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Synthesis and characterization of micro-sized polyisobutylene and evaluation of its toxicological effects on the development and homeostasis of zebrafish (*Danio rerio*)

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Rampant industrialization has led to widespread reliance on hydrocarbon polymers for various commercial applications. While these synthetic polymers, commonly known as plastics, degrade in slowly in the environments, the toxic effects of their micro-sized particles remain underexplored. In this study, we synthesized polyisobutylene (PIB) microparticles in the lab and evaluated their toxicity and accumulation in a zebrafish model. Pristine and fluorescent PIB-microplastics (MPs), with particle sizes ranging from 2 to 10 μm, were synthesized using the solvent evaporation method. Fourier-transform infrared spectroscopy (FTIR) confirmed the stability of the suspensions. Zebrafish larvae exposed to various concentrations of PIB-MPs exhibited numerous morphological and molecular changes, including delayed hatching, impaired swimming behavior, increased reactive oxygen species levels, altered mRNA levels of genes encoding antioxidant proteins, and reduced survival rates. Dissections revealed PIB-MP accumulation in the guts of larvae and adult fish within 7–21 days, causing damage to the intestinal mucosa. These findings provide insights into how contaminants like PIB can induce pathophysiological defects in aquatic fauna and pose potential health hazards to humans.

Keywords Polyisobutylene, Microplastics, Characterization, Ecotoxicity, Zebrafish

Abbreviations

MPs	Microplastics
PIB	Polyisobutylene
PIB-MP(s)	Polyisobutylene microplastic(s)
SDS	Sodium dodecyl sulfate
LC	Lower concentration
HC	Higher concentration
ddH$_2$O	Double distilled water
ROS	Reactive oxygen species
DCFDA	2′,7′-Dichlorofluorescein diacetate
FTIR	Fourier-transformed infrared spectroscopy
DLS	Dynamic light scattering
SEM	Scanning electron microscopy
PCR: RT-qPCR	Quantitative reverse transcription polymerase chain reaction
FITC	Fluorescein isothiocyanate

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gsr	Glutathione reductase
gstp1.2	Glutathione S-transferase pi 1.2
sod1 or cu/zn sod	Superoxide dismutase 1, soluble
sod2 or mn sod	Superoxide dismutase 2, mitochondrial
GIT	Gastrointestinal tract

Polyisobutylene (PIB) is widely utilized in various industries due to its one-of-a-kind elastomeric macromolecule that exhibits superior gas and moisture imperviousness, high damping, and chemical and oxidative stability[1]. PIB-containing materials are used in a variety of commercial applications, such as tire inner liners and tubes, sealants, adhesives, condenser caps, pharmaceutical stoppers, and chewing gums[2] Several in vitro reports on PIB toxicities, showed that it has low or no toxic effects on cultured cells (e.g., [3]). About two decades ago, Camphuysen et al.[4]. reported that PIB killed over 1000 seabirds in the North Sea. In another experiment by Iversen[5], who examined the impact of different oils used to impregnate paper-insulated power cables and their synthetic substitutes, notably PIB, on tumor promotion. No evidence of tumor promotion was found after the hairless skin of mice (Male and female hr/hr Oslo strain mice) were treated with 7,12-dimethylbenz(α)anthracene (DMBA) and then exposed to PIB. According to the author, he concluded that 40% of PIB oil may have decreased the number of tumors when compared to DMBA alone or DMBA and 20% PIB oil. In addition, the author claimed that PIB raised the mortality rate at larger dosages, but no supporting evidence was provided. Synthetic plastic materials, such as PIB, are known to yield microplastics (MPs) in the aquatic and terrestrial environment either through weathering or degradation[6,7]. Plastic granules less than 5 mm in diameter are referred to as microplastics (MPs). They are produced at these proportions for commercial reasons as well as for the fragmentation of larger pieces, making them ubiquitous. MPs such as polyethylene, polypropylene, polystyrene, and polyurethane have been detected in human placenta and meconium samples, with only the latter identified as airborne fallout in the operation area, suggesting possible contamination[8]. The host body interprets MPs as foreign materials inside tissues, triggering local immune responses. MPs have been detected in the intestines of humans and marine organisms[9]. Indeed, it has been proposed that MPs, if swallowed by humans, may accumulate and cause localized toxicity by initiating and/or increasing immunological responses, thereby weakening the body's defenses against pathogens and affecting how energy reserves are utilized[10].

We have recently reported the presence of PIB-MPs in water sampled from the Cauvery River in southern India[11]. This is of great concern because the river provides potable water for over 150 million humans and animals, and has long-sustained fishing and irrigation.

While several research focused on other types of MPs in recent times, PIB-MPs toxicity have received little to no attention in any animal models. In this present study, we have addressed the ecotoxicity and human health effects of PIB-MPs. To model this in the laboratory, we synthesized and characterized micro-sized PIB. Further, we evaluated its biological effects on zebrafish (*Danio rerio*), an effective vertebrate model organism for toxicity testing[12]. We demonstrate that PIB-MPs can perturb mitochondrial function, which generates reactive oxygen species (ROS) and causes apoptosis, eventually leading to widespread tissue damage. Hence, our findings bolster the accumulating evidence that MPs cause toxicity at all levels of biological complexity, and pose a threat to marine and terrestrial ecosystems. Moreover, our observation of the bioaccumulation of PIB-MPs provides valuable insights into the potential human health hazards from plastic pollution of waterbodies[13], and future water treatment strategies.

Results
Synthesis and characterization of the polyisobutylene microplastic material
The PIB-MPs were characterized by dynamic light scattering (DLS) and scanning electronic microscopy (SEM). First, analysis of the hydrodynamic size of the PIB-MPs by DLS showed that all the particles exhibited varying sizes of 290.6 nm (SDS-PIB 80 mg), 452 nm (SDS-PIB 50 mg), and 291.6 nm (SDS-PIB 2000 mg) (Table 1). All the PIB-MPs exhibited zeta potentials higher than − 30 mV, which testifies to their stability in dispersion[14] (Fig. 1). SDS exhibited a zeta potential of − 46.3 mV, the PIB-MPs exhibited 50.1, 35.6, and 58.1 mV for SDS-PIB 80 mg, SDS-PIB 500 mg, and SDS-PIB 2000 mg, respectively. We hypothesized that the variation in the zeta potentials of the PIB-MPs could be due to the nature of the PIB, the repulsive forces, and agglomeration in suspension. Indeed, SE micrography indicated that the PIB-MPs exhibited irregular morphology, with varying degrees of agglomeration at different concentrations (Fig. 2A–C). To further support what was observed in the SEM, all the PIB-MPs showed a particle size of less than 1 μm (Fig. 2D–F) in DLS results.

All the synthesized MPs exhibit the characteristic C=C backbone peak at 1655 cm^{-1} and the $=CH_2$ and $-CH_3$ are peculiar to the peaks of PIB as reported previously[14,15] (Fig. 3). However, compared to SDS-PIB 2000 mg, the PIB-MPs at SDS-PIB 80 mg and SDS-PIB 500 mg were stable without causing any shift in the chemical bonds of PIB. Similarly, the Raman spectroscopy showed the same peaks for the synthesized 80 mg and 500 mg PIB-MPs.

S/No.	Sample	Mean ± SD of particle size (μm)
A	PIB-SDS 80	290.6 ± 49.90
B	PIB-SDS 500	452.0 ± 49.30
C	PIB-SDS 2000	291.3 ± 44.35

Table 1. Average particle size of PIB-MPs analyzed by DLS.
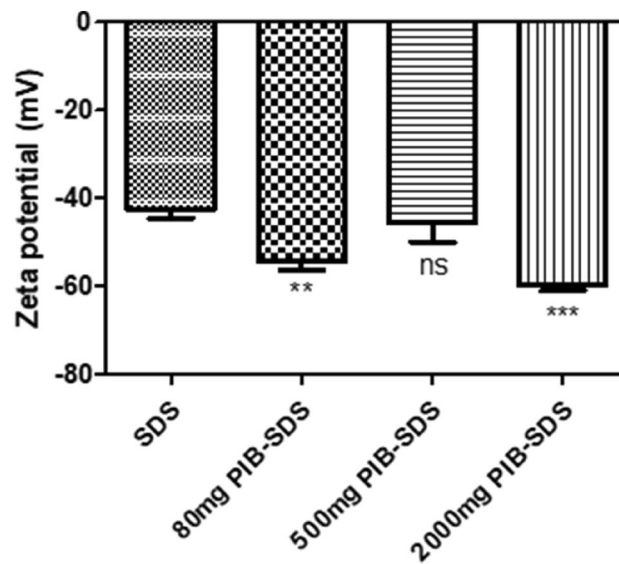
Fig. 1. Zeta potential of samples evaluated by dynamic light scattering (DLS) shows inherentstability of 500 mg PIB-MP with SDS. Number of replicates (N) = 3.

However, the 2000 mg PIB-MP peaks were completely different from the other syntheses, instead being similar to SDS. Together, we inferred the 500 mg PIB-MP synthesis with a peak at 715.942 cm^{-1} wave number as the most suitable compared to others (Supplementary Fig. 4).

Exposure to polyisobutylene microplastics causes developmental defects and mortality

To test whether exposure to PIB-MPs affects the development of zebrafish, we checked the hatching and mortality rates of larvae every 24 h for 7 days. Whereas the hatched larvae developed normally in the control, exposure to PIB-MPs, caused malformations that increased in a concentration-dependent manner in zebrafish embryos and adults (Fig. 4A,B). The developmental abnormalities observed, in both the larvae and the adult zebrafish, include abnormal lateral spinal curvature. Moreover, compared to the controls, we observed delayed hatching in the treatment group (6.0 µg/ml): about 20% of embryos hatched only on the 7th day post fertilization (dpf) (Fig. 4C). The mortality rate increased in the treatment groups as compared to the controls (Fig. 4F). However, in adults, no mortality was observed except for erratic swimming and spinal curvature after 21 days of treatment.

Exposure to polyisobutylene microplastics induces locomotor defects and anxiety-like behavior

Meta-analysis has revealed that environmentally relevant concentrations of MPs influence the locomotor activity of aquatic fauna[16]. Therefore, we asked whether exposure to PIB-MPs might produce a similar phenotype. Zebrafish larvae in the treatment group covered approximately only 1 cm distance (Supplementary Video 1), suggesting that treatment with PIB-MPs alters the swimming activity of zebrafish larvae.

Exposure to polystyrene micro- and nanoparticles was recently found to induce anxiety-like behaviors in adult mice[17]. Because zebrafish is a useful animal model of anxiety fear-inducing stimuli, such as novelty, in which an animal is confronted with an unfamiliar object or environment, we measured the mobility and inquisitiveness of zebrafish adults using a novel tank test[18]. Adult fish, upon exposure to HC PIB-MP, showed a significant reduction in their swimming activity when compared to the controls. The fish exposed to HC PIB-MP swam slowly at the bottom of the tank, without exhibiting any tendency to swim up or sideways, as compared to the controls, whereas those in the LC PIB-MP treatment group swam only to one side of the tank (Supplementary Video 2A). In humans, anxiety is often characterized by hyperarousal and/or social phobia[19]. We determined the sociability of zebrafish by examining their propensity to interact with conspecifics after three weeks of PIB-MPs exposure. We did observe a significant difference in the interaction between the experimental and control groups (Supplementary Video 2B). Moreover, fish administered with LC PIB-MP acted like they were hyperactive. Together, our findings suggest that exposure to PIB-MPs induces anxiety-like behaviors in adult zebrafish, with implications for mental and neurological disorders in humans.

Polyisobutylene microplastics accumulate in and damage the gastrointestinal tract

It is hypothesized that ingestion of MPs might affect the brain through perturbation of the gut-brain axis[17]. We asked whether the neurological defects observed upon exposure to PIB-MPs might be traced back to intestinal damage. Fluorescence microscopy, in zebrafish larvae, after 7 days of exposure to PIB-MPs, revealed that the microparticles had accumulated in the gut, mostly in the proximal and middle intestine (Fig. 5). The accumulation of MPs was found to correlate with the treatment concentration (Fig. 5). In the adults, confocal microscopy, after 21 days of PIB-MPs ingestion, revealed that the microparticles had accumulated along the entire length
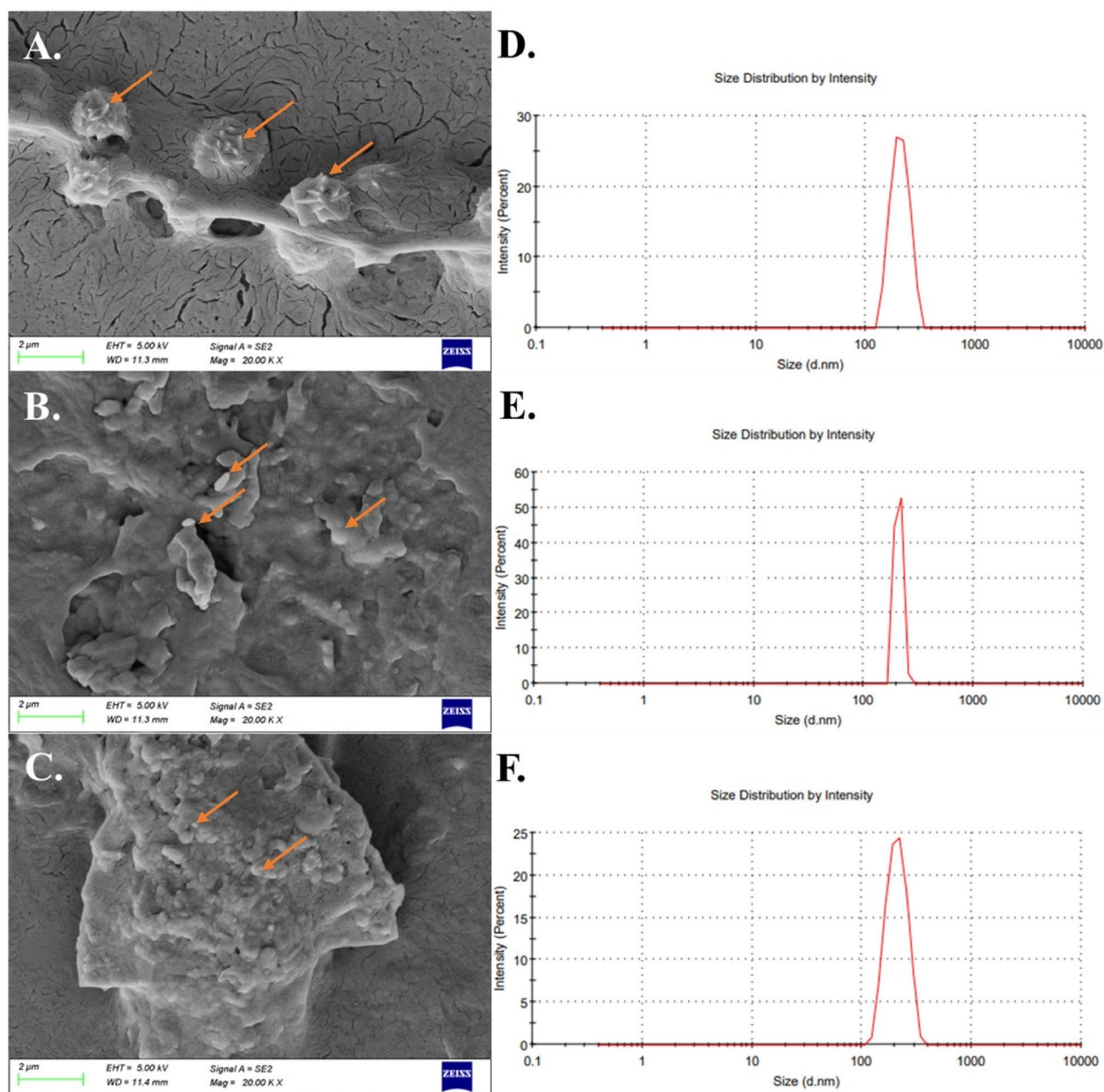
Fig. 2. SEM images and hydrodynamic sizes of PIB-MPs at the different concentrations (**A**) 80 mg/ml, (**B**) 500 mg/ml, (**C**) 2000 mg/ml, and (**D–F**) the corresponding hydrodynamic sizes, respectively. Arrows indicate PIB-MPs.

of the gut, with the accumulation being more pronounced in the fore- and midgut (Fig. 6A). We validated the accumulation by spectrophotometrically quantifying PIB-MPs filtered out from digested guts (Fig. 6B).

It has been reported that ingestion of different micro- and nanoparticles leads to various injuries in the intestine, such as direct physical damage, increased intestinal permeability, and increased local inflammatory response[20]. First, we asked whether the accumulation of PIB-MPs in the gut induces histopathology of intestinal tissues. Hematoxylin and Eosin (H & E) staining of the gut revealed histopathological changes such as mucosal damage and vacuolation in the treatment group (Fig. 6C). Second, we asked whether ingestion of different PIB-MPs increases local inflammatory response. Confocal microscopy revealed increased expression of potent proinflammatory cytokines, Tumor necrosis factor alpha (TNF-α) in the gut and tail areas of zebrafish larvae that had been exposed to PIB-MPs after 96 h post fertilization (hpf) when compared to the control group (Supplementary Fig. 5). Together, our results suggest that exposure to PIB-MP induces intestinal damage in zebrafish, which may explain the behavioral phenotypes observed.
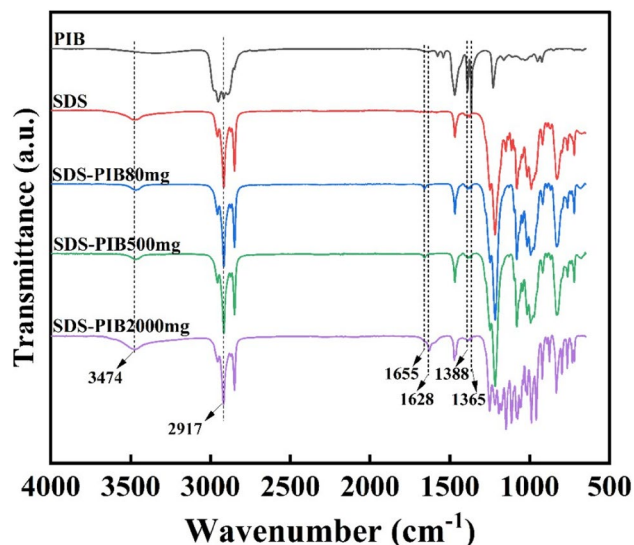
Fig. 3. FTIR spectra of prepared suspension after evaporation of chloroform show that PIB-MPs have notable peaks at 1366 and 1388 wavenumber (cm^{-1}). These peaks are similar to the peak of PIB-bulk material which are not found in SDS.
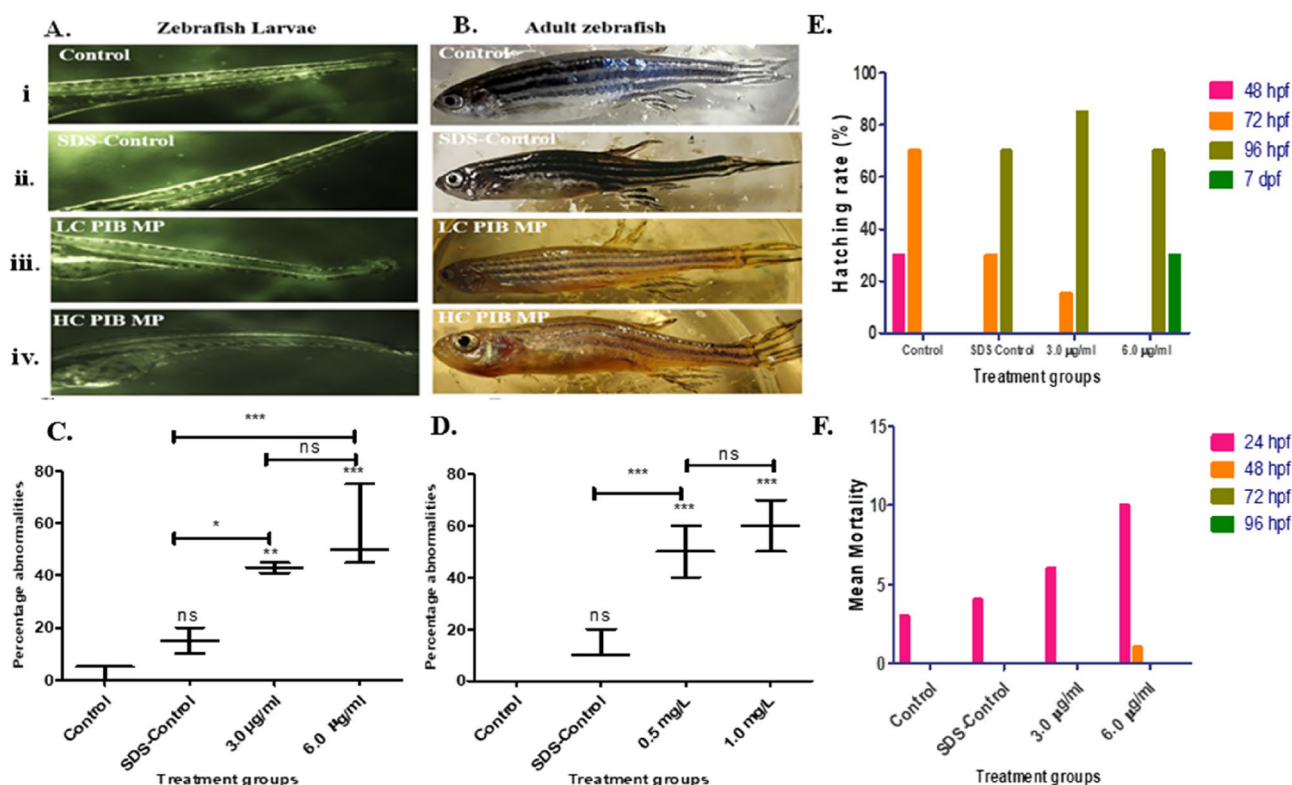
Fig. 4. Developmental toxicity test shows PIB-MP induced hatching rate, increased mortality, and induced developmental defects in zebrafish after 7 dpf. (**Ai** and **Bi**) Control larvae and adult zebrafish showing normal curvature of the spine. (**Aii** and **Bii**) SDS-Control images of larvae and adult zebrafish showing normal curvature of the spine. (**Aiii** and **Biii**) LC-PIB-MP treated groups showed abnormal curvature of the spines in both larvae and adult zebrafish. (**Aiv** and **Biv**) HC PIB-MP treated groups showed abnormal curvature of the spines in both larvae and adult zebrafish. (**C** and **D**) Percentage abnormalities of larvae and adult zebrafish exposed to various concentrations of PIB-MP. (**E**) The hatching rate (%) at 48–7 dpf of zebrafish treated with HC PIB-MP (6.0 μg/ml) shows delayed hatching compared to the control. They hatched completely after 7 dpf when left in the media containing HC PIB-MP (**F**) The mean mortality rate increases in the zebrafish treated with PIB-MP compared to the controls. The asterisks indicate significant differences from the control group (determined by Dunnett post hoc comparison, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n = 10; N = 3). Error bars represent the standard deviation; Scale bar: 100 μm, n is the number of fish per group while N is the number of replicates.
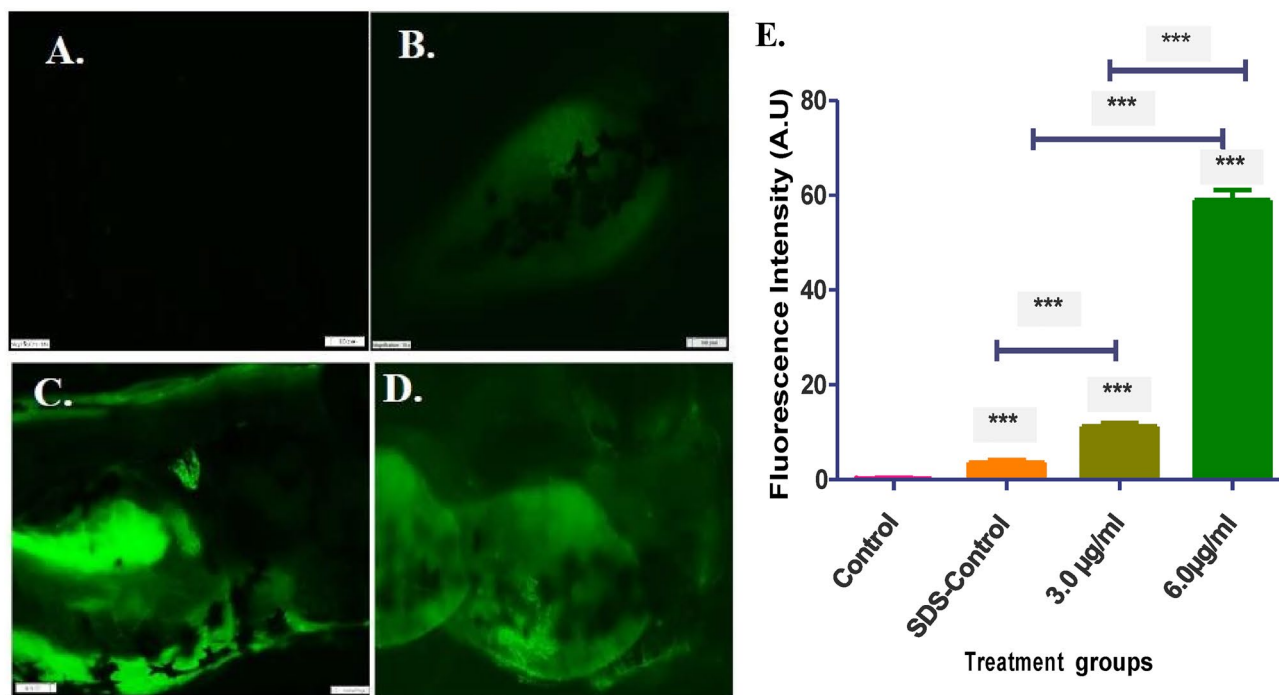
Fig. 5. Zebrafish larvae exposed to various concentrations of PIB-MP for 7 days. (**A**) Control group (without exposure to either fluorescently tagged SDS or PIB-MPs) (**B**) SDS-Control group (exposed to fluorescently tagged SDS after 7 dpf). (**C**) Treatment group (exposed to LC (3.0 µg/ml) of fluorescently tagged PIB-MPs). (**D**) Treatment group (exposed to (6.0 µg/ml) HC of fluorescently tagged PIB-MPs). Both the groups treated with fluorescent PIB-MPs show the accumulation of the PIB-MPs in the gut regions of the zebrafish larvae. (**E**) Quantitative analysis of the accumulation of the fluorescently tagged PIB-MPs and SDS in the gut regions of zebrafish larvae compared with the controls. The asterisks indicate significant differences from the control group (***$p < 0.001$). (n = 10; N = 3); Scale bar: 100 µm.

Exposure to polyisobutylene microplastics culminates in oxidative stress and apoptosis

We hypothesized that the organ damage observed is a consequence of oxidative stress caused by the MPs. Studies indicate that MPs can perturb mitochondrial integrity and dynamics that eventually lead to the generation of different types of reactive free radicals, which induce DNA damage, and compromise the antioxidant defense pool[21]. Therefore, we measured the intracellular reactive oxygen species (ROS), and changes in mRNA levels of genes encoding antioxidant proteins. In larvae from the treatment group, we observed, as evidenced by 2′,7′-dichlorofluorescein diacetate (DCFDA) staining, increased production of ROS, and, using quantitative reverse transcription PCR (RT-qPCR), robust changes in the expression of *glutathione reductase* (*gsr*); *glutathione S-transferase pi 1.2* (*gstp1.2*); *superoxide dismutase 1, soluble* (*sod1*); and *superoxide dismutase 2, mitochondrial* (*sod2*) (Fig. 7). Prolonged oxidative stress by ROS can cause apoptosis in the cells, and, indeed, apoptotic cells were observed in the head region of the fishes exposed to PIB-MPs (Supplementary Fig. 6).

Finally, we conclude that exposure to PIB-MPs induces oxidative damage and apoptosis, suggestive of impaired mitochondrial function.

Discussion

Plastic pollution is a worldwide environmental issue with growing health concerns. PIB is known as a non-toxic, and non-aggressive substance[4], however, its degradation into pieces of tiny or small particles (nano or micro sizes) has not been studied. In order to break or suspend plastic polymers, it is frequently necessary to combine various polymer species with ionic and/or non-ionic surfactants as well as additional dispersion chemicals. Surfactants such as Sodium dodecyl sulfate (SDS) help to reduce surface tension and stabilize suspensions[14]. Due to its wide applications in varieties of products, the biological impacts of PIB degradation are required. According to CIR[22], no data were available on the absorption, distribution, metabolism, or excretion of PIB. In this study, we synthesized and characterized micro size PIB and determined its toxicological effects on the development of zebrafish (*Danio rerio*). Zebrafish offers many advantages as a research model, including rapid development, optical transparency, a large number of offspring, and an excellent vertebrate model for toxicological research. Experiments carried out on the hairless skin of mice found no evidence of tumor promotion after exposure to PIB post-treatment with 7,12-dimethylbenz(α)anthracene (DMBA), an immunosuppressor and a powerful organ-specific laboratory carcinogen[5]. In analogous studies testing for acute oral toxicity, different blends of hydrogenated PIB were shown to be harmless, in mice, at doses up to 89.608 g/kg, and, in rats, at doses of 5.0 g/kg, respectively[22,23]. Moreover, when tested for irritant and corrosive effects, different mixtures of hydrogenated
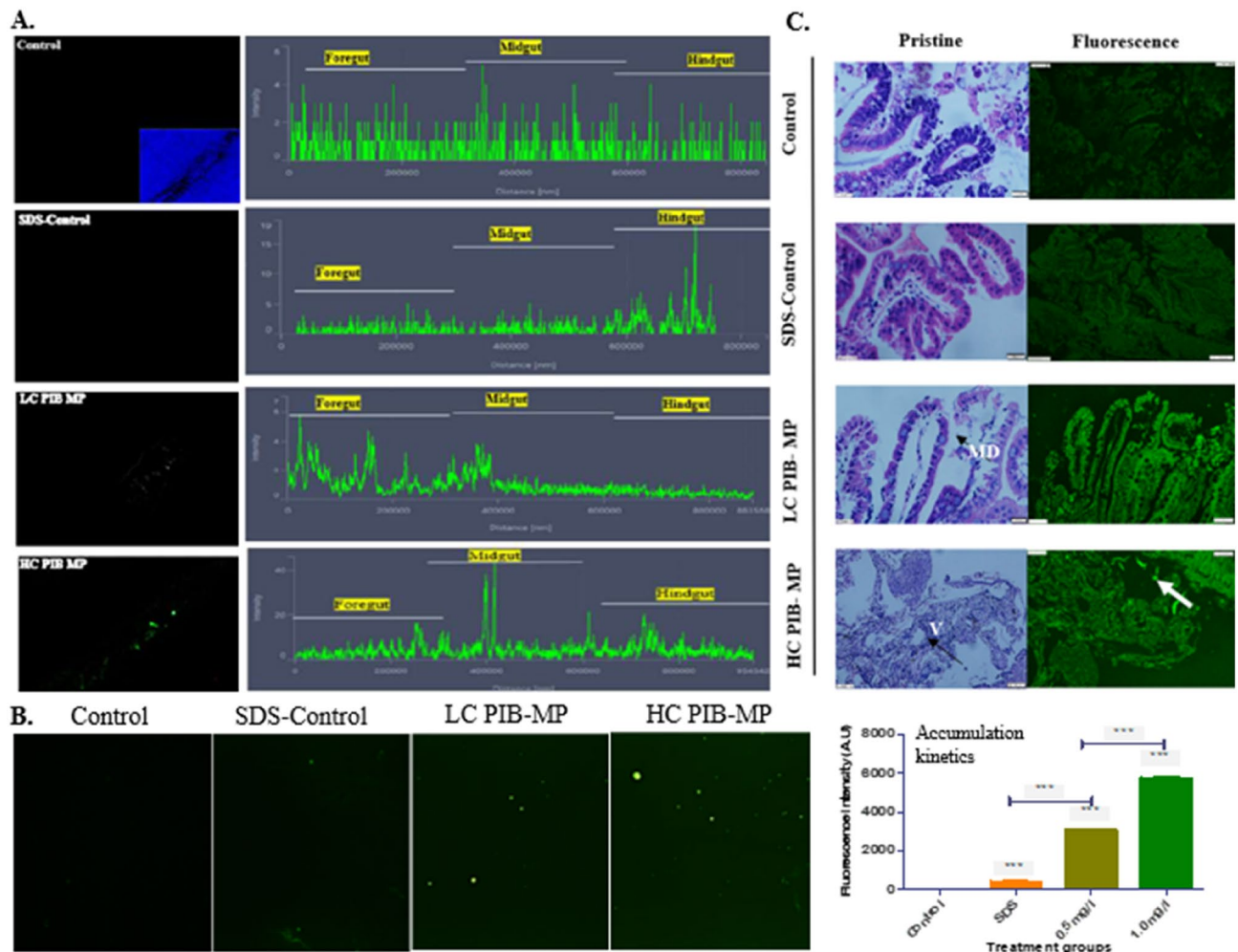
Fig. 6. (**A**) Accumulation of fluorescent PIB-MPs in the gastrointestinal tract of adult zebrafish. PIB-MPs accumulated more in the midgut than the proximal (foregut) and distal intestines (hindgut) in the highest concentration. (**B**) Gastrointestinal tract digested with KOH showed the size distribution of PIB-MPs after exposure to adult zebrafish. The asterisks indicate significant differences from the control group (***$p < 0.001$). (**C**) Representative images of the gut sections with H&E staining at 10× magnification. Histological changes including vacuolization (V) and mucosal damage (MD) are marked. The green fluorescence indicates the accumulation of fluorescent PIB-MPs in the gut tissue of the adult zebrafish after 21 days (n = 5; N = 3). LC PIB-MP (0.5 mg/l); HC PIB-MP (1.0 mg/l).

PIB exhibited no eye inflammation in either cleaned or unwashed rabbit eyes, nor any evidence of corneal or iris damage[22].

These laboratory findings, however, are in contrast to the observations of the ecotoxicological effects of PIB. Ships that carry PIB may 'flush', or wash, their tanks at sea, and PIB, upon coming into contact with water, forms a waxy, glue-like substance right below the surface. This is extremely hazardous to sea birds, and over two decades ago, more than a thousand seabirds in the North Sea were reported to have been demobilized and killed by sticking to PIB[4]. Since then, and many bird deaths later, in 2013, the International Maritime Organization (IMO) announced an agreement to classify high-viscosity PIB [Poly(4+)isobutylene (molecular weight > 224)] as Category X for carriage by ships, prohibiting the discharge of cargo residues into the sea.

Although MPs may be ingested by aquatic organisms and accumulate in their bodies, there is scant evidence about the possible hazardous impacts they might have. It is known that the absorption of plastic particles of micro- and nano-sizes via the gastrointestinal system can take place in a matter of hours[24]. Unlike the pristine PIB, our mode of synthesis, by the solvent evaporation process, produces micro-sized PIB, which facilitates their easy ingestion, and subsequent assimilation, through mixing with the fish meal. Our findings reveal that, in vivo, PIB-MPs can result in developmental defects, and altered behavior, and cause tissue damage within the digestive tracts of fish by inducing oxidative stress and disrupting the immune system. The results from our investigations are in line with the findings from other marine fauna and teleost species, hinting that the cellular processes downstream micro- and nanoparticles are conserved[25–29]. For example, our observation that PIB-MPs induce activation of the immune response has implications for human health: elevated levels of TNF in the intestines might contribute to colorectal cancer (CRC) and colitis-associated CRC development[30].
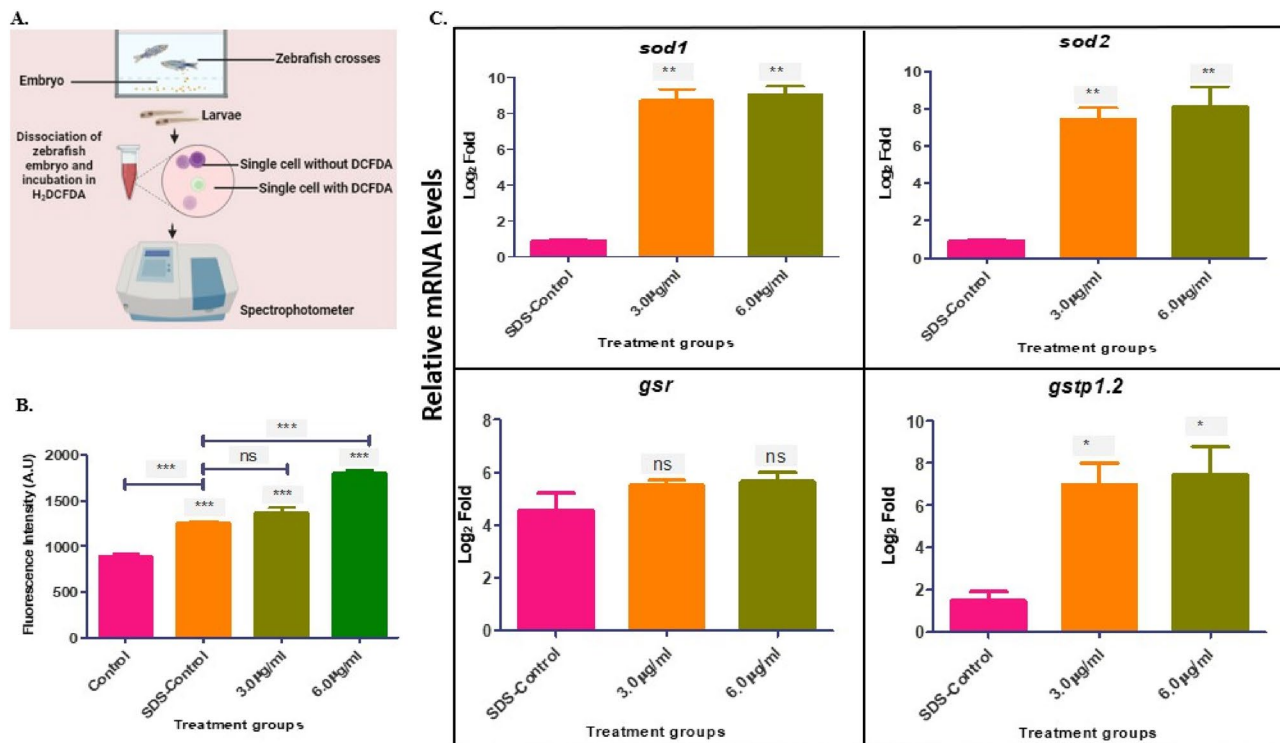
Fig. 7. Single-cell ROS detection method and increased expressions of the antioxidant genes (*sod1, sod2, gsr,* and *gstp1.2*) in zebrafish exposed to various concentrations of PIB-MPs. (**A**) Graphical summary of single-cell ROS detection method. (**B**) Quantification of H$_2$DCFDA intensity. (**C**) Expression of different antioxidant genes in fish exposed to various concentrations of PIB-MPs. The asterisks indicate significant differences from the control group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; (n = 30; N = 3). β-actin (control).

During the behavior tests including the novel tank and the social interaction in this study, distinct patterns of locomotor activity and exploratory behavior were noticed between the control group and the groups that were exposed to different concentrations of PIB-MPs.. The reduced locomotor activity found in this study may reflect studies from mice that showed ROS overproduction generated by polystyrene MPs disrupts the regeneration of skeletal muscles by directing the fate of satellite cells[31]. On the other hand, given how MPs have been shown to induce gut microbiota dysbiosis[32], and the gut microbiome has been implicated in neurological disorders[33], we hypothesize that an alteration in the gut microbiota potentially affects the functioning of the brain, leading to the anxiety-like behavioral repertoire. According to literature, there is strong evidence that gut and brain are bidirectional. This bidirectional relationship is often influenced by multiple pathways, such as the enteric nervous system (ENS), immune pathways, autonomic nervous system (ANS), neural pathways etc.[34].

Although previous studies on PIB have shown that it is stable, the thermogravimetric analysis (TGA) results in our study revealed that PIB can become unstable in the presence of detergents/surfactants. The degradation of PIB began at around 400 °C and reached 100% degradation above that temperature. SDS, employed to stabilize the synthesized PIB-MPs, decomposes only at around 300 °C. The weight loss of PIB-MPs (A, B, and C) produced with SDS began at below 40 °C and reached 100% degradation at 300–450 °C (Supplementary Fig. 7). Therefore, this could be one of the reasons for its stability in the environment[11] resulting in toxicity. As a result, more research is needed to understand the ambient concentrations present around the world, as well as their half-life, fate, and bioavailability through diverse media (soil, food, water, and so on). In addition, future research should compare the physical and chemical properties of the lab-prepared PIB emulsion to those found in the environment, as well as the degradation of consumer/commercial products.

Materials and methods
Synthesis of polyisobutylene microplastic
PIB-MPs were synthesized using the solvent evaporation method as described by Dastbaz and Ashrafizadeh[14] with slight modification. 80 mg of solid chunks of PIB (Santa Cruz Biotechnology, catalog no.: sc-255434) was added to 20 ml of chloroform (CHCl$_3$), and, in a different beaker, 2 g of sodium dodecyl sulfate (SDS) was added to 40 ml of double-distilled water (ddH$_2$O). The mixture of PIB and CHCl$_3$ was stirred intermittently every 10 min for 2 h for proper dissolution. The mixture was poured into the SDS solution, which separated into two phases: the lower contained SDS and ddH$_2$O, while the upper phase contained PIB and CHCl$_3$. After being sonicated for 30 min, the mixture assumed a colloidal form. The beaker was then covered with perforated aluminium foil (to allow CHCl$_3$ to evaporate), and placed on a magnetic stirrer overnight. The mixture was transferred to a glass Petri dish and left in the oven overnight at 61.2 °C (the temperature at which CHCl$_3$

evaporates). The pristine, dry PIB-MP was collected and placed in a glass container for further analysis (Supplementary Fig. 1). The fluorescently tagged PIB-MP was synthesized with slight modification. About 2 mg of fluorescent dye (Fluorescein isothiocyanate (FITC)) was added to the SDS solution, forming two phases: the lower phase contained a solution of SDS and FITC while the upper phase contained PIB and $CHCl_3$ solution.

Characterization of polyisobutylene microplastics
PIB-MP synthesized was characterized using the following methods. The shape and structure of the PIB-MPs were determined using a field emission scanning electron microscope (Zeiss Gemini Ultra55) operated at 5 kV.

The chemical nature of the synthesized dry, powdered samples of PIB-MPs was analyzed by FTIR spectroscopy to determine the functional groups, performed in Attenuated Total Reflection (ATR) mode in the PerkinElmer Spectrum One system. The transmittance of the samples was recorded at 32 scans/sample over the range of 650–4000 wavenumber (cm^{-1}).

For measurements under the LabRAM HR Evolution confocal Raman microscope, 5 µg PIB-MP was inserted into the silicon wafer. A 785 nm laser was used to ignite the sample, and a spectral resolution of 1 cm^{-1} was used to gather the spectrum between 500 and 3500 cm^{-1}. To lessen the impact of local errors, at least three points were measured.

The zeta potential and hydrodynamic size of the PIB-MPs were measured using the Malvern Zetasizer (version 7.13).

The fluorescent particles synthesized were dispersed in a cleaned glass slide and imaged using a confocal microscope (Zeiss LSM 510 META), and all images were processed using ImageJ-win64 software (version 1.52p) (Supplementary Fig. 2).

Zebrafish husbandry
This study is part of Project No. CAF/Ethics/684/2019 approved by the Institutional Animal Ethics Committee (IAEC) of the Indian Institute of Science, Bangalore, India before execution. All methods were performed in accordance with the OECD Test Guidelines (TGs) for testing chemicals. Healthy adult zebrafish (about 6 months old and 0.30 ± 0.022 g in wet weight) were maintained in glass aquaria of 30 l capacity, in continuously aerated water, containing 2 mg/l instant ocean salt, at 28 °C under a 14/10 h light/dark photoperiod cycle, at the Zebrafish Facility of the Developmental and Biomedical Genetics Laboratory (DBGL), Indian Institute of Science, Bangalore, India. Fish food (Tetra, Melle, Germany) and live *Artemia nauplii* (INVE Aquaculture, Thailand) were provided to the fish twice a day, in the morning and evening. Water quality metrics, such as pH, dissolved oxygen, and total hardness, were monitored and kept at ideal levels in aquaria using industry-standard procedures.

Treatment design for the exposure to polyisobutylene microplastics
The experimental design for this study is shown in Supplementary Fig. 3. The concentrations selected for the present study were determined by probit analysis. Zebrafish embryos (n = 10) at 6 hpf were cultured in a medium containing different concentrations of PIB-MP [3.0 µg/ml ($\sim 3.0 \times 10^1$ particles/ml) representing lower concentration (LC), and 6.0 µg/ml ($\sim 6.0 \times 10^1$ particles/ml) representing high concentration (HC)] for 7 days to ascertain if exposure to PIB-MP induced developmental toxicity in a six (6) well plates. Next, adult zebrafish (n = 5) were treated with PIB-MPs. Briefly, they were randomly distributed between 30 l glass tanks, each of the four (4) tanks housing five fish. Prior to the treatment, they were starved for 24 h. The adult zebrafish were fed with either pristine or fluorescently tagged PIB-MP [0.5 mg/l ($\sim 2.7 \times 10^2$ particles/l) representing LC and 1 mg/l ($\sim 5.4 \times 10^2$ particles/l) representing HC], which were mixed with their feeds for 21 days in 30 l tanks. About 18.6 µg/ml SDS control and a standard control with no microplastics ($1 \times E3$ media) were used as the two controls. The test solution in each tank was refreshed every 48 h. During the experiment, the tanks were continuously aerated to maintain the dispersion of the particles in water. Three replicate tanks were used for each sample. It should be noted that the adult fish selected for this study were firstly acclimatised for a week and fasted for 24 h beforeallocated to the control and microplastics treatment groups for 21 days exposure under semi-static conditions. Prior to dissection, the fish were not fed on the last day for proper digestion (if possible) to avoid false negative results[32] and each fish were rinsed thoroughly to remove particles from the skin, and then the intestines of each replicate were rinsed and pooled in the case of the gut for the uptake and accumulation experiments.

Developmental toxicity test
A stereomicroscope (Olympus SZ51) was used to examine the exposed zebrafish embryos, once every 24 h, for the first 96 hpf to check for evidence of morphological defects, hatching periods, and mortality[11,35–37].

Histopathology of the gastrointestinal tract
Prior to dissection, the fish were not fed on the last day, and each fish was rinsed thoroughly to remove superficial particles from the skin. The intestines were sampled on the 21st day post-treatment, and five samples from each group were processed, along with 5 histological sections prepared from each sample. After the removal of the guts, the guts were rinsed in phosphate-buffered saline (PBS, pH 7.4) and digested in 10% potassium hydroxide (KOH) for 14 days. The excised tissues gastrointestinal tract (GIT) fixed in 10% formalin for 24 h were dehydrated in ethanol gradient, cleared in xylene, and then embedded in paraffin, as previously described[32,38,39]. 4–5 µm-thickness sections were prepared using a rotary microtome (Leica RM), stained with hematoxylin and eosin (H&E), mounted on a glass slide, and imaged using a fluorescence microscope (Olympus BX51). All figures were processed using ImageJ-win64 software (version 1.52p). All experiments were done in triplicate.

Behavioral test

To examine the swimming prowess of zebrafish larvae (7 dpf), a swimming assay plate was developed using a plastic Petri dish (100 mm diameter × 15 mm high). In the plate, a circular paper marked with concentric circles, with radii 0.5 cm apart, was placed at the bottom. The dish was filled with 1 × E3 medium. A swimming assay was carried out using 10 larvae; they were released one by one at the central circle using a micropipette, and made to move to the edge of the plate naturally, or by touching with a single-hair paintbrush (maximum thrice) to provoke an escape response from the center. All the swimming experiments were recorded using a standard camera (SM-A730F/DS) for further analysis. The total distance traveled by larvae, along with the time taken to achieve the task, was quantified.

For the adult zebrafish, novel tank and social interaction tests were carried out as previously described[40]. In these assays, untreated adult zebrafish and PIB-MP-exposed fishes were placed in a 30-L experiment tank filled with 1-L of water. The standard camera (SM-A730F/DS) on standby was used for video recording in all the tests mentioned above, and, later, the videos were processed for fish movement tracking and activity analysis based on methods previously described[41,42].

Single-cell ROS detection

Single-cell ROS detection was done using a standard protocol with slight modifications[11,43]. Embryos were cultured for 96 h in different concentrations of PIB-MP.

Ten larvae were taken from each group and rinsed three times with cold PBS. The concentration of DCFDA (Sigma-Aldrich, catalog no.: D6883) for each well was 6.5 mg/ml. The plates were incubated for 30 min at 37 °C, and the fluorescence intensity was measured with a spectrophotometer (Tecan Infinite 200 PRO) and a flow cytometer (BD Accuri C6).

Gene expression analysis

RT-qPCR experiments were performed using cDNA prepared from zebrafish tissues. Amplifications were carried out with gene-specific primers (Table S1) using DyNAMo HS SYBR Green qPCR kit (Thermo Scientific, catalog no.: F410L) following the manufacturer's protocol. Fluorescence intensities were recorded and analyzed in an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The relative changes in gene expression level were calculated and compared with the expression level of a housekeeping gene, *actin, beta 1* (*actb1*).

Cell death analysis

Modifying the standard procedure[44], after 96 h of exposure to the water samples, 10 zebrafish larvae were selected from the well plate and rinsed thrice with 1 × E3 medium for 5 min before being incubated in 2 mg/ml Acridine Orange dye (Sigma-Aldrich, catalog no.: A6014) solution for 20 min at room temperature. Before imaging, larvae were sedated for 1 min over ice, mounted using propyl gallate mounting medium, and imaged using a fluorescence microscope (Olympus BX51). ImageJ-win64 software was utilized to process all figures (version 1.52p).

Immunohistochemistry

About 10 larvae, at 4 dpf, were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight and then transferred to 100% methanol for 24 h at − 20 °C. The larvae were gradually rehydrated with 75%, 50%, and 20% methanol, incubating for 10–15 min at each step, and then washed twice with PBS. The fish were incubated in a blocking buffer of 3% bovine serum albumin (BSA) (HiMedia Labs, catalog no.: MB083) solution made in PDT (1 × PBS, 0.3% Triton-X, 1% dimethyl sulfoxide) for 1.5 h. Depending on the experiments, the following primary antibodies were used: polyclonal antibody to Caspase 3 (CASP3) (Cloud Clone Corp., catalog no.: PAA626Hu01) at 1:150 dilution, and polyclonal antibody to TNF (Cloud Clone Corp., catalog no.: PAA133Hu01) at 1:100 dilution. The primary antibody was then washed thrice with PBST (1 × PBS, 0.3% Triton-X), 15–20 min for each wash, followed by incubation with Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen, catalog no.: A11034) at a concentration of 1:200 in the blocking buffer for 2 h. Cells were counterstained with Hoechst 33258 nuclear stain (Invitrogen, catalog no.: H3569) at a concentration of 1:10,000 for 40 min, and (if required) the skeletal muscles or myomeres were stained with phalloidin, and then washed thrice with PBST, 15–20 min for each wash, before mounting using VECTASHIELD Antifade Mounting Medium (Vector Laboratories, catalog no.: H-1000-10) and imaged with Leica SP8 laser confocal microscope.

Data analysis

GraphPad Prism (version 8.0) and Origin 2021 software were used to generate each graph. Oneway analysis of variance (ANOVA) and Dunnett's multiple comparison posthoc test were used in the statistical analysis of the data to determine if there was a significant difference between the control and treatment groups. At the $*p < 0.05$ threshold, statistical significance was achieved. Each set of data was presented using the mean and standard deviation.

Ethical approval

This project is part of Project No. CAF/Ethics/684/2019 that was approved by the Institutional Animal Ethics Committee (IAEC) before execution.

Consent to participate

All authors have read and approved the final manuscript.

Data availability

All the relevant data are included in the article.

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