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PMMA Microplastics Induce Sublethal Cardiovascular and Developmental Effects During Early Development of Zebrafish (*Danio rerio*)

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Abstract

Microplastics are increasingly recognized as emerging contaminants with potential toxicological effects on aquatic organisms. Among widely used industrial polymers, poly(methyl methacrylate) (PMMA) has been detected in environmental matrices, raising concerns regarding its biological impacts. This study evaluated the developmental toxicity of 40 µm spherical PMMA microplastics in zebrafish (*Danio rerio*) using a standardized early-life stage exposure (3 hpf–6 dpf). Before exposure, particles were physicochemically characterized by Fourier Transform Infrared Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FESEM), and micro-Raman spectroscopy (µRaman). Embryos were exposed to nominal concentrations ranging from 0.5 to 20 mg/L, and endpoints including survival, hatching rate, spontaneous movements, heart rate, morphology, and locomotor activity were assessed. PMMA exposure did not induce significant mortality or morphological abnormalities at any tested concentration. However, significant sublethal effects were observed, including accelerated hatching at 20 mg/L and increased heart rate across exposure groups, further supported by a positive concentration–response trend across vessel-level values, indicating altered developmental timing and cardiovascular function. These findings demonstrate that PMMA microplastics, despite low acute toxicity, can induce early physiological disturbances in a vertebrate model. The results highlight the importance of incorporating sensitive functional endpoints into hazard assessment frameworks and contribute to a more comprehensive evaluation of the environmental risks associated with microplastics traditionally considered to have low intrinsic reactivity.

Keywords: poly(methyl methacrylate) (PMMA); microplastics (MPs); toxicity; zebrafish embryo-larval stage; neurobehavioral endpoints

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1. Introduction

The intensification of plastic production and consumption over recent decades has markedly increased environmental release, with approximately 60% of all plastic ever produced already discarded and accumulated in landfills and natural ecosystems [1]. Due to their durability, resistance to degradation, and broad industrial applicability, plastics persist in the environment and progressively fragment into smaller particles classified as microplastics (MPs) (<5 mm) and nanoplastics (NPs) (<100 nm) [2,3]. These particles have been widely detected in aquatic systems, sediments, soils, wastewater, and even the atmosphere, evidencing their global distribution [4–7]. Environmental factors such as ultraviolet radiation, mechanical abrasion, and biological activity drive continuous fragmentation, generating particles with diverse sizes, shapes, and chemical compositions, which complicate methodological standardization and toxicological interpretation [3,8]. From an ecotoxicological standpoint, micro- and nanoplastics (MNPs) are of increasing concern because they can be ingested across trophic levels, bioaccumulate, and act as vectors for organic contaminants and toxic metals [9,10], while also inducing oxidative stress, inflammation, metabolic disturbances, and developmental and reproductive impairments in aquatic organisms [11–13].

Among widely used polymers, poly(methyl methacrylate) (PMMA) is notable for its advantageous physicochemical properties, including optical transparency, mechanical strength, and thermal stability, which support its broad application in dental, medical, and industrial fields [14–16]. Despite its lower production volume compared to major commodity thermoplastics, PMMA exhibits considerable resistance to environmental degradation, which may enhance its persistence in natural systems [17,18] and promote prolonged interactions with environmental microorganisms [19]. Although PMMA is frequently included in comparative microplastic studies, most research has focused on more prevalent polymers such as polystyrene, polyethylene, and polypropylene, resulting in a limited understanding of PMMA-specific toxicity. Studies addressing the isolated effects of PMMA MNPs, particularly during early developmental stages of aquatic organisms, remain scarce [20]. Nevertheless, available evidence indicates that PMMA nanoparticles (NPs) can induce mortality and pericardial edema during zebrafish early development [21] elicit pronounced gut inflammation in zebrafish larvae [22] and significantly impair somatic growth in Xenopus laevis [23]. This limited and fragmented evidence constrains a comprehensive understanding of PMMA's toxicological profile and hinders accurate assessment of its environmental risk. In this context, the zebrafish (*Danio rerio*) is a well-established model for environmental toxicity studies due to its rapid embryonic development, external fertilization, embryo transparency, and high conservation of physiological pathways relative to higher vertebrates [24–26]. These features enable the integrated assessment of lethal and sublethal endpoints, including morphological, physiological, and behavioral responses, within a short experimental timeframe.

Given the limited understanding of the specific effects of PMMA MPs, the present study aimed to systematically evaluate their impact on early zebrafish development. We hypothesized that, despite their low intrinsic chemical reactivity, PMMA MPs would not induce pronounced acute toxicity but could induce measurable sublethal effects during early development. To test this hypothesis, endpoints including mortality, hatching rate, morphological alterations, as well as physiological and locomotor functions, were assessed to provide a comprehensive characterization of the potential ecotoxicological risks associated with this polymer.

2. Materials and Methods

2.1. Characterization of Poly(methyl methacrylate) Microparticles

Poly(methyl methacrylate) (PMMA) microparticles, commercially acquired from Lamberti Microbeads® Gallarate, VA, Italy (40 µm diameter, with sphere diameter variation < 5%), were morphologically and chemically characterized before biological assays.

2.1.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were obtained in triplicate using a PerkinElmer Spectrum 100 spectrometer (universal ATR with diamond/ZnSe crystal) Shelton, CT, USA, within the range of 4000–650 cm^{-1}, with a sampling interval of 1 cm^{-1} and 12 scans per spectrum. The accessory included a pressure arm with force indicator. Background spectra were collected with a clean crystal before each analysis. Measurements were performed at the Spectroscopy Laboratory of the School of Chemistry at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) [8].

2.1.2. Field Emission Scanning Electron Microscopy (FESEM)

Sample morphology and structure were examined by FESEM. Micrographs were obtained using an FEI Inspect F50 microscope, Hillsboro, OR, USA under an accelerating voltage of 20 kV, operating in high-resolution mode to detail surface characteristics and particle structural integrity. Analyses were conducted at the Central Laboratory of Microscopy and Microanalysis (LabCemm) at PUCRS [27].

2.1.3. Micro-Raman Spectroscopy (µRaman)

PMMA particles were analyzed in triplicate using µRaman spectroscopy, Ulm, Germany (WITec Alpha300 R). A 100× objective lens was employed to locate and focus the spheres on glass slides. Acquisition parameters consisted of a 5–10 s integration time with five accumulations [8,28,29]. Analyses were performed at the Interdisciplinary Center for Nanoscience and Micro-Nanotechnology (NanoPUCRS). A commercial PMMA sample (Sigma-Aldrich, Saint Louis, MO, USA, 182230, Mw ~120,000) was used as a µRaman reference material. Spectra were acquired under identical instrumental conditions.

2.2. Breeding and Embryo Collection

Adult zebrafish (*Danio rerio*), AB strain, were maintained in a recirculating system (Zebtec–Tecniplast®, Buguggiate, VA, Italy) under controlled conditions of temperature (27 ± 1 °C), photoperiod (14:10 h light:dark cycle), pH (maintained within the range of 7–8), and conductivity (maintained within the range of 500–800 µS/cm), according to standard zebrafish husbandry recommendations [30]. Fish were separated and placed in breeding tanks overnight at a ratio of 2:1 (males: females). The divider was removed the following morning to allow mating. Viable embryos were gently rinsed and selected within one hour post-fertilization (hpf) and maintained in E3 medium until the start of exposures. All in vivo procedures followed protocols approved by the Institutional Animal Care and Use Committee of PUCRS (CEUA/PUCRS, No. 12196, approved on 20 May 2025).

2.3. Preparation of Exposure Solutions for LC_{50}

E3 medium was used to minimize potential variability associated with adult system water and to ensure standardized solution preparation [31]. The medium was prepared as a 60× stock solution containing 34.8 g NaCl, 1.6 g KCl, 5.8 g $CaCl_2 \cdot 2H_2O$, and 9.78 g $MgCl_2 \cdot 6H_2O$ dissolved in distilled water to a final volume of 2 L. The pH was adjusted to 7.2 using NaOH, and the solution was subsequently autoclaved. Based on the manufacturer's specifications, a stock solution of PMMA MPs was prepared at 100 mg/L in E3 medium

using a high-sensitivity analytical balance (Sartorius Cubis, Göttingen, Germany). The suspension was maintained under continuous agitation during preparation to minimize particle sedimentation and aggregation. Macroscopic visual inspection did not reveal visible precipitation or formation of large aggregates, indicating an apparently homogeneous dispersion before the subsequent experimental procedures.

2.4. Determination of LC_{50}

The experimental design followed the Fish Embryo Acute Toxicity (FET) test according to OECD Guideline No. 236 [32]. A preliminary assessment of median lethal concentration (LC_{50}) was conducted using PMMA microplastic (MP) suspensions at concentrations of 6.25, 12.5, 25, 50, and 100 mg/L, prepared by serial volumetric dilution of a previously prepared 100 mg/L stock suspension in E3 medium. Concentration selection was based on ranges commonly employed in exploratory microplastic toxicity studies with zebrafish embryos [33,34], aiming to identify potential lethal thresholds and establish an appropriate concentration window for subsequent sublethal analyses. Zebrafish embryos (n = 20) were placed in a 50 mL Petri dish, maintained in a bio-oxygen demand (BOD) incubator with controlled temperature (26 ± 1 °C) and 14:10 photoperiod under a semi-static regime, and the solutions were renewed every 24 h. Embryos were observed daily using a stereomicroscope (Nikon®, Melville, NY, USA, SMZ 1500) and evaluated until 96 hpf, recording survival and hatching rates.

2.5. Determination of Sublethal Effects

The experiment comprised five nominal concentrations of PMMA MPs (0.5, 1, 5, 10, and 20 mg/L), plus a control group, for a total of six experimental groups. Actual PMMA concentrations in the exposure medium were not analytically quantified. Lower concentrations (0.5 and 1 mg/L) were selected based on concentrations reported for microplastic exposure in aquatic systems, encompassing environmentally informed and higher-exposure scenarios [35]. Given the limited data available specifically for PMMA microplastics, these concentrations were considered representative of plausible exposure scenarios rather than typical environmental PMMA concentrations. Higher concentrations (5–20 mg/L) were included to characterize potential hazards and evaluate concentration–response relationships under controlled experimental conditions. Although these concentrations may exceed typical environmental levels, they were selected to encompass higher-exposure scenarios reported for microplastics in aquatic systems and to facilitate hazard characterization. These concentrations should therefore be interpreted as experimental exposure scenarios rather than representative environmental concentrations of PMMA microplastics. Exposure solutions containing PMMA MPs were renewed every 24 h. Water quality control was maintained through daily monitoring of conductivity and pH (AKSO model 86505, São Leopoldo, Brazil) in both pre- and post-exposure solutions [36].

All sublethal exposure experiments were performed in polystyrene Petri dishes containing 50 mL of exposure suspension, with a density of ≤ 2 embryos/mL, and maintained in an incubator under stable temperature conditions (28.5 ± 1 °C) and a controlled 14:10 h light: dark cycle. To minimize potential background contamination, PMMA-containing laboratory materials were avoided throughout exposure procedures whenever possible. Animals were randomly assigned to the experimental groups to ensure an unbiased distribution among treatments and exposed for 6 days, following the protocol described by Pereira et al. [37]. In vivo parameters were analyzed at developmentally relevant stages commonly employed in zebrafish embryotoxicity assays, as follows: spontaneous tail coiling (STC) at 24 hpf, corresponding to the onset of early motor activity; heart rate at 48 hpf, when cardiac function is well established and reliably quantifiable; morphology at 120 hpf,

a stage suitable for the detection of developmental abnormalities after organogenesis; and locomotor behavior at 144 hpf, when larvae display stable swimming activity and neurobehavioral responses. Survival and hatching rates were monitored daily throughout the experiment (n = 120 embryos/group/experiment; 4 independent experiments) (Figure 1).

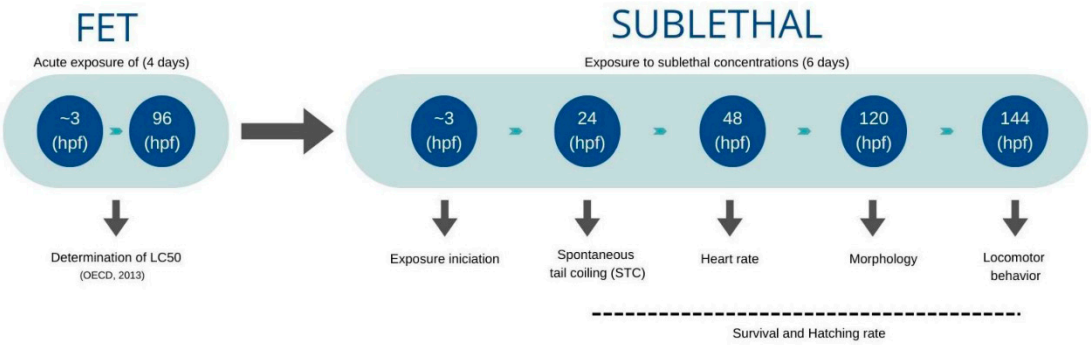


Figure 1. Experimental design of exposure to PMMA MPs. Healthy embryos were initially subjected to the Fish Embryo Test (FET) for 96 h, according to OECD guidelines No. 236 [32], to determine the median lethal concentration (LC_{50}). For sublethal effects, embryos were exposed for six days, with evaluations at specific stages of development (24, 48, 120, and 144 hpf). The analyzed parameters included spontaneous tail coiling (STC), heart rate, morphological changes, and locomotor behavior, as well as survival and hatching rates. Original design (image created using Canva).

2.6. Survival and Hatching Rate

Embryonic survival and hatching rates were monitored using a stereomicroscope (Olympus®, SZ40, Olympus®, Hachioji-shi, Tokyo, Japan) from ~3 to 144 hpf, evaluated daily (n = 120 embryos/group/experiment; four independent experiments), and expressed as a percentage over time [37–39].

2.7. Spontaneous Tail Coiling (STC) and Heart Rate

Spontaneous tail contraction frequency was quantified in embryos at 24 hpf following 2 min acclimation at room temperature. Recordings were performed for 1 min (n = 10 embryos/group/experiment; four independent experiments) [37].

Heart rate was evaluated at 48 hpf under a stereomicroscope (Olympus®, SZ40). After 2 min acclimation at room temperature, heartbeats were counted in three 10 s intervals per animal by two independent observers (n = 8 embryos/group/experiment; four independent experiments) and expressed as beats per minute (bpm) [39].

2.8. Morphological and Behavioral Assessment in Larvae

Morphometric parameters were assessed in larvae at 120 hpf, including interocular distance (µm), body length (mm), and ocular surface area (mm^2). Analyses were conducted using a stereomicroscope (Nikon®, Melville, NY, USA, SMZ 1500), with images subsequently analyzed using ImageJ software version 1.54s; National Institutes of Health, Bethesda, MD, USA (n = 8 larvae/group/experiment; four independent experiments) [39]. No immobilization techniques were used during image acquisition to prevent possible pharmacological or mechanical interference that might alter physiological parameters or obscure sublethal effects. Imaging was conducted within a brief time frame and only when the larvae were naturally at rest; any recordings displaying movement or visual distortion were excluded from the analysis.

Larval locomotor behavior was evaluated at 144 hpf, including mean velocity (cm/s), total distance moved (cm), absolute turn angle (deg), and mean meander (deg/cm). Animals were placed in 24-well plates for recording, acclimated for 1 min, and filmed for five minutes using a high-resolution digital camera (AltaVision®, Basler ace, model scA1300-

60gc), Ahrensburg, Schleswig-Holstein, Germany [37,39]. Locomotor pattern parameters were analyzed using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands) (n = 18 larvae/group/experiment; three independent experiments).

2.9. Statistical Analyses

All statistical analyses were conducted using GraphPad Prism (version 8.0.2; GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was used to assess data normality across all in vivo methodologies. The Log-Rank (Mantel–Cox) test was applied to the survival rate, and data were expressed as mean ± CI 95%. Two-way ANOVA, followed by Dunnett's post hoc test, was used to analyze hatching rate, and data were expressed as mean ± standard error of the mean (SEM). Non-parametric results were analyzed using the Kruskal–Wallis test, followed by Dunn's post-hoc test, and data were expressed as medians and interquartile ranges. Parametric data were evaluated by one-way ANOVA followed by Dunnett's post-hoc test, and data were expressed as mean ± standard deviation (SD). The adopted significance level was $p \leq 0.05$.

To address the hierarchical structure of the experimental design, in which treatment was applied at the vessel level, and individual organisms within the same vessel share the same exposure medium, a complementary set of analyses was performed, treating the vessel as the experimental unit. Four independent experiments were conducted for all in vivo endpoints except behavioral analyses, with one vessel per treatment per experiment. Behavioral analyses were performed in three independent experiments, with one vessel per treatment per experiment. Individual observations within each vessel were averaged to produce a single vessel-level value per endpoint, time point, concentration, and experiment before statistical modelling, thereby controlling for pseudoreplication [40,41]. The experiment was included as a fixed blocking factor to account for between-experiment variability. Treatment-versus-control comparisons were performed using Dunnett-adjusted contrasts [42] applied to linear models fitted to vessel-level values. To evaluate whether endpoints changed monotonically with increasing concentration, concentration–response trend analyses were conducted by fitting a linear model with log1p-transformed concentration (log1p[mg/L]) as a continuous predictor and experiment as a blocking factor, of the form: vessel value ~ *log1p(concentration)* + *experiment* [43]. Log1p transformation was applied to linearize the concentration–response relationship while accommodating the zero-concentration control group. The slope coefficient from each model was used as the primary effect size. As a non-parametric complement, Spearman rank correlations between concentration and vessel-level endpoint values were calculated. These analyses were conducted in R (version 4.4.1) using the packages *dplyr*, *ggplot2*, *broom*, and *emmeans*.

3. Results

3.1. Morphological, Structural, and Chemical Characterization of Particles

The analysis of the particles shown in Figure 2 demonstrates homogeneous particle distribution, predominantly spherical morphology, and smooth surfaces. The field-emission scanning electron microscopy (FESEM) reveals the absence of structural collapse or fractures. Higher magnification images allow the observation of a relatively uniform size distribution, with an average diameter of approximately 40 µm, indicating a controlled and reproducible manufacturing process. The lack of extensive agglomeration indicates limited particle aggregation under the conditions evaluated (Figure 2A). Figure 2B presents images obtained by micro-Raman microscopy (µRaman), further confirming the spherical shape of the particles as well as their homogeneous distribution, with well-defined boundaries between individual spheres. The micrometric measurements observed in the images are consistent with the technical specifications provided by the manufacturer, reinforcing the

reliability of the material characterization. The chemical characteristics of the material were analyzed by FTIR and subsequently confirmed by μRaman spectra. FTIR analysis was performed in the range of 4000–650 cm^{-1}, with 12 scans at room temperature, as shown in Figure 2C. A higher number of scans was not required due to the high purity of the material, in agreement with studies on acrylic polymers, considering the well-defined characteristic bands and the absence of spurious signals [28]. Regarding the observed bands, a peak at approximately 2950 cm^{-1} was attributed to the asymmetric and symmetric stretching vibrations of C–H bonds from methyl and methylene groups present in the polymer chain. The intense peak at 1721 cm^{-1} is characteristic of the stretching vibration of the carbonyl group (C=O) associated with ester functionalities, representing one of the main spectral signatures of PMMA [44]. Additionally, bands around 1141 cm^{-1} and 1238 cm^{-1} are related to C–O–C stretching vibrations, typical of acrylic esters. Other secondary bands, such as those observed at 1434 cm^{-1} and 749 cm^{-1}, correspond to angular deformation modes (bending) of CH_3 groups and out-of-plane vibrations of the polymer backbone, respectively, further confirming the chemical identity of the material [28].

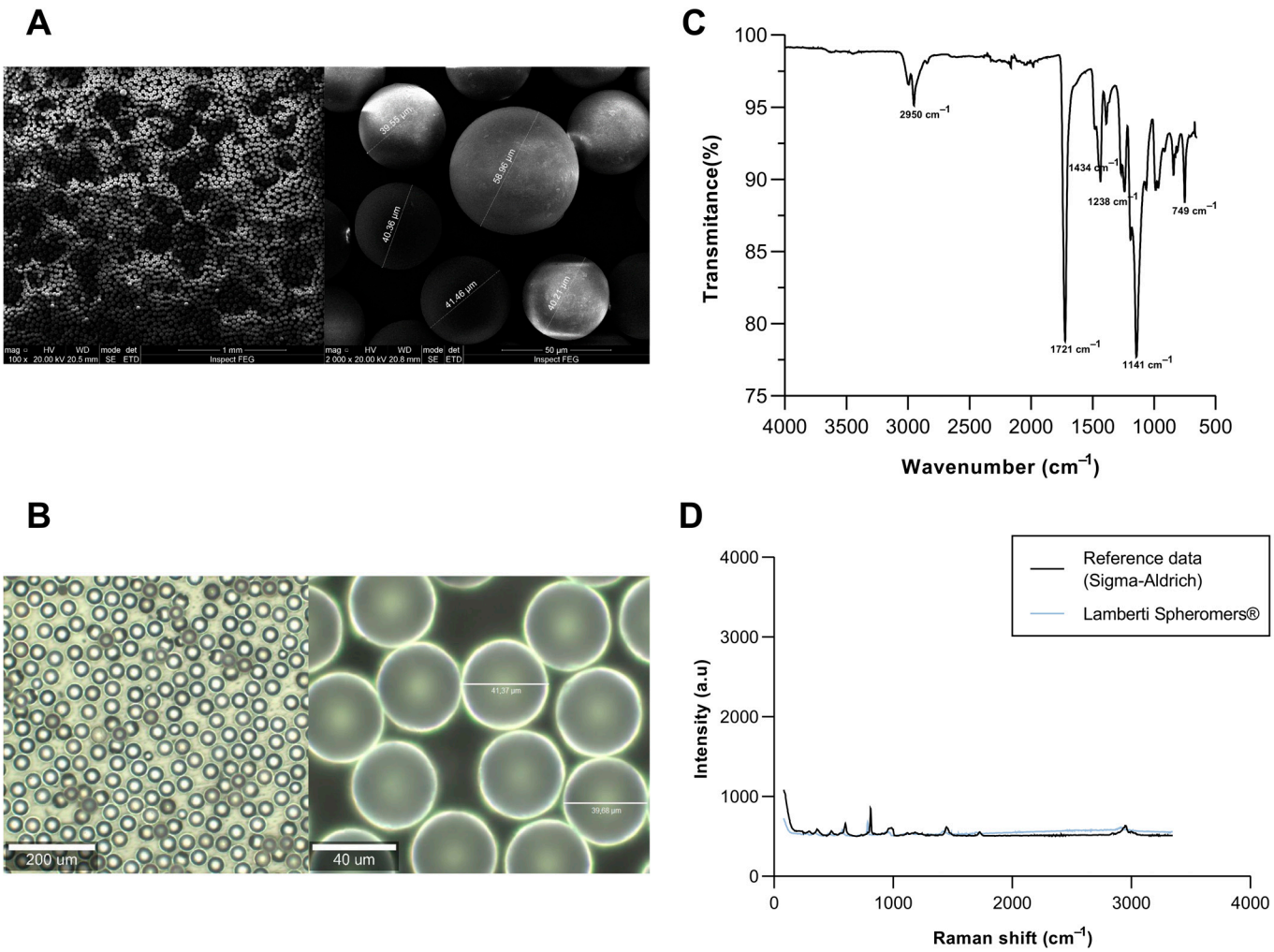


Figure 2. Morphological and chemical characterization of PMMA microparticles. (**A**) Field Emission Scanning Electron Microscopy (FESEM) images showing the surface morphology and spherical shape of the PMMA microparticles at different magnifications. (**B**) Optical microscopy images obtained during μRaman analysis, illustrating particle distribution and individual particle size. (**C**) Fourier Transform Infrared (FTIR) spectrum of PMMA microparticles, highlighting the characteristic absorption bands of the polymer. (**D**) μRaman spectra of PMMA microparticles compared with reference data, confirming the chemical identity of the material.

3.2. Determination of LC_{50}

The acute toxicity assessment (LC_{50}) was conducted using the Fish Embryo Acute Toxicity (FET) test, following the protocol established by the OECD guidelines No. 236 [32]. PMMA MP concentrations of 6.25, 12.5, 25, 50, and 100 mg/L were tested. The results did not indicate significant differences in lethality among the evaluated concentrations, and no mortality of zebrafish embryos was observed throughout the experimental period. Therefore, concentrations previously reported in studies involving micro- and nanoplastics (MNPs) were adopted [45,46], with the experimental design specifically directed toward the assessment of sublethal effects.

3.3. Survival and Hatching Rate

The survival of zebrafish embryos exposed to different concentrations of PMMA MPs (0.5; 1; 5; 10; 20 mg/L) was monitored from 24 to 144 hpf. Mortality rates below 10% were considered acceptable for the control group, in accordance with the chemical testing guidelines for aquatic organisms established by the OECD [32]. As shown in Figure 3A, survival rates remained high throughout the experimental period. No concentration-dependent mortality associated with PMMA MP exposure was detected.

Regarding hatching rate (Figure 3B), the control group reached values exceeding 85% by 96 hpf, consistent with the expected developmental progression. Embryos exposed to 20 mg/L showed an earlier increase in partial hatching at 48 hpf compared with the control group ($p = 0.002$), suggesting accelerated hatching at this concentration.

After correcting the experimental unit by analyzing vessel-level values, categorical treatment-vs-control comparisons did not show significant differences after Dunnett adjustment. However, concentration–response trend analyses at the vessel level identified consistent positive associations between PMMA concentration and hatching-related endpoints at 48 and 72 hpf (Figure 3C). Positive concentration-related trends were supported by both the vessel-level trend model and Spearman rank correlation at 72 hpf (model $p = 0.003$, coefficient = 2.93; Spearman rho = 0.50, $p = 0.014$), and by the trend model alone at 48 hpf (model $p = 0.012$, coefficient = 6.18; Spearman rho = 0.38, $p = 0.068$). The complementary endpoints, hatched count and unhatched count, showed corresponding trends in the expected directions at both time points, consistent with the hatching rate results (Figure S1).

3.4. Spontaneous Tail Coiling and Heart Rate

The spontaneous tail coiling (STC) activity (Figure 4A) did not differ significantly between the control group and the groups exposed to PMMA MPs (0.5–20 mg/L), indicating the absence of detectable early motor alterations at the evaluated embryonic stage.

In contrast, embryonic heart rate (Figure 4B) was increased at 1 ($p < 0.001$), 5 ($p = 0.008$), 10 ($p = 0.002$), and 20 mg/L ($p < 0.001$) PMMA MPs compared with the control group, whereas no detectable effect was observed at 0.5 mg/L. A positive concentration–response trend was also observed in the vessel-level analysis. This pattern suggests that cardiovascular effects may emerge at concentrations around 1 mg/L and indicates a possible concentration-related response within the tested range, supported by a positive concentration–response trend in the vessel-level analysis (Spearman's rho = 0.54, $p = 0.006$).

When vessel-level values were analyzed, no individual concentration reached statistical significance compared to the control after Dunnett adjustment. However, concentration–response trend analysis confirmed a positive association between PMMA concentration and heart rate at 48 hpf, supported by both the vessel-level trend model (model $p = 0.039$, coefficient = 2.57) and Spearman rank correlation (rho = 0.54, $p = 0.006$) (Figure 4C). No

concentration-related trend was detected for spontaneous tail coiling (model $p = 0.235$), consistent with the individual-level analysis.

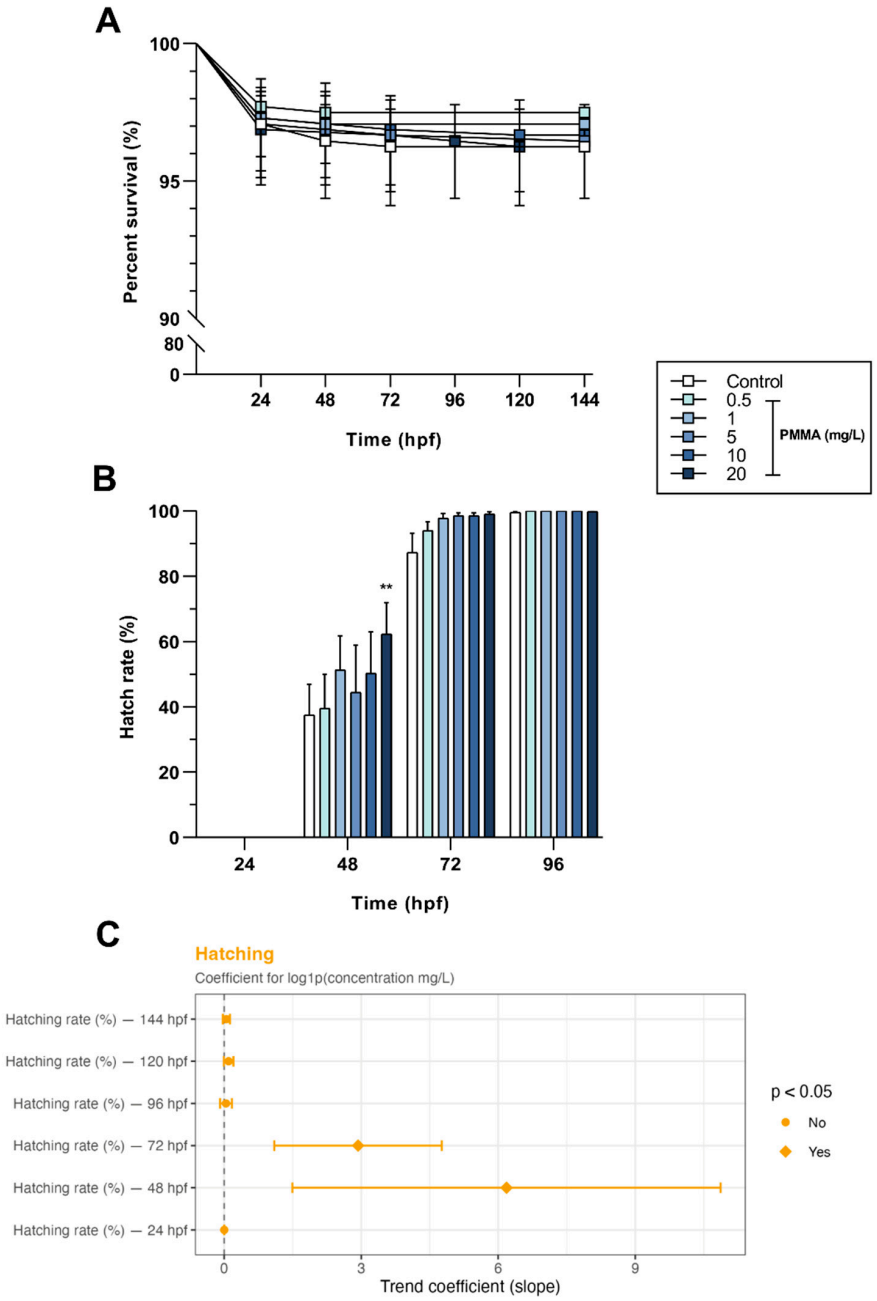


Figure 3. Effects of PMMA MPs on survival and hatching rate of zebrafish embryos and larvae ($n = 480$/group). (**A**) Survival rate of zebrafish embryos exposed to increasing concentrations of PMMA microparticles from ~ 3 to 144 h post-fertilization (hpf), expressed as percentage (mean ± 95% confidence interval), estimated using the Log-rank (Mantel–Cox) test. (**B**) Hatching rate (%) of embryos exposed to PMMA microparticles at different concentrations, evaluated over time (mean ± SEM). Statistical analysis was performed using two-way ANOVA followed by Dunnett's post-hoc test. "*" indicates significant differences compared to the control group (** $p \leq 0.01$). (**C**) Concentration–response trend coefficients for hatching-related endpoints (slope coefficient from a linear model fitted to vessel-level values, with log1p-transformed concentration log1p[mg/L]) as a continuous predictor and experiment as a fixed blocking factor. Error bars indicate 95% confidence intervals. A positive coefficient indicates an increasing hatching rate with increasing PMMA concentration.

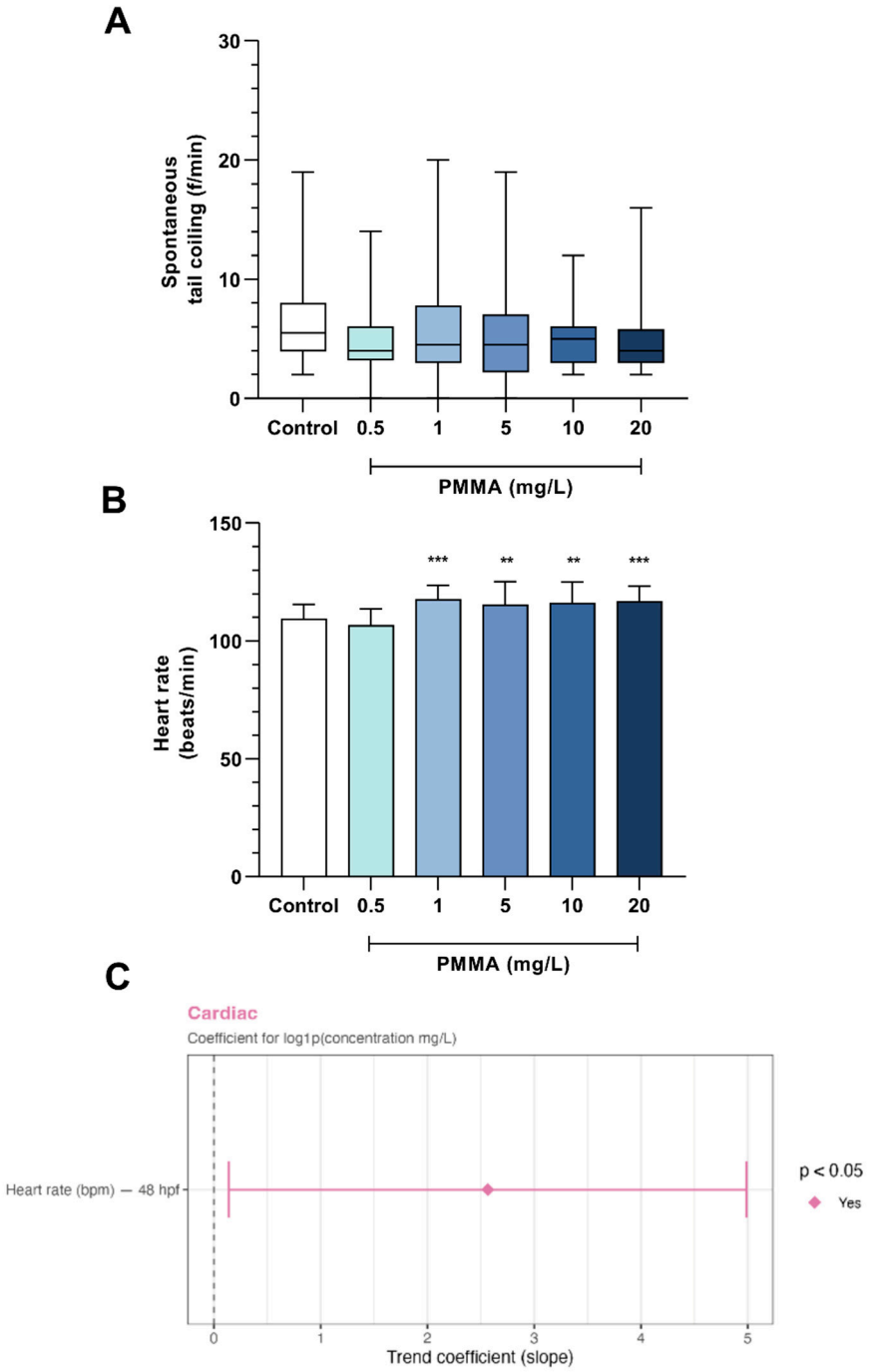


Figure 4. Effects of PMMA MPs on functional parameters of zebrafish embryos and larvae. (**A**) Spontaneous tail coiling frequency was analyzed at 24 hpf following exposure to PMMA (n = 40/group). Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post-hoc test. Boxplot bars represent data range variation from minimum to maximum and the median. (**B**) Heart rate (beats per minute) was measured at 48 hpf ($n \geq 31$/group). Statistical analysis employed one-way ANOVA followed by Dunnett's post-hoc test. Column bars represent mean ± standard deviation. "*" indicates significant differences compared to the control group (*** $p \leq 0.001$; ** $p \leq 0.01$). (**C**) Concentration–response trend coefficient for heart rate at 48 hpf. (slope coefficient from a linear model fitted to vessel-level values, with log1p-transformed concentration log1p[mg/L]) as a continuous predictor and experiment as a fixed blocking factor. Error bars indicate 95% confidence intervals. A positive coefficient indicates an increasing heart rate with increasing PMMA concentration. hpf, hours post-fertilization; bpm, beats per minute.

3.5. Morphology

Morphometric analyses comprising body length, interocular distance, and ocular surface area revealed no statistically significant differences between control and treatment groups at any evaluated concentration (Figure 5), indicating that PMMA MPs did not impair somatic growth or craniofacial morphogenesis and suggesting low structural toxicity under the experimental conditions. Vessel-level Dunnett contrasts and concentration–response trend analyses confirmed the absence of concentration-related effects on morphological endpoints (Figure S1).

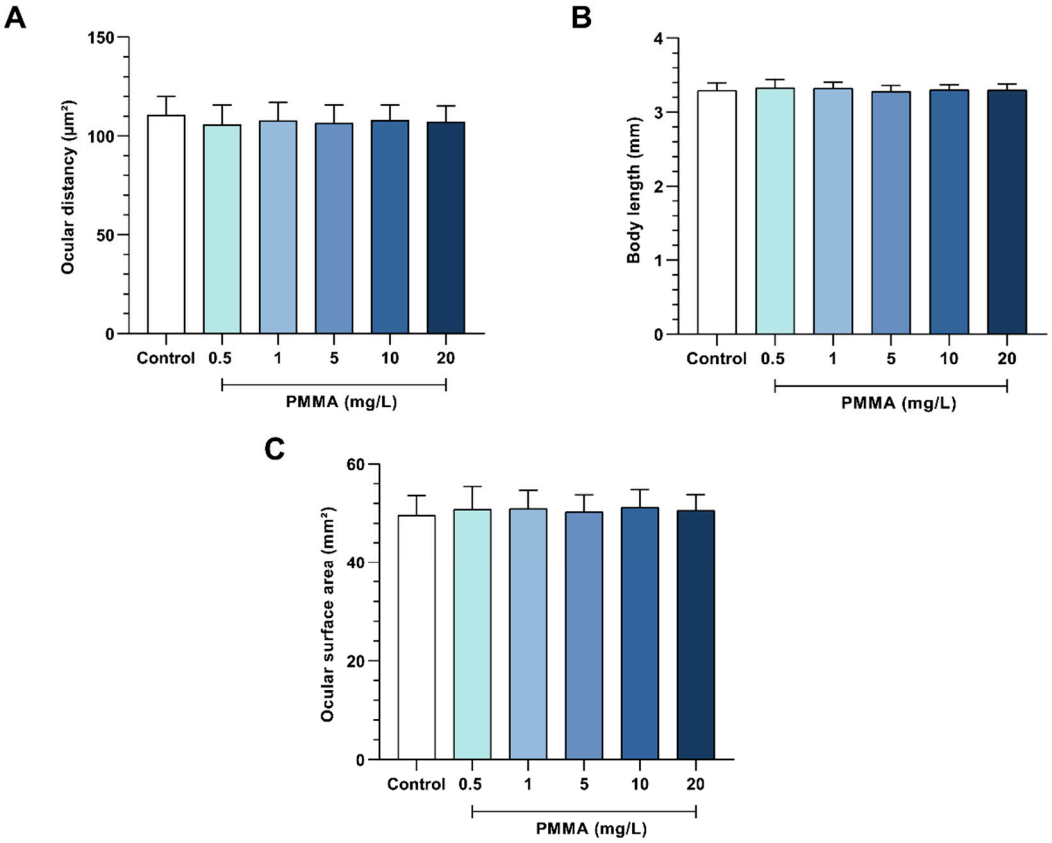


Figure 5. Effects of PMMA MPs on zebrafish larvae morphology (n = 32/group). (**A**) Ocular distance, (**B**) body length, and (**C**) ocular surface area of zebrafish larvae analyzed at 120 hpf using ImageJ software. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. Column bars represent mean ± standard deviation. No statistically significant differences were observed among the experimental groups compared to the control group.

3.6. Behavior

Behavioral assessment at 144 hpf demonstrated no significant differences in the parameters of mean velocity, total distance moved, absolute turn angle, and mean meander (Figure 6). Vessel-level Dunnett contrasts and concentration–response trend analyses confirmed the absence of concentration-related effects on locomotor parameters (Figure S1).

A summary of concentration–response trend coefficients across all endpoints and time points is presented in Figure S1, illustrating that significant positive trends were restricted to hatching rate and heart rate at 48–72 hpf.

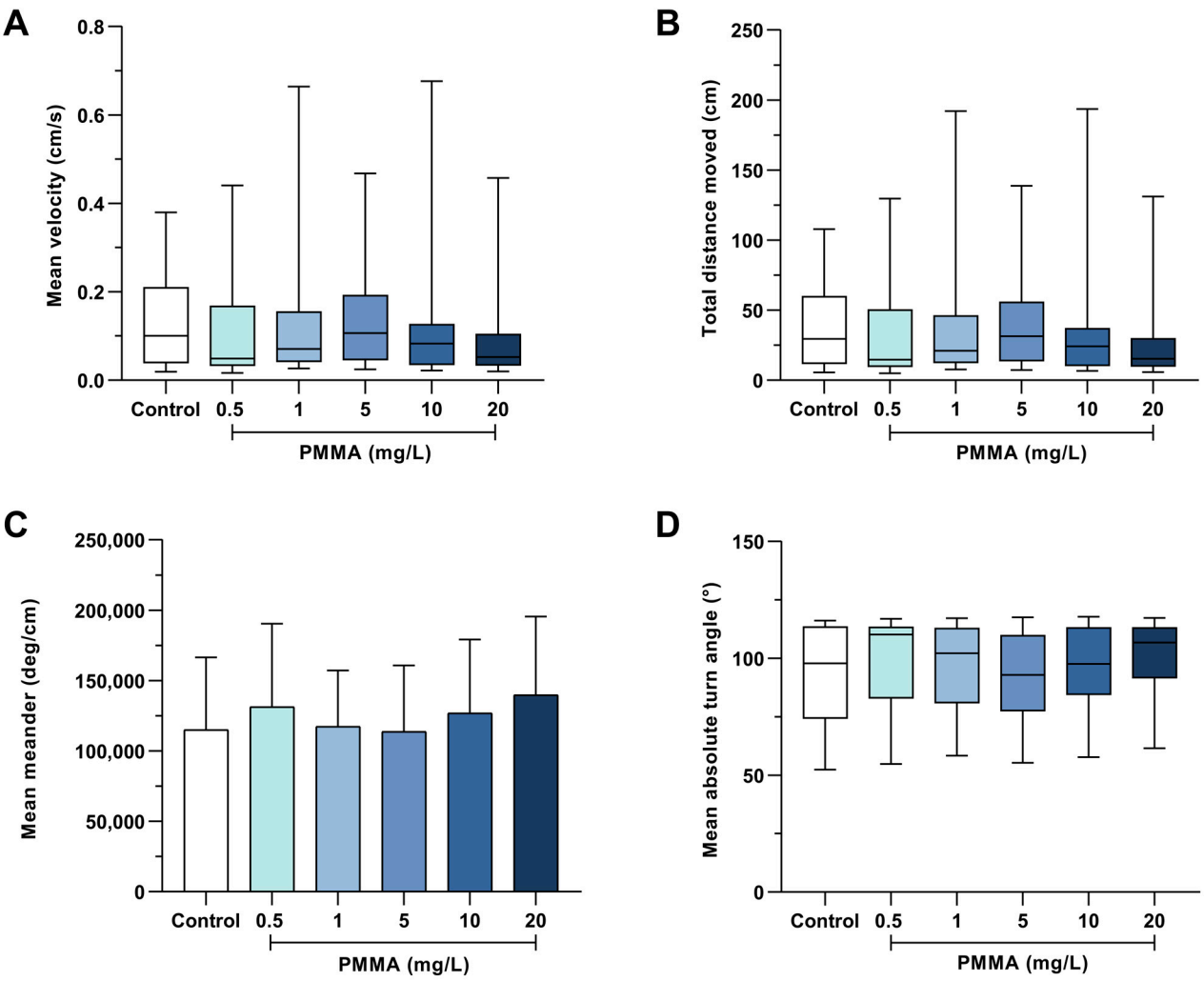


Figure 6. Effects of PMMA MPs on zebrafish larvae's behavior at 144 hpf ($n \geq 45$/group). (**A**) Mean velocity, (**B**) total distance moved, and (**D**) mean absolute turn angle. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's post-hoc test. Boxplot bars represent data range variation from minimum to maximum and the median. (**C**) Mean meander. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. Column bars represent mean ± standard deviation. No statistically significant differences were observed among the experimental groups compared to the control.

4. Discussion

PMMA is extensively used in medical, dental, optical, and household applications and has been identified in environmental matrices as well as in aquatic organisms [47,48]. PMMA is described as relatively stable and chemically inert under environmental conditions [14,15,49], characteristics that may favor its environmental persistence once released. Despite its widespread use and environmental occurrence, information regarding its toxicity in aquatic species remains scarce [50], with most available studies primarily addressing its nanometric forms rather than micrometric-sized ones [47,48,51]. In this context, the present study investigated the toxicological effects of PMMA MPs using zebrafish embryos as an experimental model.

Before the initiation of biological assays, the MPs were thoroughly characterized to ensure material identity and structural integrity. Fourier Transform Infrared Spectroscopy (FTIR) and micro-Raman spectroscopy (μRaman) confirmed the chemical composition of PMMA, while Field Emission Scanning Electron Microscopy (FESEM) verified particle

morphology and size homogeneity. This combined analytical approach strengthens the reliability of the exposure design and ensures that the observed biological responses can be attributed to well-defined and chemically validated particles.

An acute toxicity assessment was conducted in accordance with the OECD Fish Embryo Acute Toxicity (FET) guidelines No. 236 [32] to determine the LC_{50} of PMMA MPs. No significant differences in lethality were detected across the tested concentration range, and embryo mortality was absent throughout the exposure period, precluding the calculation of an LC_{50} under the present conditions. While these findings indicate low acute lethality, they do not exclude the possibility of subtle or delayed toxic effects. Indeed, the absence of mortality highlights the importance of investigating sublethal endpoints, which may provide greater sensitivity for detecting early developmental disturbances associated with MNP exposure [45,46].

Consistent with the acute toxicity assessment, the sublethal concentrations selected for subsequent analyses did not induce a decrease in embryo survival, confirming that the experimental design effectively targeted non-lethal endpoints (Figure 3A). However, the increased hatching rate observed at the highest concentration, additionally supported by a positive concentration–response trend at the vessel level, suggests that PMMA MPs may interfere with the hatching process. Therefore, the observed effects are more plausibly related to indirect interactions between MPs and the chorion surface or to changes in the surrounding microenvironment during embryonic development. In zebrafish, hatching is a regulated process involving chorionase secretion and coordinated embryonic movements [52]. It is possible that the presence of PMMA MPs alters the physicochemical characteristics of the chorion surface or modifies local exchange conditions between the embryo and the surrounding medium, thereby affecting hatching dynamics. Accelerated hatching has been proposed as an adaptive response to environmental stressors, allowing embryos to escape unfavorable conditions within the chorion [53]. In this context, the increased hatching rate observed in the present study may reflect an alteration in developmental timing associated with PMMA MP exposure. Although a stress-related developmental response could potentially contribute to this effect, this interpretation remains speculative and requires further investigation. Additionally, exposure to MPs can be associated with oxidative stress and related physiological disturbances [54,55]. However, as oxidative stress and inflammatory markers were not evaluated in the present study, these mechanisms should be considered speculative and require further investigation.

Previous studies have also suggested that microplastic exposure can be associated with oxidative stress and related physiological disturbances [54,55]. However, as oxidative stress and inflammatory markers were not evaluated in the present study, these mechanisms should be considered speculative and require further investigation.

The clarity and reliability of spontaneous tail coiling (STC) assessment support its application as a sensitive indicator of embryonic behavioral alterations [56], and it has been widely acknowledged as an effective endpoint for evaluating neurotoxic effects [57]. No significant alterations in STC were observed at any tested concentration, indicating preservation of early neurobehavioral function. The maintenance of this parameter suggests that, at this developmental stage, PMMA MPs did not measurably disrupt embryonic locomotor circuitry. In contrast, although the observed acceleration in heart rate was relatively modest, it may suggest that subtle physiological responses can occur even in the absence of evident structural abnormalities or overt behavioral impairment. The biological relevance of this response, however, remains uncertain and should be interpreted cautiously. Similar cardiovascular responses have been reported in zebrafish embryos exposed to MNPs, where increased heart rate has been associated with physiological stress and compensatory metabolic adjustments during early development [45,58]. The embryonic cardiovascular

system is particularly sensitive to redox and metabolic changes [59]. Previous studies have demonstrated that MNPs can induce oxidative stress, mitochondrial dysfunction, and metabolic alterations in zebrafish [54,60], conditions that may affect cardiomyocyte bioenergetics and the regulation of contractility. Additionally, mammalian studies report increased pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, following exposure to methacrylates, accompanied by alterations in cardiovascular parameters [61]. Although these mechanisms were not directly assessed in the present study, redox imbalance and inflammatory signaling may represent potential mechanisms contributing to the observed modulation of heart rate. However, this interpretation is based on biological plausibility and remains hypothetical, as their involvement was not directly demonstrated in the present study.

Structural malformations are frequently observed following exposure to contaminants with higher reactive potential or bioaccumulative capacity [25]. The absence of morphological alterations suggests low structural toxicity of PMMA MPs under experimental conditions.

Locomotor behavior in zebrafish larvae is considered an integrative endpoint of neurological, muscular, and metabolic function [62]. Smaller MPs with greater internalization capacity have been associated with locomotor and behavioral alterations [60]. The absence of behavioral changes in the present study may be related to particle size, low bioavailability, or limited systemic internalization of PMMA MPs.

Under the evaluated conditions, PMMA MPs exert functional rather than structural effects. This profile may be associated with the larger particle size tested, as nanometric MPs exhibit greater potential for internalization and tissue translocation [63], thereby increasing the likelihood of broader systemic impacts. Taken together, the present findings indicate that PMMA MPs display a predominantly sublethal toxicological profile in zebrafish embryos, selectively affecting physiological parameters without compromising survival or global morphogenesis. Collectively, these findings emphasize that even polymers considered chemically inert may pose functional biological challenges, underscoring the need to reassess environmental safety paradigms for emerging plastic contaminants.

Some limitations should be considered when interpreting the present findings. The study evaluated PMMA MPs with a diameter of 40 μm, and exposure concentrations were based on nominal rather than measured values. While the lower concentrations may be environmentally plausible, the higher concentrations were included to characterize potential hazards and concentration–response relationships. In addition, mechanistic biomarkers and particle uptake or tissue localization were not assessed. These aspects should be considered when extrapolating the findings to environmentally heterogeneous exposure conditions and interpreting the underlying mechanisms. Further studies addressing these aspects will help to clarify the environmental and mechanistic relevance of the observed responses.

5. Conclusions

The present study demonstrates that PMMA MPs exhibit low acute lethality and do not induce significant morphological alterations in zebrafish embryos and larvae under the tested conditions. However, exposure resulted in specific sublethal effects, particularly involving the modulation of hatching dynamics and an increased heart rate, additionally supported by a positive concentration–response relationship across vessel-level data, indicating functional physiological disturbances during early development. Although embryonic survival was not significantly reduced and most evaluated endpoints were unaffected, the observed changes in hatching and heart rate suggest that PMMA MPs may influence specific developmental and physiological processes under the conditions tested. Overall, the results reinforce the importance of incorporating sublethal and functional end-

points into microplastic risk assessment frameworks, even for polymers generally classified as having low intrinsic hazard.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microplastics5030178/s1. Figure S1: Concentration–response trend coefficients across all endpoints and time points. Each point represents the slope coefficient from a linear model fitted to vessel-level values, with log1p-transformed concentration (log1p[mg/L]) as a continuous predictor and experiment as a fixed blocking factor. Error bars indicate 95% confidence intervals. Points are colored by biological category: hatching, cardiac, survival, morphology, locomotion, and behavior. Positive coefficients indicate increasing endpoint values with increasing PMMA concentration; negative coefficients indicate decreasing values; bpm, beats per minute.

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Institutional Review Board Statement: All procedures followed the Brazilian Council of Animals Experimentation guidelines for Use of Fish in Research (CONCEA) and the Brazilian legislation (11.794/08). The study protocol was approved by the Institutional Animal Care and Use Committee of Pontifícia Universidade Católica do Rio Grande do Sul (CEUA/PUCRS No. 12196, approved on 20 May 2025).

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available in the repository under DOI: 10.5281/zenodo.18928419.

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