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Realistic microplastics harness bacterial presence and promote impairments in early zebrafish embryos: Behavioral, developmental, and transcriptomic approaches.

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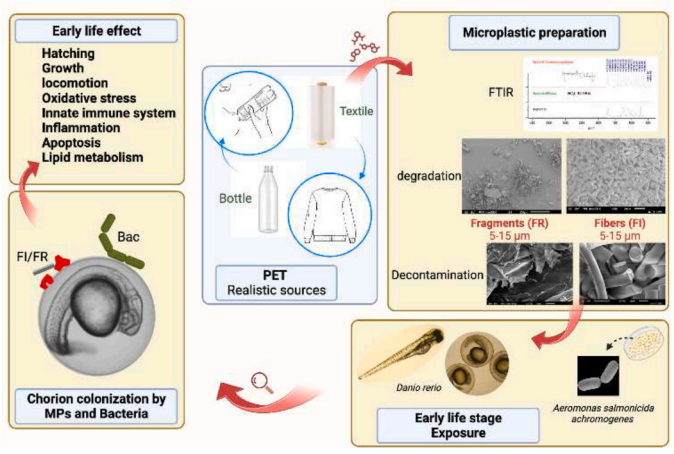
HIGHLIGHTS

- The 24 hpf zebrafish embryonic chorion can block 5–15 μm PET fragments and fibers.
- Bacteria have a high affinity for embryonic chorion.
- Triggered MPs toxicity may stem from bacterial association.
- Complex exposure may disrupt the larval antioxidant system.

GRAPHICAL ABSTRACT



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ABSTRACT

The plastisphere is a newly recognized ecosystem. However, its interaction with early life stages of aquatic vertebrates is a multifaceted issue that requires further research. This study investigated the involvement of bacteria in shaping realistic microplastics hazards in zebrafish *Danio rerio* embryos. Fish were exposed to bottle micro-fragments (FR) and textile micro-fibers (FI) of polyethylene terephthalate (5–15 μm), concomitant with *Aeromonas salmonicida achromogenes* challenge from 2h post-fertilization for 3 days. Egg chorion showed affinity for FR and FI, inducing earlier embryo hatching. However, this effect was masked by biofilm invasion. Fragments were more detrimental than fibers on developmental parameters, while bacterial presence compromised body length, eye, and yolk sac surface area. In a further finding, MPs alone increased locomotor activity in zebrafish larvae, without synergistic effect when combined with bacteria. Data showed that realistic MPs had no significant effects except for downregulated *sod* and *cyp1a* gene expression, whereas bacterial challenge inhibited larval

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potency for most of the evaluated mRNA levels (*mpx* (immune system), *apoeb* (lipid metabolism), *nfkb* and *tfa* (inflammation), *cyp* and *sod* (oxidative stress)). This study provides new insights into realistic microplastic effects under relevant conditions when combined with environmental pathogen within the first life stages of aquatic vertebrates.

1. Introduction

The prevalence of freshwater microplastics (MPs) pollution in the environment has become a major global issue, as they can have significant impacts on aquatic ecosystems and organisms. They can originate from a variety of sources, including the breakdown of larger plastic debris, called primary MPs, and the use of personal care products containing microbeads known as secondary microplastics (Sun et al., 2019). With their size between 0.1 and 1000 μm, low density, and high persistence, MPs can be ingested by aquatic organisms leading to potential bioaccumulation and biomagnification (Wang et al., 2017). They have been detected in fish gut, skin, and gills (Blettler et al., 2019; Zhu et al., 2019; Feng et al., 2019). The ingestion of microplastics by aquatic life can have cascading effects throughout the food chain, as larger predators consume contaminated prey. However, MPs accumulation can occur due to other factors, such as the waterborne exposure, and ingestion of microplastics directly. At the lower end of the size spectrum, microplastics can harbor more toxins, release more harmful by-products, or be host to exogenous pathogens (e.g., amines, phthalates, persistent organic pollutants, heavy metals, bacteria) (Geyer et al., 2017). Thus, MPs can exacerbate the hazard at contaminated sites.

Aquatic organisms can be highly vulnerable to MPs ingestion. Zebrafish are a common model organism used in research due to their transparent embryos, ease of maintenance, high-throughput sequencing, and genetic similarity to humans (Bhagat et al., 2020). They are also susceptible to the harm of microplastics, which can affect their growth, development, behavior, and overall health. As such, zebrafish have become an important model organism for investigating the impacts of microplastics on aquatic organisms. Particularly, investigating the first hours of zebrafish life is scientifically sound given the critical developmental stages during which zebrafish are quite sensitive towards environmental variations (Limonta et al., 2019). Indeed, their small size, transparency during this time frame, and the fact that development occurs entirely outside of the maternal body made them an attractive model to examine developmental processes (Yang et al., 2009; Strähle et al., 2012). Ecologically, this period reflects the real-world scenario where early-stage aquatic life is exposed to microplastics, providing insights into potential ecological risks. As reported, microplastics can interfere with feeding behavior and cause physical damage to tissues, leading to reduced growth rates and increased mortality. A study conducted by De Marco et al. (2022) found that exposure to microplastics caused developmental abnormalities in zebrafish, such as delayed hatching and serious deformities. While polyamide (PA), polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC)-MPs (70 μm) exhibit less mortality in zebrafish, they lead to gut injury (Lei et al., 2018). In a similar finding, exposure to a high dose of polystyrene (PS) (500 μg mL^{-1}) (100 nm - 200 μm) impaired intestinal immune cells in zebrafish (Gu et al., 2020), while mixing with PE (50 μm) led to alterations in the expression of immune-related genes and metabolic pathways in the liver (Limonta et al., 2019).

To provide basic knowledge about the biological effects of microplastics, studies with commercial microplastics with precisely manipulable physicochemical parameters (including size, shape, surface adhesion) are needed to assess the contribution of these parameters. However, those materials exhibit controlled size and surface chemistry, and mostly possess a spherical morphology that differ significantly from their real-life models in many physical and chemical properties. Thus, without precise exposure and material data, current risk assessments may involve unrealistic scenarios and materials that have no industrial or ecological relevance (Guimaraes et al., 2021). How these exposures might relate to the environment is therefore difficult to determine. Nevertheless, as our understanding of micro-effects advances, more environmentally relevant MPs need to be used in laboratories to provide more realistic results in *in vivo* behavioral and toxicological experiments (Abouda et al., 2022; Missawi et al., 2023; Romdhani et al., 2022; Zitouni et al., 2022). Microplastics surface is easily colonized by microorganisms that form biofilms named as "plastisphere" (Xu et al., 2019). Bacteria are commonly the first microorganisms to adhere to MPs in the aquatic compartment, often being associated with characteristic pathological lesions and causing mortality and economic loss (Lobelle and Cunliffe, 2011). Aeromonas spp. are abundant in the aquatic environment among fish pathogens, particularly zebrafish, which have demonstrated vulnerability to these bacteria (Lin et al., 2007; Cornet et al., 2020).

Yet, the full picture of fish response to microplastics associated with infectious agents is poorly understood. Our study fits in this context, assessing the fate and toxicological behavior of microplastics associated with bacteria on the freshwater model zebrafish (*D. rerio*) exposed to PET (polyethylene terephthalate) micro-fragments and micro-fibers, one of the main classes and shapes of polymers found in the aquatic environment, and *Aeromonas salmonicida achromogenes*. Based on the hypothesis that MPs may synergize with other environmental contaminants to generate greater deleterious damage, we intended to unravel the first-life effects of complex environmental pollutants on zebrafish embryos by providing details on the developmental, behavioral, and gene expression responses. The findings of this work are expected to broaden current knowledge about the true magnitude of contaminant effects on freshwater wildlife.

2. Materials and methods

2.1. PET-MPs preparation

The PET plastic items were selected based on two criteria: their shape (fragment and fiber), and their condition (before and after final product manufacturing). For that, plastic bottles and bound plastic fibers were purchased from a local market and a commercial supplier (GoodFellow, US) respectively. Sample polymer types were first confirmed with FTIR spectroscopy, then smaller particles were prepared separately. Briefly, PET bottles were cut into small pieces and dispersed in MilliQ water for grinding with the blender. The solution was sonicated (15 h at 35 kHz) and sieved on pre-set sieves (53-45-15-5 μm). Microplastics retained on the 5 μm sieve were resuspended in MilliQ water and the solution was then filtrated (in a vacuum filtration system on a 47 mm diameter, 5 μm pore size, polycarbonate filter). Finally, the microfragments were collected in a glass bottle and stored at 4 °C. Secondary PET microplastics were produced following the protocol of Von der Esch et al. (2020). However, microplastic fibers were designed following Cole (2016) using the 'cryotome' method and a step-by-step guide, complete with accompanying images is available in the cited reference. Microfibers were prepared using PET-reels of transparent, polyfilament synthetic fiber. Briefly, Fibers were tightly aligned around a custom-made spool, coated with a frozen section medium (glycol based, water-soluble freezing solution (Neg 50™, Richard-Allan Scientific), and then frozen (10 min; −80 °C). The frozen block was cut into ~10 mm lengths and sectioned at predetermined lengths (5 et 15 μm) using a cryotome. Sections were melted in a heated water bath (60 °C for 15 min), Filtered (15 μm and 5 μm) and washed with MilliQ water. Finally, the

microfibers were collected in a glass bottle and stored at 4 °C. The second step was to purify the obtained particles from any possible environmental pathogens, for which four solutions were tested (KOH 10%, KOH 20%, alcoholic (70%) KOH, and H_2O_2 10%) and the one that best retained the original shape was selected. Three replicates of each type of MPs were incubated for 4 h with agitation (125 rpm) at 25 °C. Then, samples were washed 3 times with ultra-pure water to remove any remaining solution, naturally dried and examined under a scanning electron microscope (SEM). MPs size class used in the experiment is 5–15 μm. The stock solutions of MPs-fragments and fibers were diluted to obtain a final concentration of 1000 μg L^{-1}.

2.2. Quality control and quality assurance

The bench was decontaminated by alcohol wiping of the surfaces prior to the process and as needed. Important precautions were taken to avoid cross-contamination: wearing gloves and cotton gowns, covering samples, excluding plastics contact, pre-filtering all solutions (0.45 μm), using blanks (sample-free alcohol), and rinsing equipment with pre-filtered distilled water before each use.

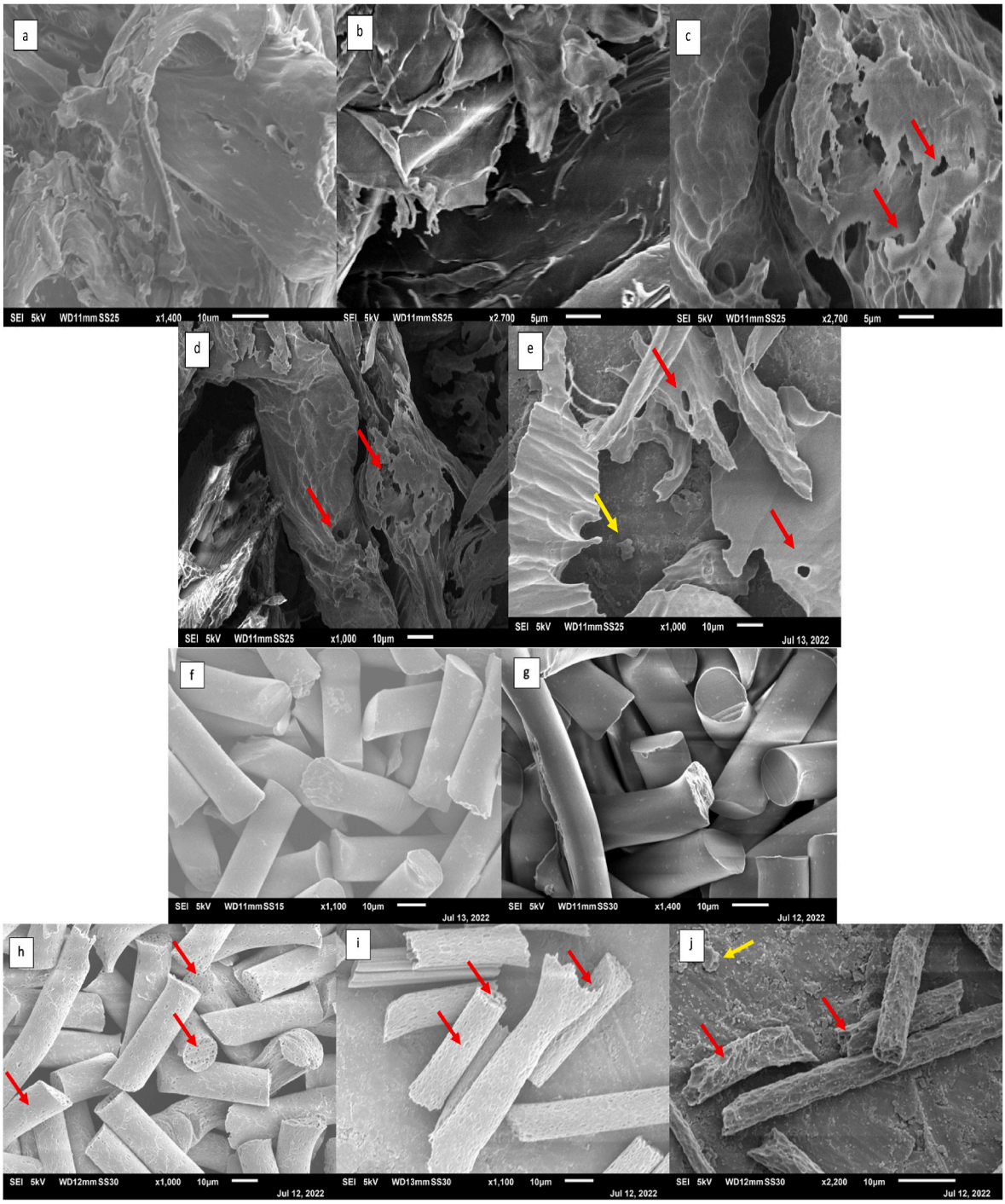

Fig. 1. SEM images of MP-Fragments and MP-Fibers after different treatments. (a,f) Control, (b,g) H_2O_2 30%. (c,h) KOH 10%. (d,i) KOH 20% and (e,j) Alcoholic-KOH. Red arrows show the holes created inside/outside the MPs. Yellow arrows show the fragmentation of MPs into smaller pieces. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Bacterial strain preparation

The bacterial strain *Aeromonas salmonicida achromogenes* used for infection was provided by the Rural Economy Center (CER Groupe, Marloie, Belgium) and was originally isolated from an infected fish showing furunculosis. The bacterial strain was identified by Biolog system and are available in the BCCM collection (Belgian Coordinated Collection of Microorganisms) under the reference number LMG p-31558. The strain was isolated from a single colony in sterile Brain Heart Infusion (BHI) solid medium (Sigma-Aldrich, Saint-Louis, MO, USA) followed by incubation at 26 °C for 18 h (Cornet et al., 2020). Bacteria were cultured until they reached 10^8 CFU mL^{-1} just prior to the experimental challenge.

2.4. Zebrafish husbandry and embryo exposure

All zebrafish husbandry and experimental procedures were performed according Belgian and European animal protection standards.

Wild type (AB circ) zebrafish adults were maintained in a ZebTec housing system (Tecnilab, Netherland) with recirculating reconstituted water maintained at 27 °C, 12:12 h (light/dark) photoperiod, pH 7.2, and 500 μS cm^{-1} of conductivity. They were fed twice daily on pellets (GEMMA Micro 300, Skretting, Netherlands) and once with freshly hatched *Artemia nauplii*. Embryos were obtained according to the standard breeding procedure in a tank designed by Lawrence (2007). Briefly, 6 adults (1:1 male-female ratio) were held overnight in the tank and induced to spawn the following morning when the light was turned on after a dark phase of 12 h. At 1 hpf, embryos were sampled, washed with filtered housing system water, and randomly transferred to Petri dishes, prior to the exposure.

To mimic the environmental scenario, exposure occurred during the first hours of post-fertilization life. During exposure, six groups of embryos were tested (Fig. 1): Control (CT); bacteria-exposed embryos at 10^8 CFU L^{-1} (CTB); MPs fragments-exposed embryos at 1000 μg L^{-1} (FR); MPs fragments-exposed embryos combined with bacteria (FRB); MPs fibers-exposed embryos at 1000 μg L^{-1} (FI); MPs fibers-exposed embryos combined with bacteria (FIB). The exposure solutions for each group were renewed once a day. Embryos and zebrafish larvae were incubated at constant temperature incubator (27 °C and 12 h light/12 h dark) until the end of the study. Zebrafish mortality in each experimental group was recorded daily throughout exposure and dead fish were immediately removed to allow for normal growth of the remaining fish. After exposure (96 hpf), zebrafish larvae were collected and randomly sampled, then stored at −80 °C for the gene expression analysis.

2.5. SEM characterization of zebrafish embryonic chorion

Five embryos from each treated group were examined by SEM (JEOL JSM-7500F) after incubation for 24 h to investigate the permeability of their chorion during the first life hours. Briefly, samples were washed with deionized water from a MilliQ system, incubated for 2 h 30 min in glutaraldehyde 2.5 % and cacodylate 0.1 M (1:1) at 4 °C, then in cacodylate 0.2 M, and immersed for 30 min at 4 °C in a medium of cacodylate 0.1 M and osmium tetroxide 1 % (1:1). Embryos were imbibed in an increasing bath concentration of ethanol (30 %–100 %), dried and dyed with a 0.5 nm gold layer. The embryo's chorion was sectioned with an autoclaved scalpel to facilitate observation of both the inner and outer membrane.

2.6. Gene expression analysis

After 96 hpf, four pools (of 10 larvae each) were used for each treatment. The total RNA was extracted with Tri Reagent solution (Ambion, Thermofisher Scientific) as described by the manufacturer. The pellet was dried and resuspended in 25 μL of RNase-free water. Total RNA concentration was quantified using a NanoDrop-2000 spectrophotometer (Thermo Scientific), and integrity was verified on agarose gels. The genomic DNA was treated with 1 U of rDNAse I (Mobio) for 15 min at 37 °C. Then, 1 μg of total RNA was reverse transcribed using the RevertAid RT kit as per the manufacturer's instructions (Thermofisher Scientific). Genes were selected for their role in the different functions of the innate immune responses: Myeloid specific peroxidase (*mpx*); Inflammatory response: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (*nfkb2*), Transferrin-a (*tfa*); Oxidative stress: Superoxide dismutase 1 (*sod1*), Catalase (*cat*), Cytochrome P450 family 1 subfamily A (*cyp1a*); Metabolism: Apolipoprotein Eb (*apoeb*), Fatty acid synthase (*fasn*), Citrate synthase (*cs*); Apoptosis: B-cell 2 apoptosis regulator (*bcl2*). Two housekeeping genes were used as reference gene: EF1α (*eef1a1*) and Beta actin (*β-actin*). Bestkeeper results showed a coefficient of correlation of 0.975 ($p < 0.001$), SD of 0.5 and 0.97 ($p < 0.001$) SD of 0.58 for *eef1a1* and *b-actin* respectively. The Bestkeeper values suggested the geometric mean. The list of specific primers used for gene expression analysis is described in Table S1. Real-time PCR reactions were carried out with Power UP sybr green mix (Thermofisher) using a 1:50 dilution of the cDNA for target and reference genes. Primers for target and reference genes were used at 625 nM. The thermal conditions used were 3 min at 95 °C of preincubation, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. All reactions were performed using Quantstudio5™ device (Applied Biosystem) and the relative gene expression was calculated according to the standard curve method based on the geometric mean of the housekeeping genes. Values for each sample were expressed as normalized relative expression (NRE), calculated with the formula NRE = Concentration of target gene/concentration of reference gene. The results are expressed as average of values.

2.7. Survival, hatch, and development

Zebrafish mortality in each experimental group was recorded daily throughout exposure. The hatching rate was conducted as an hour-by-hour monitoring during 10 h from 47 hpf to 57 hpf under a stereomicroscope (Nikon SMZ1270). The percentage of hatched eggs in each replicate (n = 5 petri dishes) was calculated. In addition, the morphology was assessed at 48 hpf (for embryos) and 96 hpf (for larvae). A total of 30 embryos per condition were randomly selected to observe the length, the yolk sac, and the eyes area under a compact Olympus inverted microscope CKX53 equipped with a 5 MP 2/3" Sony Exmor CMOS sensor camera (ToupCam Industrial Digital Camera, ToupTek Europe). Then, pictures were analyzed using Danioscope (Version 1.2.206, Noldus Information Technology BV, the Netherlands).

2.8. Heartbeat and behavior

Heartbeat rate was monitored with a compact inverted microscope Olympus CKX53 equipped with a 5 MP 2/3″ Sony Exmor CMOS sensor camera with the software Toupview (ToupTek, China) at 36 frames per second for 20s. Short videos were recorded at 48 and 96 hpf. In each experiment 10 larvae of each condition were filmed on their side to properly see the pericardial cavity and, thus, the heart. Video analysis was performed with the DanioScope software (Version 1.2.206, Noldus Information Technology BV, The Netherlands).

At 96 hpf, 20 larvae per condition were placed in 96-well plates for a swimming behavior assay at 27 °C. The DanioVision video track system (Noldus, The Netherlands) was used to record total distance moved and mean velocity during light-dark transitions (10 min light/10 min dark) across 3 cycles. Data analysis was performed using Ethovision XT 15 software (Noldus Information Technology, USA). Prior to the start of the study, larvae were acclimated for 10 min in the dark in the DanioVision video-track system.

2.9. Statistical analyses

Gene expression, morphology, heartbeat rate, locomotor activity results are presented as boxplots representing the quartile distribution of the data. Statistical analyses were performed using Rstudio software (R version 4.2.2). For each analysis, the effects of the type of MP (control, fibers or fragment), the presence or absence of bacteria, and their interaction were analyzed using a generalized linear model with the following code: glm (Variable ~ Type of MP*Bacterial challenge, family = gaussian, data = Data). Post-hoc comparisons (Tukey's test) at a 5 % significant level were performed on the factor (Type of MP or Bacterial challenge) or their interaction (Type of MP x Bacterial challenge) with General Linear Hypotheses and multiple comparisons for parametric models (multcomp R package) and BH (Benjamin-Hochberg) correction was applied. Significant differences are indicated by different letters.

3. Results

3.1. Microplastics characterization

Result of type and shape analyses of the fragmented MPs are reported in Fig. S1. The chemical compositions of both micro-fragments and micro-fibers were identified as PET (92.95 % and 87.54 % respectively). Fragments were characterized by irregular shapes. Based on the FTIR spectra, only standard peaks of PET were registered, confirming that the chemical composition of the microplastics was not altered during our first step of preparation.

The microplastic samples were soaked separately in four different solutions for decontamination (Fig. 1). This process allows the release of any adherent pathogens to strictly focus on the toxicity of the bacteria tested, while maintaining the original shape and size. No microbial structure was detected with all the tested solutions. Results shown in Fig. 2 reveals that after treatment with H_2O_2 30 % (Fig. 1b and g), both

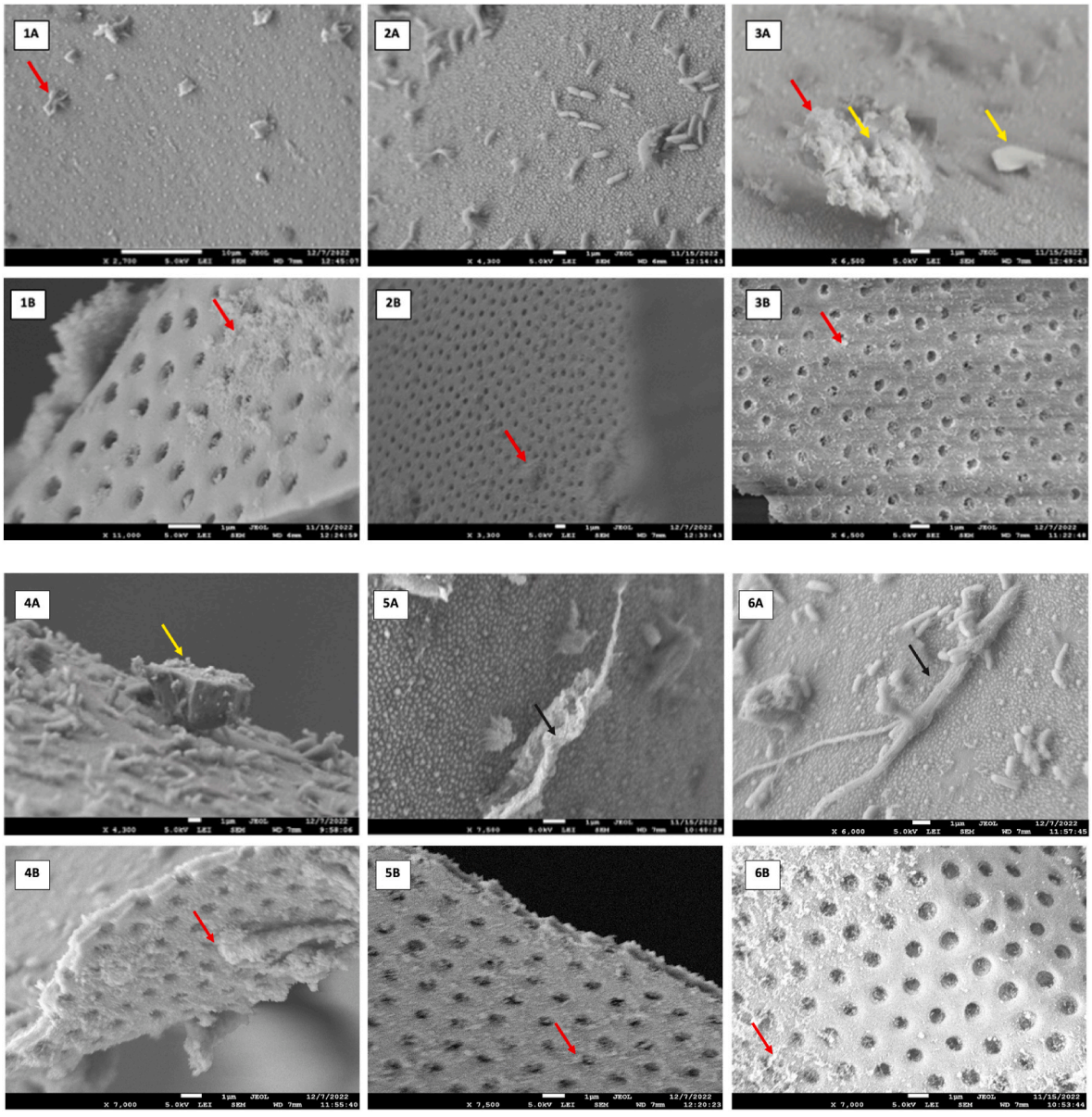

Fig. 2. SEM images of the outside/inside-membrane surface of zebrafish embryo after exposure to MPs for 24 h (1A) Control ext (1B) Control int (2A) Bacteria ext (2B) Bacteria int (3A) Fragment ext (3B) Fragment int. (4A) Fragment + Bacteria ext (4B) Fragment + Bacteria int (5A) Fiber ext (5B) Fiber int (6A) Fiber + Bacteria ext (6B) Fiber + Bacteria int. Red arrows: embryonic organic matter. Yellow arrows: MPs-fragment. Dark arrows: MPs-fibers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fragments and fibers structures were not altered following 4h of incubation, when compared to the control samples. The H_2O_2-treated PET-MPs had smooth surface. However, the other treated PET-MPs were wrinkled. Obviously, KOH 10 % (Fig. 1c and h) KOH 20 % (Fig. 1d and i) and alcoholic-KOH (Fig. 1e and j) were more aggressive and altered the structure of both fragments and fibers. The most corrosive solution was alcoholic KOH, which fragmented the microplastics into smaller pieces, thereby changing the size range of the MP stock sample. Overall, the H_2O_2 decontamination method was used to purify the MP samples prior to the filtration step.

3.2. Microplastics and bacteria embryonic chorion adhesion

The distribution of PET-MPs in the chorion membrane upon 24h exposure of zebrafish embryos to different particle shapes (fragments and fibers) in the presence/absence of bacteria is shown in Fig. 2. There was no evidence of microplastics or bacteria in the embryos of the blank control group. As shown in Fig. 2, microplastics were attached to the surface in all MPs-exposed groups, while no MPs appeared inside the yolk sac. The same observations were evident when exposed to bacteria, which were gathered only on the surface of the chorion membrane and could not penetrate inside the interior of the embryo. In addition, FR-PET and FI-PET were overlaid with bacteria, which can promote biofilm formation and increase their proliferation and attachment to the membrane.

3.3. Concomitant effect of bacterial challenge and microplastic exposure

3.3.1. Modulation in early developing zebrafish and behavioral repertoire

Exposure to PET-MPs harboring or not bacteria had no significant effect on survival. The hatching rate (%) was recorded every hour from 47 to 57 hpf (Fig. 3). The corresponding statistics showed that almost 100 % of the untreated control embryos hatched by 72 hpf. Exposure to fibers (FI) and fragments (FR) resulted in an increase in hatching rate compared to the control, reaching 100 % of eggs hatched earlier (52 hpf and 54 hpf respectively, $p < 0.001$). Exposure to bacteria alone reduced hatching, but the difference was not significant ($p > 0.05$). In contrast, when microplastic exposure alone was compared with that in conjunction with bacteria, the effect was reversed, and the rate of hatching was significantly slowed down ($p < 0.003$). The delay was more pronounced in the FIB-exposed group (fiber + bacteria).

3.3.1.1. Developmental parameters. The average body length, eye size, and yolk sac area are shown in Fig. 4. Treated groups did not exhibit any significant effect at 48 hpf. Significantly lower body, eye, and vitellus size ($p < 0.0001$) were observed in 96 hpf larvae exposed to FB microplastics, comparatively to the untreated larvae. These parameters were slightly lower in FI-exposed larvae (96 hpf) than in the blank control group, but only the vitellus aera decrease was significant. Bacterial challenge significantly shortened the body length and reduced the eye and vitelline surface area of zebrafish larvae. Compared to the single exposure of PET-MPs, the effect on zebrafish larvae exposed to FI/FB and bacteria was highly visible vs control ($p < 0.0001$).

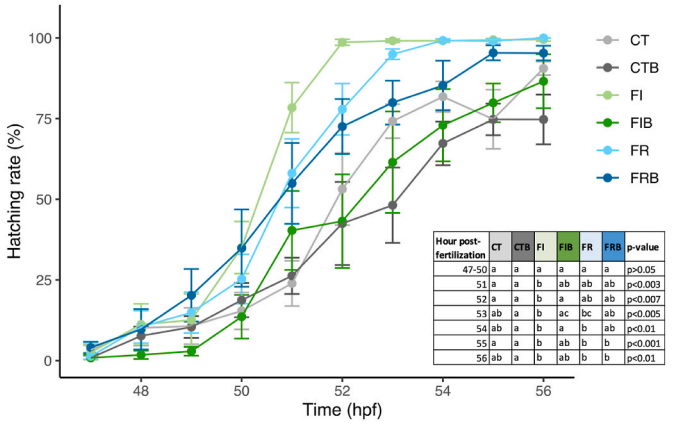

Fig. 3. Hatching rate of zebrafish larvae exposed to microplastics and bacteria, alone or combined. (CT = control, CTB = bacteria, FI = fibers, FIB = fibers + bacteria, FR = fragments, FRB = fragments + bacteria). Data are expressed as mean ± S.E.M. Different letters indicate significant differences between groups, within each time period ($p < 0.05$).

3.3.1.2. Heartbeat rate analysis. The heartbeat rate was recorded at 48 and 96 hpf (Fig. 5). In the CT group, zebrafish larvae registered 148.24 ± 20.87 beats per min (BPM) at 48 hpf, and around 172.68 ± 17.26 BPM at 96 hpf. Compared to untreated group, an increasing trend of the BPM was noticed in all exposed groups. This increase was significant ($p < 0.001$) in zebrafish exposed to bacteria. However, PET-MPs did not alter heart function ($p > 0.05$). At 48 hpf, under the combined exposure of MPs and bacteria, heart rate significantly increased ($p < 0.001$). No difference was registered between the combined and single MPs exposure groups.

3.3.1.3. Behavioral Repertoire: swimming performance. To assess larval swimming performance under several stimuli, the total distance moved was analyzed in zebrafish exposed to PET-MPs and bacteria (Fig. 6). During the white light routine, untreated larvae moved 197.82 ± 29.35 mm per 10 min in the light and 1362.38 ± 609.60 mm per 10 min in the dark. While all treated larvae exhibited normal behavior during the dark period ($p > 0.05$), the total distance moved was significantly increased in FI and FR treated samples compared to the control during the light period ($p < 0.01$). Following tapping test, in the first 6 s of tapping, the total distance moved per second of FI and FR exposed groups was significantly shorter *vs* control group ($p < 0.001$). In addition, with fragment-MPs, the concomitant exposure to the bacteria induced an increase in locomotor activity, while no significant effect of bacterial challenge was reported in FIB stimulation condition.

3.3.2. Expression of target genes

The expression of several genes related to innate immune system (*mpx*); inflammation (*nfkb2, tfa*); oxidative stress (sod1, cat, cyp1a); metabolism (*apoeb, fasn, cs*) and apoptosis (*bcl2*) in zebrafish larvae is presented in Fig. 7. The levels of cyp1a and sod were significantly decreased in larvae of all exposure groups compared to the control ($p < 0.002$), while no significant effect occurred in *bcl2, cat, cs* and *fasn* expression ($p > 0.05$). In contrast, *apoeb, mpx, nfkb2* and *tfa* expressions were significantly downregulated in larvae of bacteria-exposed groups compared to the control, and no significant changes in the expression of the above indicators were observed when combined with fibers or fragments, and in microplastic-exposure groups.

4. Discussion

Microplastic pollution is an emerging issue of global concern as it threatens the aquatic environment. Their toxicological monitoring is carried out mostly with commercial MPs rather than realistic MPs. In this study, two widespread forms of MPs, *i.e.*, fragment and fiber, were used for the synthesis of PET-MPs, considering their environmental relevance. Plastic bottles and textile fibers, which better represent the physicochemical properties of common plastic products, were used to provide more environmental relevance considering their application in daily activities. Additionally, the mechanical degradation protocol mimics the main breakdown route of polymer in nature. The MPs produced might therefore more accurately reproduce the physiological interactions and biological effects of plastic items under ecological conditions. The SEM figures revealed the surface morphology of the pre-prepared FI and FB. The CT-MPs surface appeared smooth, and no

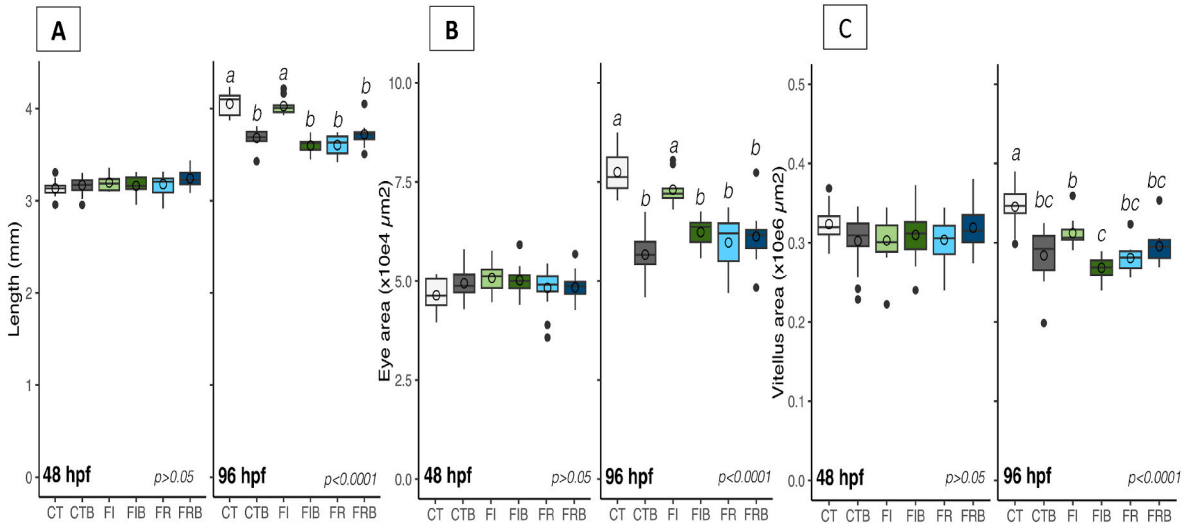

Fig. 4. Boxplot representing the Length (mm), Eye surface (µm2) and Yolk sac surface (µm2) of zebrafish larvae exposed to microplastics and bacteria, alone or combined (from 47 hpf to 56 hpf). (CT = control, CTB = bacteria, FI = fibers, FIB = fibers + bacteria, FR = fragments, FRB = fragments + bacteria). Empty circles represent the mean value for each group and plain circle represent outliers. Different letters indicate significant differences between groups, within each time ($p < 0.05$).

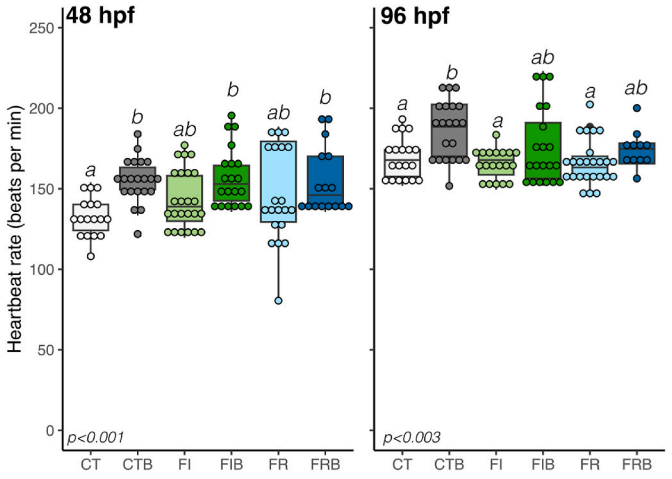

Fig. 5. Boxplot representing the heartbeat at 48 and 96 hpf of zebrafish larvae exposed to microplastics and bacteria, alone or combined. (CT = control, CTB = bacteria, FI = fibers, FIB = fibers + bacteria, FR = fragments, FRB = fragments + bacteria). Circles represent the value for each individual. Different letters indicate significant differences between groups, within each time ($p < 0.05$).

obvious cracks were observed. In contrast, the decontamination step with various KOH solutions enhanced MPs damage, as pore generation and development of cracks and small wrinkles. Hydrogen peroxide appeared to be non-aggressive to both fragments and fibers with a structure similar to untreated MPs, so it was selected for the final step of MPs-stock preparation. Bio-decontamination using these solvents is a low-temperature method that provides an alternative to thermal disinfection, which is often inappropriate, particularly when heat-sensitive materials must be treated. Interestingly, several studies have shown that H_2O_2, environmentally non-toxic agent, has bactericidal properties against gram-positive and gram-negative bacteria (Ríos-Castillo et al., 2017).

Although the increasing research on the behavior of MPs and their hazards, there is a huge gap about a critical hotspot, the "plastisphere" or the microbial communities on plastic litter. Given their small size, rough hydrophobic surfaces, and long half-lives, MPs display a high affinity to microbial colonization. Several taxa are more abundant in biofilms on plastics than free-living bacteria in the surrounding environment (Amaral-Zettler et al., 2020; Dussud et al., 2018). Pathogens, both genera associated with fecal contamination and free-living bacterial genera widely linked to pathogenicity, like *Pseudomonas*, *Vibrio*, and *Aeromonas*, were observed adhered to MPs (Silva et al., 2019; Wu et al., 2019; Kirstein et al., 2016). A recent study by Viršek et al. (2017) showed that among all bacteria species identified in microplastics sampled from the North Adriatic Sea, *Aeromonas salmonicida* represent the highest number of OTUs. However, whether realistic MPs associated with *A. salmonicida* exacerbate the health status of zebrafish has not been reported until now. Therefore, the current work aimed to unveil the effects of realistic MPs, particularly under the circumstance of coexisting with bacteria, during the first hours of zebrafish development, since they are quite susceptible to contaminants in which any alteration in their early stage can trigger significant disorders in their adulthood, as well as at the population level.

Toxicity is based on the effects of toxin accumulation (Huang et al., 2007). SEM investigation and observed membrane morphology revealed that FI-PET, FR-PET (5–15 µm) and bacteria were attached to the external embryonic chorion and were not associated with membrane damage. Consistent with our findings, different types of MPs (>5 µm) were observed on the chorionic surface of zebrafish embryos, potentially owing to the pore size of the chorionic membrane, which varies between ~500 and ~700 nm (Pitt et al., 2018; De Marco et al., 2022; Prata et al., 2022). This adhesion may lead to membrane disassembly, as in the work of Zhang et al. (2020), who reported an irregular membrane layer after exposure to PS-MPs. The same explanation is given for bacterial attachment only to the outer membrane surface, a fact attributed to their larger size (0.8–2 µm) compared to the pores. The zebrafish embryonic chorion is a specialized biostructure that encloses embryos until larval hatching and is also considered a potential barrier to uptake exogenous substances (Duan et al., 2017). Here, FI and FR-MPs may bend the transport function of the membrane at the adsorption site, thereby reducing oxygen supply, leading to stimulated muscle movement, and ultimately resulting in the effects of accelerated hatching rate (Malafaia et al., 2020). The precocious hatching of embryos exposed to PET-MPs, although unexpected, is consistent with existing evidence on fish embryonic development, which is enhanced by occurrence of contaminants and changes in water quality. Based on comparable studies, premature larvae may be due to altered metabolism and/or heavy metal/MPs

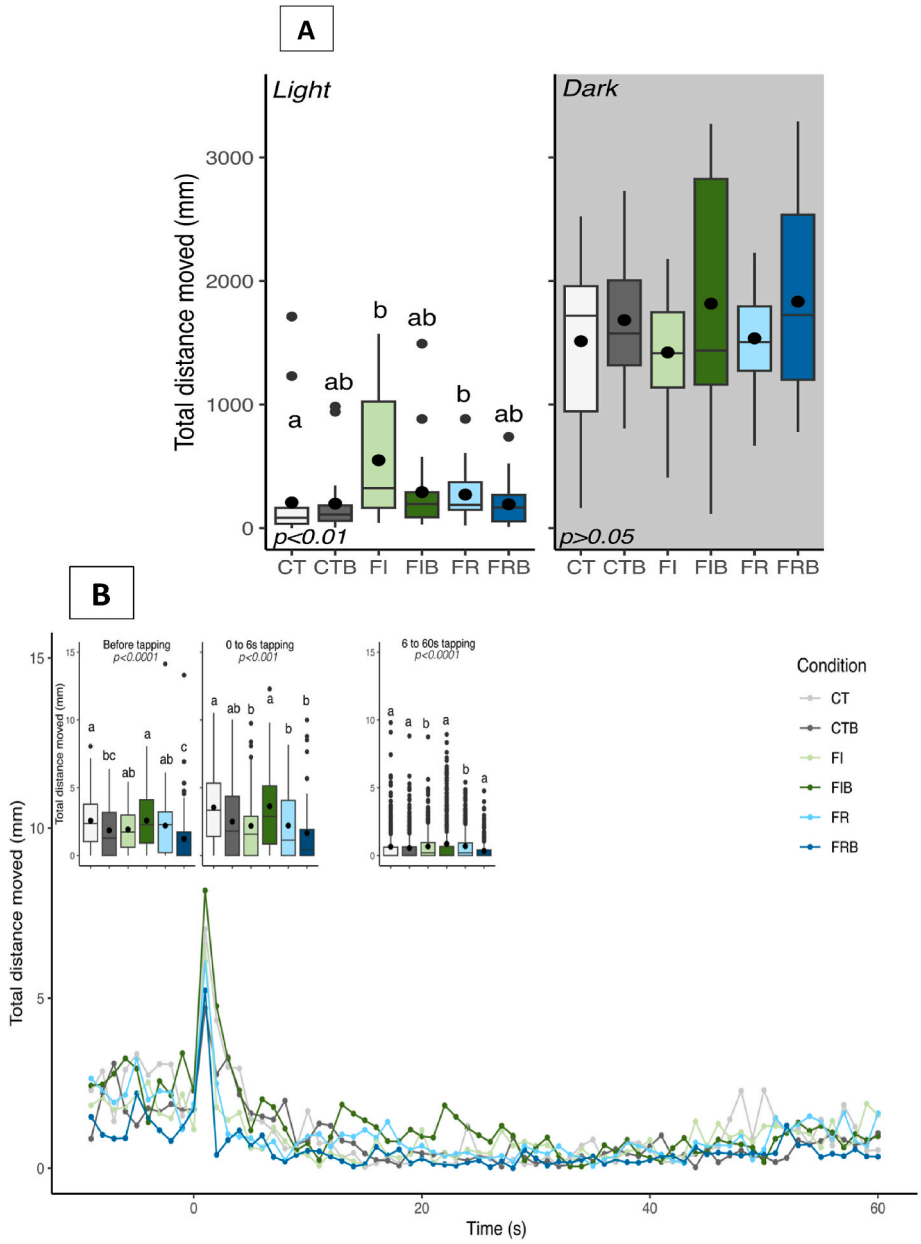

Fig. 6. Boxplot representing locomotor activity of zebrafish exposed to microplastics and bacteria, alone or combined, during white light routine (A), and tapping (B) stimulation. (CT = control, CTB = bacteria, FI = fibers, FIB = fibers + bacteria, FR = fragments, FRB = fragments + bacteria). Different letters indicate significant differences between groups ($p < 0.05$).

induced embryo hypoxia (Jezierska et al., 2009; Malafaia et al., 2020) or chorionic injury (Stouthart et al., 1996). More interestingly, it is noteworthy that the existing literature has reported a reduction in egg hatchability when using commercial MPs (Duan et al., 2020; Zhang et al., 2020; Cheng et al., 2021; De Marco et al., 2022), in contrast to our present work where we adopted realistic MPs. However, no synergistic effect was observed when MPs (FI and FR) were combined with *A. salmonicida*. The hatching rate was significantly delayed compared to the groups exposed to single MPs ($p < 0.003$) and its profile was closer to the control ($p > 0.005$). A possible explanation is that bacteria may hide the effects of MPs when adsorbing to their surface. Additional evidence that may be related to the last hypothesis is the antibacterial properties of amniotic and chorionic membrane layers that were tested to inhibit the growth of 7 bacterial strains: *E. coli, Bacillus cereus, Klebsiella pneumonia, Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus, and Shigella flexneri* (Zare-Bidaki et al., 2017). The limited data provided are not sufficient to fully understand the variation in hatching exposed to realistic MPs and bacteria and require further analysis to establish the underlying mechanisms behind this early life stage impairment.

Effects on prior and post-hatch embryos have been monitored. In the instant case, only bacterial challenge of embryos showed an insignificant enhancement of heartbeat rate at 48 h (alone and combined with MPs) and 96 h (alone). In addition to clogging pores, bacteria may lead to oxygen depletion in the medium, imposing hypoxia on all larvae in the medium. The assessment of growth in toxicological assays is common because it is an easily monitored parameter that reflects critical molecular and cellular responses (Newman, 2009). Our data show that the growth of zebrafish larvae declined with the body length, eye, and vitellus area decreasing. Similar larval length reduction was noted after exposure to ZnO-NPs combined with commercial PS-NPs, and to PS-MPs (Bai et al., 2010; Chen et al., 2017; Rabezanahary et al., 2023).

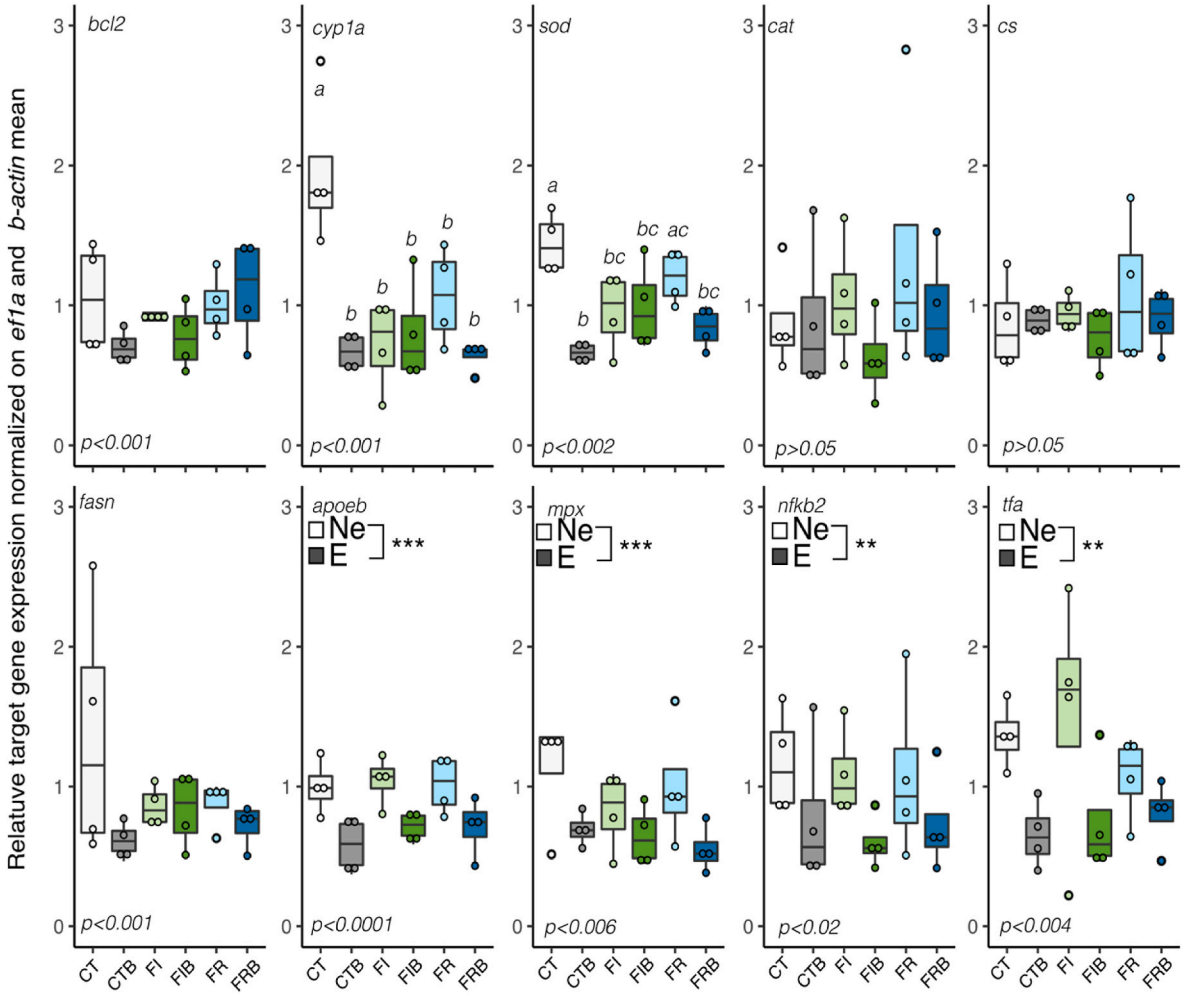

Fig. 7. Expression of genes related to innate immune system (*mpx*); inflammation (*nfkb2*, *tfa*); oxidative stress (*sod1, cat, cyp1a*); metabolism (*apoeb, fasn, cs*); apoptosis (*bcl2*) in zebrafish larvae exposed to microplastics and bacteria, alone or combined. (CT = control, CTB = bacteria, FI = fibers, FIB = fibers + bacteria, FR = fragments, FRB = fragments + bacteria). Different letters indicate significant differences between groups ($P < 0.05$). Significant differences between challenged (E) and unchallenged (NE) groups independently of MPs exposure are indicated by ** ($p < 0.01$) or *** ($p < 0.001$).

However, this impairment was only recorded at 96 hpf (after hatching) and no effect was registered at 48 hpf (before hatching). This is an important finding which may relate to the protective role of the chorion and the perivitelline fluid (PVF). The study by De la Paz et al. (2020) showed that the PVF is a primitive inherited extraembryonic immune system that protects the embryos from external environmental threats before hatching, in which they evidenced the reduction of bacterial virulence by enzymatic modification of their cellular envelope and/or by blocking the action of proteases required to penetrate the embryonic epithelia. Thus, larvae protect themselves by their chorion and PVF before their immune system develops. A decline in growth is typically consistent with a shift of energetic resources toward different physiological functions, such as inflammatory response or hypoxia control (Salerno et al., 2021). Consequently, reduced resources are responsible for slower swimming and impaired behaviors (Jakubowska et al., 2022).

Alterations in locomotor activity are widely used as endpoints of neurotoxicity since they are 10–100 times more sensitive than mortality to the ecotoxicological impacts of pollutants (Frese and Braunbeck, 2023). In the context, our study found that under light stimulus, PET fibers and fragments increased the total distance moved by zebrafish larvae, suggesting that realistic microplastics can cause impaired locomotor activity. This behavior may reflect anxiety in stressed zebrafish. A correlation among both aberrant neurogenesis and altered motor neuron axons and behavioral disorders during larval development has been previously demonstrated in multiple reports, as synaptic activity induced by neurons and motor neurons enhances the development of swimming behavior (Jiang et al., 2018; Fan et al., 2022). Other research has recorded an inhibition of locomotor ability after exposure to PS microbeads (1 and 5 μm) (Qiang and Cheng, 2019; Rabezanahary et al., 2023). However, under tapping stimulus, our data revealed that MPs caused hypoactivity in zebrafish larvae, and the behavioral effects caused by PET microfibers were more pronounced. A similar observation was also noted by Chen et al. (2017), who suggested that the depletion of AChE activity and oxidative stress were responsible for the decreased activity. Reduced swimming activity in zebrafish leads to lower feeding activity, consequently reducing their growth and predator avoidance potential, and hence their adaptability to natural habitats (Mattsson et al., 2015). Thus, further analysis on early life stage zebrafish is required to explore the involved underlying mechanisms, so we extended our study to investigate the molecular underpinnings of realistic MPs exposure and bacterial challenge.

The innate immunity, effective within the first few days post-fertilization, is the primary protective barrier for larvae facing environmental threats such as microplastics and pathogen infection, whereas the adaptive immunity development is initiated not earlier than 2 weeks post-fertilization (Ordas et al., 2011). In early-infected larvae, bacterial challenge had only a significant reduction in the level of the neutrophil marker *mpx*, which plays a crucial role in neutrophil

microbicidal activity. As such, we cannot exclude that their reduced mRNA level is related to a lower amount of immune cells. Bacterial challenge downregulated innate immune system related gene expression, suggesting that larvae were stressed, and their immune system was repressed. In line with the current data, Aksakal and Ciltas (2019) and Krishnaraj et al. (2016) have previously shown a stress-induced decrease in the expression of this gene in zebrafish exposed to copper oxide and titanium dioxide nanoparticles. However, overproduction within infected larvae may also trigger oxidative stress, which can lead to injury to host tissues (Pattison et al., 2012; Nybo et al., 2019). The decline in *mpx* level was paralleled by a similar downregulation of *nfkb* and *tfa* genes in the infected larvae, pointing to a distinct inflammatory response versus the MPs-exposed groups. The *tfa* gene is crucial for iron transport and iron-regulated hormone expression and is involved in the immune response against bacterial challenge (Fraenkel et al., 2009; Rojo et al., 2007). A marked depletion of the transferrin protein concentration in blood plasma of radiation accident victims has been observed and suggested as a potential mutagenic factor (Nylund et al., 2014). Nfkb regulates genes involved in immunity, antimicrobials, and cytokines (van der Vaart et al., 2012). The inhibition of *nfkb* gene expression has been documented in numerous studies proving its efficacy in attenuating iNOS expression and cytotoxic NO generation and in blocking immuno-cell activation (Chuang et al., 1996). Impaired immunity would make individuals more vulnerable to infection-induced mortality (Dhanagovind et al., 2021). Oxidative stress and inflammatory responses are strongly interrelated and frequently evaluated to define distinct disorders. ROS generation may induce oxidative stress, mitochondria impairment, enhanced inflammatory cytokines, and pro-apoptotic factors that could initiate cellular mortality (Banerjee and Shelver, 2021). Superoxide dismutase (SOD) is an antioxidant detoxification enzyme implicated in the metabolism of superoxide anions. In particular, sod 1 (Cu/Zn sod) combines Cu and Zn and localizes to the cytoplasm and intermembrane space of mitochondria (Wang et al., 2018). Here, a down-regulation in mRNA expression of the mentioned gene involved in antioxidant response was reported after bacterial infection alone or associated to MPs. Cytochrome P4501a (*cyp1a*) exhibited similar impairment in all exposed groups, possibly due to stimulation of MPs/pathogen-mediated ROS production. An interesting find in our study is the affected area of the larval yolk sac. Under normal conditions, this area is expected to be fully resorbed by 5 dpf (Martínez et al., 2019). This significant decrease could be explained by the dysregulation of yolk lipid catabolism and transport, causing an increase in yolk sac consumption (Wang et al., 2019). Our study revealed a significant down-regulation of *apoeb* gene levels upon exposure to bacteria. Apoeb is one of the apolipoprotein genes involved in lipid transport. Thus, one major factor responsible for the reduced lipid deposition in zebrafish larvae caused by bacteria is the aberrant function of fatty acid transport. Innate immune system, inflammation, lipid metabolism, and oxidative stress, four intimately linked processes, were among the most affected processes, with a strong downregulation of several genes.

5. Conclusion

The present study applied an innovative approach to compare the differential outcomes associated with two shapes of realistic microplastics upon environmental pathogen challenge within the first few hours of the zebrafish life-stage. The findings indicate that both MPs can induce markedly different impairments, and compared to single exposure, bacterial challenge amplified their potency for most evaluated endpoints. The occurrence of interactive responses among environmentally relevant microplastic and bacterial loads provides evidence that microplastics have minor effects relative to other pollutants, but we believe that further interactive research is required to improve our comprehension of the circumstances under which stronger adverse responses may be exerted. The advantage of the current research is that we pointed out that the use of commercial microplastics may overestimate their hazards, which is highly meaningful in instructing the public to consider the toxic effects of MPs in an informed manner. The use of realistic concentrations should be the major objective to have more environmental relevance. However, due to the scarcity of information on environmental concentrations of small MPs and the ever-increasing production of plastics, higher concentrations are often chosen to simulate higher levels of microplastic pollution in water bodies. These concentrations could potentially be found in some highly contaminated areas where waste management is almost non-existent.

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CRediT authorship contribution statement

Omayma Missawi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Charlotte Wouters:** Data curation, Methodology, Software. **Jérôme Lambert:** Data curation, Methodology. **Mutien-Marie Garigliany:** Formal analysis, Validation, Visualization, Writing – review & editing. **Patrick Kestemont:** Formal analysis, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Valérie Cornet:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.141107.

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