fertilization (hpf) in somite phase (Kimmel et al. 1995), to evaluate three concentrations of PS-MPs: T1 = 3.848, T2 = 0.384, and T3 = 0.038 $\text{mg} \cdot \text{L}^{-1}$;

approximately 3.84×10^{-6} , 3.84×10^{-7} , and 3.84×10^{-8} g/mL particles of PS-MPs mL^{-1} , respectively (Guimarães et al. 2021). The number of particles was determined by visual counting (in triplicate) using a 10X objective. Briefly, we homogenized the MPs in their original solution and collected 10 μL that were placed on a slide, after placing the cover slide, we sectioned it in four quadrants for counting. While the concentration of particles per mg was determined using the formula $\delta(4/3\pi r^3)$ (Santos et al. 2020) - where r corresponding a radius and δ the density of the particle -, as well as the concentration formula (m/V) - where m corresponds to the mass of the total particles and V the volume -. The size of the PS-MPs used in this study is relevant because there is a lack of information about the negative effect in embryos and larvae of *D. rerio* and in other organisms using MPs of diameters longer than 50 μm in laboratory settings.

The concentrations of PS-MPs used in this study were chosen based on the concentration of microplastics previously reported for aquatic and freshwater environments (Eriksen et al. 2014; Koelmans et al. 2019; Malafaia et al. 2020; Guimarães et al. 2021).

The Bioassay was performed using a 24 well culture plate with 24 eggs/well and 2 mL of watered MPs [PS-MPs (100 μm) + purified water], in a static aquatic system during 120 h. The exposure test covered two stages of development: embryonic (~0 to 48 h) and larvae (~49 to 120 h) (Kimmel et al. 1975). The chorion was removed during the exposure time and after the eggs' hatching. The negative control (Ctrl) or the reference group was composed of embryos in purified water with no MPs. Both treatment and control groups were tested in triplicate and maintained at a 14:10 light/dark photoperiodicity and at 26 ± 1 °C. Embryos and larvae were monitored daily, evaluating their survival rate = (living organisms / total organisms) * 100 -, and the hatching rate = (eggs hatched / total eggs) * 100 -, at 24, 36, 48, 72, 96, and 120 h equivalent to 36, 48, 60, 84, 108, and 132 hpf.

The PS-MPs exposure effects were evaluated using three different analyses: General Morphological Score (GMS), biometrics, and Integrated Biomarkers Response version 2 (IBv2) Index. The development (embryonic and larvae) of *D. rerio* was evaluated using the GMS score through the observation of 12 selected endpoints (detachment of the tail, somite formation, eye development, movement of embryo/larvae, circulation of blood, heartbeat, pigmentation of head/body and tail, pectoral fin, protruding mouth, hatching and swim bladder inflated) according to the predetermined score and photographs by Beekhuijzen et al. (2015) and adjusted by Li et al. (2018) every 24 h until 120 h. A final GMS score was obtained from the sum of the final endpoint score during the exposure time, where the "Ctrl" gave a maximum

theoretical score, and lower-ranking scores indicate a development delay (Beekhuijzen et al. 2015).

At the end of the experiment (120 h), larvae were collected for the morphological evaluations. The biometric analysis included 12 dorsal and lateral morphologic parameters of head, spine, and tail (S1) (Selderslaghs et al. 2009; Beekhuijzen 2015; Malafaia et al. 2020). These structures were selected based on previous reports about the effect of MPs in the sensorial, physiological, and skeletal structures of zebrafish larvae using smaller MPs than those reported in this study (Malafaia et al. 2020). At the same time, dorsal and lateral photographs were taken from nine larvae per treatment (three individuals per triplicate) to record the effect of MPs.

To resume the biological response of the PS-MPs exposure, we used the IBR v.2 index, proposed by Sanchez et al. (2013). This is a modified IBR version from that proposed by Beliaeff and Burgeot (2002). This index was estimated using the 12 morphological parameters recorded in the biometric analysis. Briefly, the IBRv.2 index baseline of zero is created first from the results of the "Ctrl" group to compare it with the standardized differences obtained from the treatments. For this, log-transformed data are first standardized using the mean and standard deviation. Subsequently, the data of the treatments are subtracted from the standardized data from the "Ctrl" to obtain a deviation value, represented it in a star diagram with a positive and negative response magnitude in stressful conditions compared to normal conditions (Maulvault et al. 2018). Finally, the IBR values are obtained from the sum of the absolute data of deviation values.

The normality and homoscedasticity of the data were tested using a Shapiro-Wilk and Bartlett test, respectively. The GMS score and biometrics, and the effects of different densities of MPs were analyzed using non-parametric confidence intervals (at 95%) with the method of adjusted bootstrap percentile (BCa) (Davison and Hinkley 1997), with $B=1,000$. BCa is a second-order interval that uses an accelerated bias-corrected method that corrects for bias and skewness in the distribution of estimates (Chernick and LaBudde 2011). We considered a significant difference when the overlapping of upper or lower limits was greater than 50% between treatments and Ctrl as between treatments (Cumming and Finch 2005). Analysis of the data was done using the boot package (v1.3-27; Canty and Ripley 2021). and results were plotted using ggplot 2 (Wickham 2013) in R software (version 3.6.1).

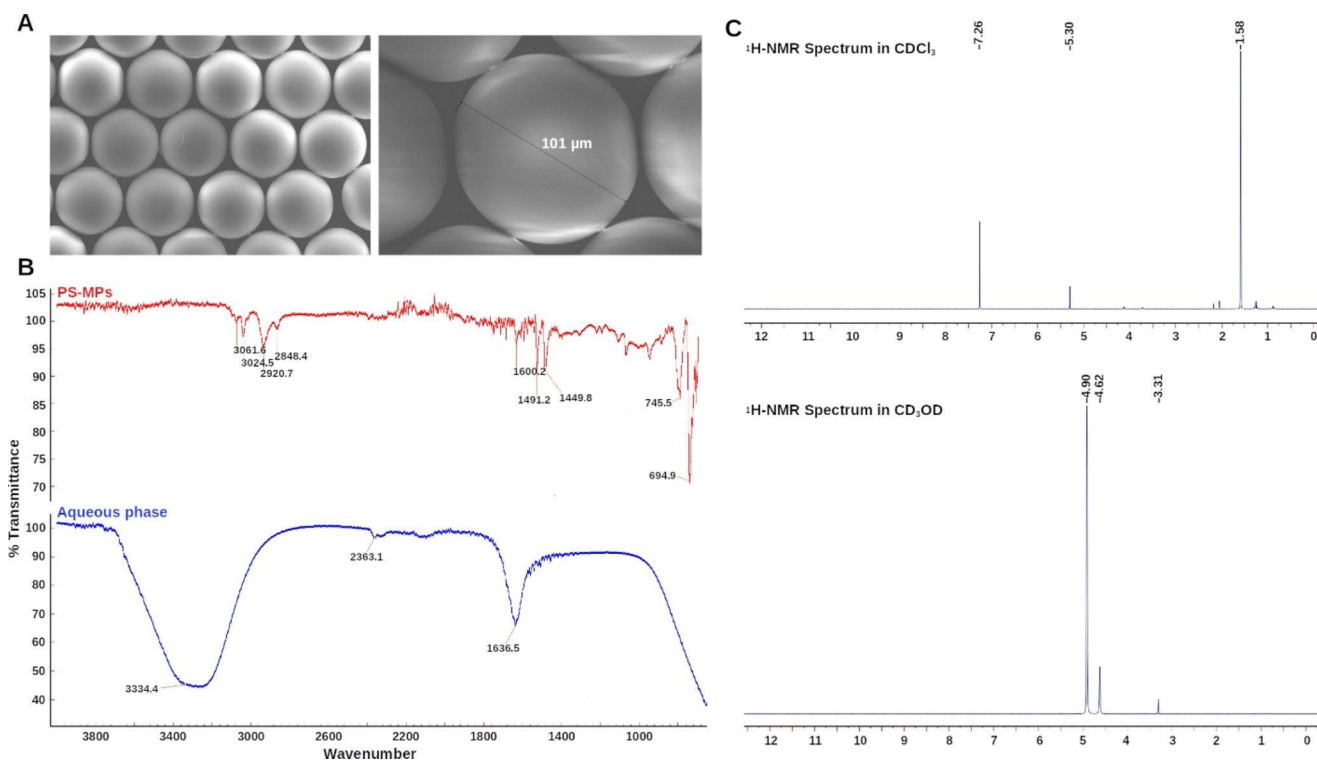


Fig. 1 Characterization of PS-MPs by Scanning Electron Microscopy (SEM) at 15.0KV in a STEM mode. (A) A Round and spherical shape with sizes of approximately 100 μm of diameter: left $\times 250$ at 100 μm . Right $\times 700$ at 10 μm . (B) Infrared Spectroscopy (IR) spectrum of polystyrene microplastic (PS-MPs), and aqueous suspension (AS). B) Red line: IR spectrum of the PS-MPs registered in a range of 600 to 4000 cm^{-1} where absorption peaks are observed at 3061.6 and 3024.5 cm^{-1} corresponding to aromatic C-H stretching. The peaks at 2920.7 and 2848.4 cm^{-1} correspond to the existence of methylenes. There are also absorption peaks at 1600.2, 1491.2, and 1449.8 cm^{-1} due to aromatic C=C stretching. These absorption peaks indicate the existence of benzene rings. The peaks at 745.5 and 694.9 cm^{-1} corre-

spond to C-H out-of-plane bending and indicate that there is only one substituent in the benzene ring. Blue line: IR spectra of the AS matches the spectrum of water. There, three fundamental vibrations are found, the absorption peak at 3334.4 cm^{-1} corresponding to O-H asymmetrical stretching, the absorption peak at 1636.5 cm^{-1} corresponding to H-O-H scissoring and the peak at 2363.1 cm^{-1} belonging to a combination band. (C) Nuclear Magnetic Resonance (NMR) of aqueous suspension (AS) of the PS-MPs reagent. Top: ^1H -NMR Spectrum of the AS at 600 MHz in CDCl_3 where the residual chloroform is observed at 7.26 ppm, residual dichloromethane at 5.30 ppm and water at 1.58 ppm. Bottom: ^1H -NMR Spectrum in CD_3OD where the residual methanol is observed at 3.31 and 4.62 ppm and water at 4.90 ppm

Results and Discussion

SEM microscopy showed MPs with a perfectly smooth surface with sizes ranging between 99.5 and 101 μm (Fig. 1 A). The Energy-dispersive X-ray spectroscopy (EDXS) analysis showed the dominance of carbon and detected the presence of oxygen (98.72 and 1.28% of atomic percentage, respectively). These amounts indicate that there is not another chemical element (S1). In the IR spectrum of these PS-MPs, we observed the presence of aromatic C-H and C=C stretching corresponding to the existence of methylene, and benzene rings, respectively. Also, a band shows the C-H out-of-plane bending which indicates that there is only one substituent on the benzene ring. All this are components of polystyrene $\text{C}_8\text{H}_6\text{n}$. These results show that the absorption bands match to PS (red line) (Fig. 1B). We also, analyzed by IR the AS where microplastics are suspended, and the result shows an infrared absorption spectrum which fully

matches with the spectrum of water, presenting an O-H asymmetrical stretching band, an H-O-H scissoring band, and a combination band (blue line) (Fig. 1B). To further analyze whether the AS contains any dispersants or preservatives, we used NMR, which is a more sensitive technique. For this purpose, ^1H -NMR spectra were acquired in both deuterated chloroform (CDCl_3) and deuterated methanol (CD_3OD) since the chemical shifts of the water signals in these solvents are well identified (Fig. 1 C). The chemical shift of water in ^1H -NMR is perfectly identified in the solvents employed, so we conclude that hardly the control group exposed to the components of this AS would be damaged (Fig. 1 C).

Morphological deformities (i.e., pericardial edema, yolk sac edema, ingestion, and spinal deformity) have been some of the main features reported in zebrafish embryos and larvae exposed to MPs < 50 μm (Zhao et al. 2021; Cormier et al. 2021; De Marco et al. 2022; Lu et al. 2022; Tarasco et al.

2022). The present work is an additional contribution to the adverse effects of MPs in fish using MPs of 100 μm . During the experiment no mortality was observed in embryos and larvae analyzed. In other studies, the exposure to low density PE ranging from 5 to 18 μm , low mortality was observed (Karami et al. 2017), and no mortality was observed with MPs with ranges of 11 to 13 μm (Cormier et al. 2019). But the sublethal effect observed in this study is consistent with previous reports with PS of 45 μm (Chen et al. 2017), and MPs with a size range of 1 to 5 μm (Santos et al. 2021a; Santos et al. 2021b). In this study we observed a delay in the hatching process that is crucial during embryogenesis. In the three treatments, eggs started to hatch after 36 h (48 hpf) and at 72 h (84 hpf) a 100% of hatching was observed. In the “Ctrl” group, eggs started to hatch at 24 h (36 hpf), with 100% of hatching at 48 h (60 hpf). MPs were not able to pass the chorionic membrane that have pore sizes between 0.5 and 0.7 μm (Beekhuijzen 2015). The chorionic acts as a biological barrier, but the continues contact of the zebrafish eggs with the spherical MPs would have obstructed the chorion pores, potentially altering the gas exchange as reported in eggs exposed to PE of 23 to 55 μm in size (Malafaia et al. 2020), changes of water quality (Alderdice et al. 1958), presence of heavy metals (Jeziarska et al. 2009), and chorionic membrane damage (Southart et al. 1996).

Here, we evaluated the embryo to larval development through selected parameters previously defined in the GMS analysis. In the exposed group we observed a lower score with respect to the “Ctrl” group through time. Some of the main endpoints that were altered were a delay in the presence of pectoral fin, mouth protrusion, and the swim bladder inflated; as well as a deficit of pigmentation in the eyes, head-body, and tail sections. The same has been reported in *D. rerio* exposure to certain nanomaterials (Pereira et al. 2019). The lower GMS final score in treatments of PS-MPs exposure, was confirmed by statistical estimates - T1: 69 ± 0.95 [CI 95% (67.00–71.00)]; T2: 67 ± 1.24 [CI 95% (64.00–68.00)]; and T3: 63 ± 0.66 [CI 95% (62.00–65.00)] compared to the “Ctrl”: 71 ± 0.46 [CI 95% (70.00–72.00)] -. After 120 h of exposition, we evaluated the morphometric of parameters and some of them present alterations (Fig. 2). Among the sensorial parameters, the Ocular area (OA) and the Minimum interocular distance (MIID) showed an inverse trend as the MPs concentration increases (Fig. 2 A & B). In semi-static conditions, eggs exposed to PE showed a decrease in parameters of the optical system (Malafaia et al. 2020). In this study we did not used gene expression analyses, but in zebrafish exposed to PS of 45 μm in size, an up-regulation of the *zfrho* visual gene (Chen et al. 2017), related to neurotoxicity with possible effect in the locomotor behavior was observed (Chen et al., 2012). Visual impairment could lead to behavioral defects (Kashyap et al.

2007), that would affect development and survival (Haug et al. 2010). We also observed a decrease in the proboscis's length (PL) (Fig. 2 C). This could be caused by the constant contact of the larvae while swimming near to the PS-MPs. It was also noticeable an augmentation of the Vitelic sac's area (VSA) in the experimental group compared to the “Ctrl” group (Fig. 2D). Similar effect was reported in exposure to PE of 23 to 55 μm in semi-static conditions where edema formation in the VSA of larvae was observed (Malafaia et al. 2020). This occurred in response to alterations in the osmotic exchange affecting the permeability of the fish tissues to allow the entrance of liquid. The edema formation has been observed in response to toxicological stressors, for example, during exposure to crude oil (Bonatesta et al. 2022). Alterations in the VSA have been also observed in response to exposure to PE of 1 μm of size (Qiang and Cheng 2019), and in response to exposure to nanomaterials (Pereira et al. 2019). In this study, the augmentation of the VSA in response to exposure to PS-MPs of 100 μm is relevant, because it indicates a delay in the development of an organ that contains the essential nutrients for its development during the first days of life until autochthonous feeding.

In the case of the skeletal structure, the Head's height (HH) was small in all the treatments while the Head's depth (HD) was smaller only in T3 (Fig. 2E & F). These alterations can be related to the constant external contact of the larvae with the PS-MPs (agglomerates at the bottom of the well) either after hatching or remaining swimming laterally or in the bottom through the MPs particles, or by holding their body vertically with its head down by the absence of the swim bladder which were completely inflated at 120 hpf (Kimmel et al. 1995). In other studies, it has been reported that MPs of 1 μm of size are able to enter into the head of *D. rerio* larvae (Santos et al. 2021a). Also, the tail length (TL) and the relation to the Anus-to-mouth distance (AMD) were shorter than the “Ctrl” (Fig. 2G & H). The same was observed with smaller MPs where the lack of proportion in the morphology can have adverse effects in the correct development (Malafaia et al. 2020). Alternatively, the parameters that were not statistically significant in this study were the Maximum interocular distance (MAID), Swim bladder area (SBA), Head's width (HW) and Spine's length (SL) (Fig. 2I, J, K & L).

Moreover, the radar plots show the results of the IBRv.2 index highlighting that the VSA and HH were the highest and lower morphological endpoints observed here (Fig. 3 A). By using this schematic approach, it is easy to observe the variability of the endpoints in each treatment with respect to the “Ctrl” group. However, it is important to notice that the results of IBRv.2 index depend on the parameters evaluated and the level or multilevel selected by the researcher. For

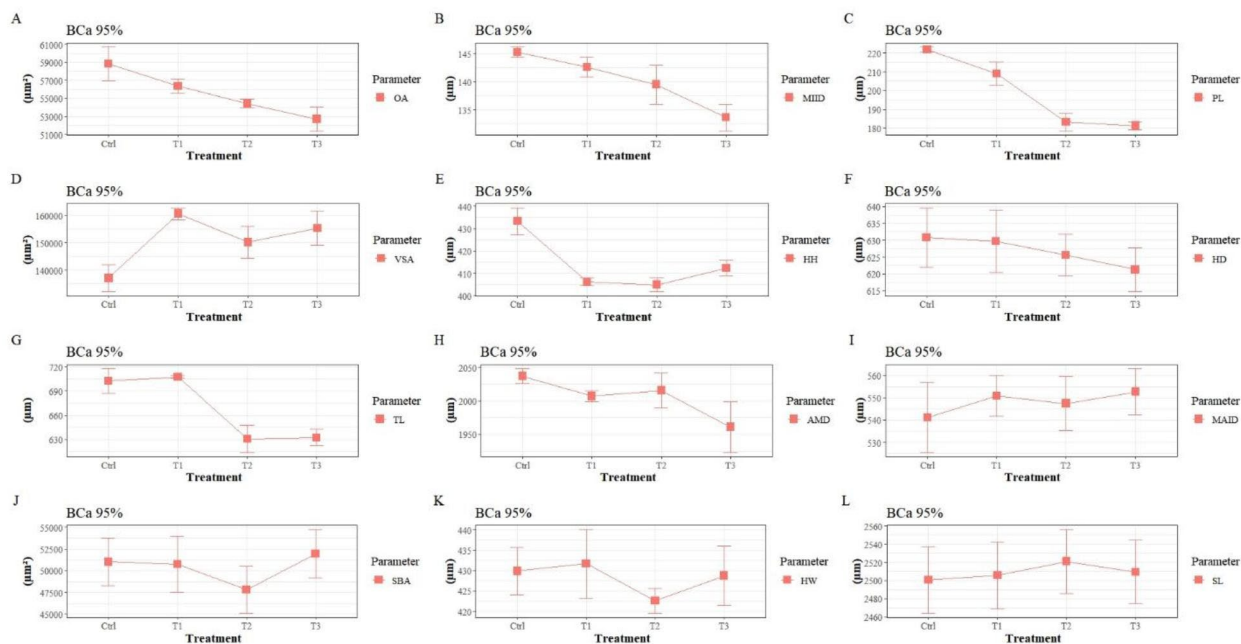


Fig. 2 Confidence intervals using the method of adjusted bootstrap percentile (BCa) of the biometrics parameters. OA: Optical area, MIID: Minimum interocular distance, HD: Head deep, HH: Head height, PL: Proboscis length, VSA: Vitelic sac area, TL: Tail length,

AMD: Anus-mouth distance, MAID: Maximum interocular distance, SBA: Swim bladder area, HW: Head width, and SL: Spine length. Ctrl: 0, T1: 7×10^1 , T2: 7×10^2 , and T3: 7×10^3 particles number of PS-MPs mL^{-1} . Mean, standard error, and CI values in Table S1

example, in guppy fish exposed to PS of 32 to 40 μm in size, it was observed a higher index in the catalase activity related to the oxidative stress and the lower index was addressed to growth (Huang et al. 2020). And in *D. rerio* larvae co-exposed to MPs + Cu, the oxidative stress was the highest value (Santos et al. 2021a; Santos et al. 2021b). In this study, it is noticeable that the impact of the PS-MPs produced an effect of concentration-dependent manner denoting a dose-effect response, which was observed through a pattern of correlation between the GMS scores and IBRv.2 indexes. The decrease of the final GMS score showed a delay in development thought the estimated mean value $\text{GMS}_{T1} = 69 \pm 0.95$, $\text{GMS}_{T2} = 67 \pm 1.24$, and $\text{GMS}_{T3} = 63 \pm 0.66$; while the IBRv.2 index showed an increase in the response's magnitude with values of $\text{IBR}_{T1} = 7.8$, $\text{IBR}_{T2} = 13$, and $\text{IBR}_{T3} = 15$. This effect in tendency suggests the effect of MPs is proportional to the number of exposure particles (LeMoine et al. 2018; Qiang and Cheng 2019). As the effects were not associated with organic pollutants, it is necessary to evaluate other mechanisms involved in the phenotypic alterations observed, and friction of eggs with PS_MPs and swimming larvae could have affected these morphologic parameters. This is a topic that deserves further investigations because there is still controversy of the environmental relevance of using pristine MPs/NPs with uniform shape/size (Savoca et al. 2017). Some authors provide clear evidence that pristine and contaminated microplastics have the potential to

affect the development of zebrafish. For example, Tarasco et al. (2022) observed that after long-term exposures to MPs, there were an increased incidence of skeletal deformities, specifically in the caudal fin region. The same would have occurred after short time exposures reported in this study. In the same way, De Marco et al. (2022) reported that exposure of zebrafish embryos to 10 μm pristine PS particles for up to 120 hpf increased the incidence of pericardial edema, spinal curvature, and column deformation.

Another feature observed here is that fish mistakenly eat MPs confounding it as food (Savoca et al. 2017). Indeed, the ingestion of $\text{MPs} \leq 50 \mu\text{m}$ has been described widely from larvae and adults of zebrafish and tend to accumulate in the gastrointestinal tract, disseminate in the body and expel it (LeMoine et al. 2018; Qiang and Cheng, 2019; Malafaia et al. 2020), with effects at transcriptomic and genetic levels (Mazurais et al. 2015; LeMoine et al. 2018). In the gastrointestinal tract they interfere with feeding and absorption of nutrients (Zhao et al. 2021), cause epithelial detachment and mucus hypersecretion (Limonta et al. 2019), and reduction of the swimming efficiency (Qiang and Cheng, 2019). In this study we observed larvae ingesting MPs (Fig. 3B). Although here we did not evaluate any metabolic effect, the presence of MPs of this size would have detrimental effect on fish larvae because it could cause occlusion.

Finally, after this comprehensive evaluation using pristine MPs, at relevant environmental concentrations, we

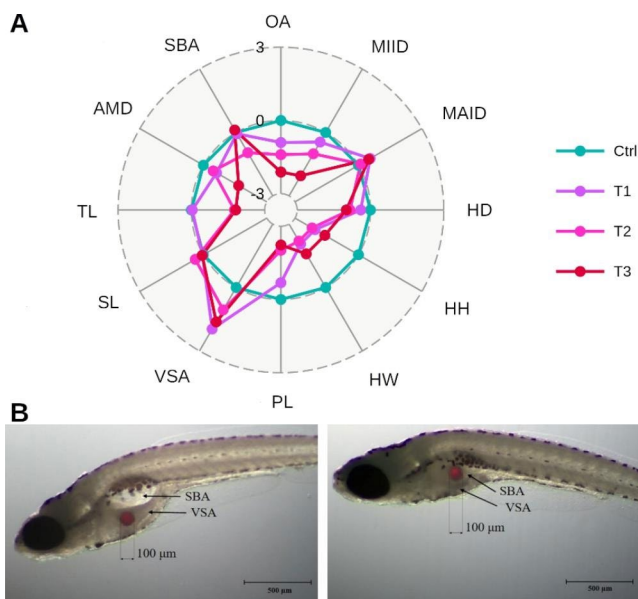


Fig. 3A Radar plot of Integrated Biological Response Index analysis (IBRv.2). Area above zero (positive values) indicates a size increase and below zero (negative values) indicates a size decrease for each morphologic parameter as biomarker compared to the reference group (Ctrl) OA: Optical area, MIID: Minimum interocular distance, MAID: Maximum interocular distance, HD: Head deep, HH: Head height, HW: Head width, PL: Proboscis length, VSA: Vitelic sac area, SBA: Swim bladder area, SL: Spine length, TL: Tail length and AMD: Anus-mouth distance. Ctrl: 0, T1: 3.84×10^{-6} , T2: 3.84×10^{-7} and T3: 3.84×10^{-8} particles number of PS-MPs mL^{-1} 3B) Polystyrene microplastic (100 μm) ingested by *D. rerio* larvae. With (left), and without (right) swim bladder inflated observed in T3 (7×10^3 particles number of PS-MPs mL^{-1}). VSA: Vitelic sac area; SBA: Swim bladder area. Stereomicroscopy at 30x and a scale of 500 μm

propose the use of an integrative approach to add to the current tests in laboratory and wild organisms because in natural settings, these MPs are co-exposed to other pollutants like heavy metals, drugs, and organic matter as well as the additives used during their production.

Conclusion

Results from this study highlight the relevance of a detailed evaluation of the effects of plastic microparticles with size of 100 μm on the developmental fate of fish during their early life stages. We observed more negative effects in the head in response to the constant contact with the spherical beads of PS. It is likely that these alterations would possess fitness costs to adulthood and would have a negative effect during their life, but it is a topic that deserves further investigation. The ingestion of MPs of 100 μm by fish larvae can occur. Therefore, future investigations should consider including the screening of particles of 100 μm and above in fish larvae. Further works must consider alternative exposure schemes as well as the inclusion of molecular

approaches to decipher the molecular basis of the morphological alterations reported here.

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