



Environmental microplastics accumulate in gonads in a sex-dependent manner and alter reproductive success in zebrafish (*Danio rerio*)

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ABSTRACT

Microplastics (MPs) are increasingly associated with physiological disruptions in aquatic organisms, yet the biological responses to environmentally sourced particles remain underexplored. This study investigated the reproductive toxicity of environmentally derived MPs collected from the Ikopa River (Antananarivo, Madagascar) in *Danio rerio*. Zebrafish were chronically exposed to cryomilled riverine MPs (1.2–50 µm) at concentrations of 100 and 1000 µg/L for 66 days, with daily reproductive assessments conducted over the final 21 days in accordance with OECD Test Guideline 229. Microplastic accumulation in gonadal tissue was assessed, along with subcellular responses via enzymatic assays in gonads and proteomic profiling in liver samples. Reproductive toxicity was evaluated through gonadal histology, fecundity, and fertility rates. MPs accumulated in gonads in a sex- and concentration dependent manner, with the highest levels in males exposed to 1000 µg/L (177.88 ± 102.65 particles/mg tissue, mean $\pm$ SD, $n = 4$). Despite MPs accumulation, no histopathological lesions were observed. However, significant oxidative stress and energy metabolism disruptions were identified in the liver, suggesting hepatic dysfunction as a potential driver of reproductive impairments. Furthermore, six polychlorinated biphenyl (PCB) congeners ranging from dozens to hundreds of ng/g MPs, and seven polybrominated diphenyl ether (PBDE) congeners in the range of a few ng/g MPs were detected on MPs surfaces, which may exacerbate toxicity via apoptosis inhibition. These findings provide novel mechanistic insights into how environmentally relevant MPs impair reproductive function in fish. The results underscore the necessity of incorporating environmental microplastics into toxicity testing frameworks to ensure accurate ecological risk assessment.

1. Introduction

Microplastics (MPs) are plastic debris with a size ranging from 1 µm to 5 mm (Frias and Nash, 2019). By origin, microplastics can be classified as primary microplastics, designed to have a microscopic size, originate from industrial and commercial applications (Cole et al.,

2011), and secondary microplastics, which result from the weathering process of larger plastics, such as physical abrasion, photo-oxidation by ultraviolet B exposure, or biodegradation by microorganisms (Li et al., 2020). Numerous polymers, shapes, and microplastic particles can be found in the aquatic environment (Kameda et al., 2021). Once released, each particle can be exposed to the abiotic factor in the environments

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(UV light, abrasion) or interacts with other pollutants and biota to form a brand-new toxicant particle. Essentially, size, shape and polymer type play a crucial role in microplastic toxicity. The microplastic size seems to be the main predictor of toxicity (Cui et al., 2024). Studies have shown that smaller microplastic (1–10 μm) are bioavailable and more likely to cross biological barriers, inducing severe oxidative stress and inflammation (Zhou et al., 2023). In addition, it is worth noting that shape might also influence the toxicity of microplastics. Qiao et al. (2019) observed that fibers caused more severe intestinal toxicity compared to fragments and beads, due to their higher accumulation potential. Besides, polymer types also exhibit different levels of toxicity; polystyrene microplastics, with similar size and identical shape, showed a lower No Observed Effect Concentration up to 10-fold lower compared to amino formaldehyde, and 10- to 100-fold lower compared to polyamide when exposed to zebrafish (*Danio rerio*) (Cui et al. 2024).

Apart from the diversity of MPs found in the environment, their characteristics add a layer of complexity to microplastic toxicity assessment. Indeed, some authors have already pointed out the discrepancies between experimental designs and environmental conditions, leading to the lack of representativity in the *in vivo* approach. For example, Burns and Boxall (2018) noticed that despite polyethylene being the most dominant polymer reported in environmental samples, most *in vivo* studies used polystyrene as test material. Besides, inconsistencies have been also found in terms of shapes used in experimental studies. In 2024, Cui et al. by reviewing 116 microplastic toxicity assessment studies, noticed that spheric particles are the commonly used particle shape, even though fragments and fibers are the most dominant particles found in the environment. Bucci et al. (2020) have also asserted that 83 % of the concentration and 80 % of the size of the MPs used *in vivo* experiments are environmentally irrelevant.

Efforts have already been made to address this issue of representativity in experimental studies of MPs by using aged or weathered microplastics as test materials (Missawi et al., 2021, 2023). Importantly, many organisms showed a preference for aged microplastics bearing biofilms over pristine ones (Vroom et al., 2017). Moreover, significant changes in lysozyme, antimicrobial and antiprotease activities were noticed in Pearl danio (*Danio albolineatus*), with male being more affected than female once exposed to 1 $\mu\text{g/L}$ of weathered polyethylene for 40 days (Harikrishnan et al., 2024). Hutton et al. (2024) have also found that the Inland silverside (*Menidia beryllina*) exposed to a cryomilled polyester microfibrils at a concentration of 30 particles/mL displayed neurotoxic responses similar to those found in fish exposed to nanoparticles. In European perch (*Perca fluviatilis*), diet with aged polyethylene MPs (sized 100–180 μm) exhibited more pronounced oxidative stress responses compared to those exposed to virgin MPs (Michailidou et al., 2024). Overall, the aged MPs appear to be more toxic, especially due to the change in surface properties. Furthermore, in the aquatic environment, MPs might leach their additives in water through aging (Jang et al., 2021), or adsorbed pollutants from the water (De Frond et al., 2019), which also contributes to a potentiating effect of their toxicity.

At a sub-organismal level, chronic exposure to MPs induces an excessive oxidative stress, which ultimately leads to inflammation, tissue damage, and functional impairment (Wang et al., 2024). Eventually, those impairments may lead to individual-level effects, such as bioenergetic imbalances (Wang et al., 2024) and/or reproductive toxicity (Junaid et al., 2023). Studies have demonstrated that reproduction capability of freshwater fish seems to be more sensitive to MPs than marine fish (Wang et al., 2024). Understanding and assessing fish reproductive health in response to pollutants is crucial for evaluating long-term ecological risks and ensuring the sustainability of aquatic ecosystems. Data on the reproductive toxicity of environmental MPs remains scarce, and studies have also demonstrated that their toxicity may vary depending on their geographical source (Cormier et al., 2022), which is associated with the local pollutants sorbed on the particles. Furthermore, a recent study has shown that microplastic concentration

increase during the rainy season in tropical regions, which coincides with the reproductive period of most freshwater fish (Rabezanahary et al., 2024). This study aims to assess whether environmental MPs collected from the urban Ikopa River (Antananarivo, Madagascar) translocate into fish gonads and induce oxidative damage, potentially impairing reproductive functions under chronic exposure conditions. Polymer types were determined with micro-Raman spectroscopy, sorbed pollutants and weathered surface were analyzed with Gas Chromatography-Ion Mobility-Mass Spectrometry (GC-IM-MS) and Scanning electron Microscope (SEM). Under controlled conditions, the goal is to highlight driving factor of toxicity of environmental MPs. Accordingly, juvenile zebrafish (*Danio rerio*) were exposed for 68 days, in which the last 23 days were dedicated for reproduction assessment. Enzymatic biomarkers such as Malondialdehyde (MDA) on gonads, along with proteomic analysis and vitellogenin content in the liver were used to assess the sub-organismal response to microplastic exposure. At the individual level, reproductive toxicity was assessed via gonadal histology, fecundity rates, and fertility success.

2. Material and methods

2.1. Environmental plastics sampling and preparation

Environmental plastic particles were collected using a plankton net (500 μm mesh size) at depths ranging from 0 to 50 cm, during the dry season (October 2023) and the rainy season (February 2024), from Ikopa River at the downstream of the city of Antananarivo, Madagascar. After each sampling campaign, microplastics (from 500 μm to 5 mm) and mesoplastics (greater than 5 mm) from the collecting bucket were manually picked with forceps and stored in a glass jar at -20°C waiting for the cryomilling. Each particle was weighed, photographed and examined under Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR, Spectrum Two™, Perkin Elmer) with a spectral range 4000–600 cm^{-1} , resolution: 4 cm^{-1} and 1 accumulation to determine the polymer types. Thereafter, spectra were analyzed with BioRad KnowItAll® Informatics System Raman ID Expert (2025) software. Particle surfaces were also examined under a SEM (JEOL JSM-6010LV). (Information about collected micro- and mesoplastics are detailed in the Fig. S1).

For the exposure, a stock of MPs was prepared by cryogenic grinding as described in Gardon et al. (2022). Briefly, a batch of ± 2 g of environmental plastic pieces (meso- and microplastics collected from the Ikopa river) and 2 cm diameter steel ball were added into a grinding jar (50 mL), all cooled in liquid nitrogen. Afterwards, the jars underwent a 5-min grinding process at 30 kHz using a Mixer Mill mm 400 (Retsch™). The resulting microplastic powder was suspended in filtered 70 % ethanol for decontamination and passed through a 50 μm stainless steel sieve. The sieved material was then washed with filtered distilled water (0.45 μm). The fraction < 50 μm was filtered through 1.2 μm polycarbonate membrane filters (Isopore™, Merck Millipore) in a vacuum filtration system (CVP-14, mrc-Lab Equipment). Each batch undergoes 6 grinding cycles. The sieving procedures were conducted in an extractor hood utilizing clean and rinsed glassware. The obtained 1.2–50 μm microplastic powder was dried and conserved in glass tube.

2.2. Fish husbandry and exposure

In order to obtain the fish cohort, 800 embryos (wild type, zebrafish AB circ strain) were kept under standard condition until the perfect age. At 2 months post fecundation, 240 juveniles were randomly selected and kept under standard conditions in a semi-static system. Thereafter, the fish were randomly placed in 12 glass tanks (20 fish per tank) equipped with an aeration and under a 12:12 h light/dark cycle. Each tank contained 6 L of reconstituted reverse osmosis water at a temperature of 27°C , pH 7.2, and conductivity of 450 $\mu\text{S/cm}$. After two weeks of acclimation, juveniles were exposed for 68 days to 3 sets of nominal

concentrations (CT = 0 µg/L, C100 = 100 µg/L and C1000 = 1000 µg/L) of environmental derived MPs. Prior the reproduction assessment, fish were exposed for 45 days, during which three groups of 4 replicates were formed (a replicate is one glass tank containing 20 fish). Each replicate was randomly placed in the exposure system. The solutions for each group were replaced twice a day (2×4 L). The fish were fed *ad libitum* twice a day with commercial pellets (GEMMA Micro 300, Skretting, Netherlands). After 45 days of exposure, 9 couples were formed per condition for the 23 days of reproduction phase, and the rest was euthanized using buffered MS-222 (Tricaine methane sulfonate, C10H15NO5S; CAS 886–86–2) at a concentration of 250 mg/L (500 mg/L NaHCO_3). The liver and one gonad were sampled, dry frozen and stored at -80°C for biomolecular analysis and the other gonad was directly placed in Davidson solution for histological analysis. During the experiment, the concentration of nitrite, nitrate and ammoniac were assessed weekly.

2.3. Ethics statement

All zebrafish husbandry and experimental procedures were approved by the Animal Ethics Committee of the University of Namur (ethical protocol: UN KE 24/425) and conformed to Belgian and European animal protection standards.

2.4. Microplastic characterization

2.4.1. Stock solution

One milligram of environmental derived MPs was resuspended in 10 mL of distilled water filtered with cellulose nitrate filters ($\varnothing = 47$ mm, pore size = 0.22 µm, Whatman, UK). Then, the solution was sonicated for 20 min to ensure the microplastics dispersion. Afterwards, 1 mL was vacuum filtered on a Mixed Cellulose Ester (MCE) filter (0.8 µm, 25 mm $\varnothing$, SKC Inc.). The filter was fixed on a large microscopy slide (75 $\times$ 52 mm) and sealed in a Petri dish waiting for the micro-Raman analysis. Two replicates were made for the microplastic characterization in the stock solution.

2.4.2. Microplastics in gonads

After 45 days of exposure, gonads were sampled in order to assess the presence of MPs. This exposure duration covers the critical period of sexual maturation in wild-type zebrafish (AB), which begins at around 45 days post-fertilisation (dpf) and is generally complete by around 90 dpf (Singleman and Holtzman, 2014). Prior to dissection, each fish was thoroughly rinsed with filtered water. Eight gonads (4 testes, 4 ovaries, one gonad per fish) per condition were sampled and directly placed in a safe-lock tube (Eppendorf® Safe-Lock Tubes, 1.5 mL – Microtubes). The weight of each gonad was determined before its digestion. The microplastic extraction was adopted from the protocol of Phuong et al. (2016). Briefly, each gonad was poured in 100 mL Erlenmeyer flask by rinsing the safe-lock tube with 25 mL of a solution of potassium hydroxide (KOH) 10 %. Thereafter, samples were incubated in an incubator shaker (Jeio Tech IST-4075R) for 24 h at 150 RPM (revolution per min) at 40°C . Finally, the solution was vacuum filtered on a MCE filter (0.8 µm, 25 mm $\varnothing$, SKC Inc.) The filter was fixed on a large microscopy slide (75 $\times$ 52 mm) and sealed in a Petri dish waiting for the micro-Raman analysis.

2.4.3. Blank processing

To cope with microplastic environmental contamination, a procedural blank was made for both stock solution and MPs in gonad analysis. For the stock solution, the blank was made of 10 mL of filtered water and 10 mL of filtered ethanol 70 %. For the microplastic in gonad, 3 replicates of empty safe-lock tube (same lot as containing the samples) were rinsed with 25 mL of filtered KOH 10 %, thereafter the solution was poured in a 100 mL Erlenmeyer flask and incubated with all the samples in the incubator shaker. Finally, each solution was vacuum filtered on a MCE filter (0.8 µm, 25 mm $\varnothing$, SKC Inc.). Then, the filter was fixed on a

large microscopy slide (75 $\times$ 52 mm) and sealed in a Petri dish waiting for the micro-Raman analysis. As our aim was to identify microplastics present in gonads, all polymers identified in the blanks were subtracted from the dataset (Shruti and Kutralam-muniasamy, 2023).

2.4.4. Micro-Raman and spectra analysis

After the sample preparation, microplastic particles were directly analysed on the filter surface with micro-Raman spectroscopy using DXR3xi Raman Imaging Microscope, operated by Thermo Scientific™ OMNIC™xi Software. Particle identification was performed by scanning 15 regions of 0.55 mm² resulting in an analyzed sample area up to 4 % for microplastics in gonads and 30 regions of 0.55 mm² per filter resulting in an analyzed sampled area up to 8 % for the stock solution. An example of an image of a scanned region is displayed in supplementary data Fig. S2. In each region, particles greater than 1 µm were automatically detected through 50x objective using the automatic detection setting. Subsequently, the size and coordinates of each particle were determined. Thereafter, Raman spectra were recorded for each particle with one laser (wavelength = 532 nm, laser power = 4.0 mW, exposure time = 0.16667 s (6 Hz), number of scans = 5, image pixel size = 1 µm). Spectrum range was set from 150 cm⁻¹ to 3500 cm⁻¹. Spectra were analyzed based on the available database of known compounds using BioRad KnowItAll® Informatics System Raman ID Expert (2025) software. Intensity correction and/or baseline spectrum adjustments were made in order to get a better comparability. For MPs in gonads, identified polymers were crosschecked with our own database made from spectra of polymers found in the stock solution (supplementary data Fig. S3).

2.4.5. GC-IM-MS analysis of persistent organic pollutants (POPs) sorbed on MPs

The chemical analysis of the POPs sorbed on the MPs was performed by GC-IM-MS (Muller et al., 2023), using the isotope dilution method. The MP sample was prepared as follows: A total of 78,2 mg of the mix of MPs was weighted and placed into a glass recipient. The sample was spiked with 2 ng of a ¹³C standard mixture of indicator polychlorinated biphenyls (PCBs 28, 52, 101, 138, 153 and 180) and 2 ng of a ¹³C standard mixture of polybrominated diphenyl ethers (PBDEs 28, 47, 99, 100, 153, 154 and 183). The mixture was allowed to stand at room temperature for several hours to ensure complete adsorption of the standards onto the MP surfaces. Subsequently, 2 mL of trace contaminant-grade n-hexane was then added (Biosolve, Dieuze, France). The solution was vortexed for 30 s and left to rest for 20 h to facilitate extraction of sorbed contaminants. After 20 h, 100 µL of the supernatant was transferred onto a Na₂SO₄ column previously conditioned with n-hexane. The analytes were then eluted with 2 mL of n-hexane and collected into a clean test tube. This step was carried out to remove traces of water and to filter out any residual pieces of MPs. The eluate was then concentrated to ~200 µL using a hot water bath (40°C) and a gentle stream of N₂. The solution was then quantitatively transferred to a GC vial containing 100 µL of keeper solvent (n-nonane). The solution was once again concentrated to 100 µL with a gentle stream of N₂. Finally, 2 ng of ¹³C PCB 80 recovery standard were added. The sample vial was sealed and stored at 4°C until instrumental analysis. A procedural blank was prepared in parallel using the same protocol. Measurements were performed on a commercial timsTOF Pro 2 mass spectrometer (Bruker, Bremen) equipped with a Scion 456-GC connected to an atmospheric pressure chemical ionization source for sample introduction and ionization (GC-APCI II, Bruker, Bremen). Injections were conducted in splitless mode (injection temperature 275°C , 1 µL). The GC column was a low polarity Rxi-5Sil MS column (30 m $\times$ 0.25 mm $\times$ 0.25 µm, Restek). Helium (grade 6.0, Air Liquide, Belgium) was used as the carrier gas. Ion mobility separations were performed by trapped ion mobility.

2.4.6. Morphological and elemental analysis of MPs

After the cryomilling, MP samples were transferred to a SEM combined with an energy dispersive X-ray spectrometer for further analysis (SEM-EDS) (supplementary data Fig. S4). A total of 6 samples of the microplastics stock (5 scanned particles/sample) were screened. The analyses were conducted using microscopes JEOL 6010LV Scanning Electron Microscope with Energy Dispersive X-ray Spectrometer (QUANTAX SDD energy-selective X-ray microanalyser). To avoid contamination by chemical artefacts introduced by the metal coating, the samples were first analysed without the gold coating step, after checking that there was no sample charge under the electron beam. The chemical composition spectra of the investigated fragments were finally compared with those available in the available database.

2.4.7. Quality assurance/Quality control

We adopted several measures to avoid contamination during the analysis. The water and ethanol used for all analyses were filtered with cellulose nitrate filters ($\varnothing = 47$ mm, pore size = 0.22 μ m, Whatman, UK). All equipment was rinsed three times with filtered water before use. Special care was taken to clean the vials used for the digestion of the gonads. Prior to digestion, a KOH base wash was performed. Briefly, 50 mL of 10 % KOH was incubated in each glass vial in the incubator shaker for 24 h at 50 °C at 150 RPM. Thereafter, vials were rinsed (3 times) thoroughly using filtered deionized water and finally dried in oven. During the digestion procedures, vials remained covered. During the dissection, equipment (forceps, scissors) were rinsed with SDS (Lab Solutions Low-Foaming Liquid Detergent), deionized water and ethanol between each fish. In addition, 100 % cotton lab coats and particle-free nitrile gloves and sleeves were used during manipulation. All manipulation was performed under a vertical laminar flow hood. For the micro-Raman analysis, all surfaces were cleaned with Milli-Q water before and after work and long exposure to air were avoided for each sample as they were covered immediately after mounting.

2.5. Toxicity endpoints

2.5.1. Pre-reproduction phase endpoints

2.5.1.1. Gonad histology. Fresh ovarian tissue (one gonad per fish) (n; CT = 8, C100 = 7, and C1000 = 11) from females exposed to MPs for 45 days were fixed in Davidson's solution for 24 h and then stored in formalin at ambient temperature for one week. Prior to dehydration, the gonads were washed in clear water and submerged in 70 % methanol for 24 h. The tissues were dehydrated overnight using a HistoCore PEARL system (Leica Biosystems). Fixed ovaries were embedded in paraffin and subsequently sectioned at 8 μ m thickness using a microtome. Gonadal sections were stained with haematoxylin-eosin-saffron and examined under an Olympus CKX53 microscope at 4 $\times$ magnification. The ovarian follicle stages were assessed according to the OECD Guideline 123 (OECD, 2010) classification system. Follicles were counted at the following stages: *peri* nucleolar oocyte (PO), cortical alveolar oocyte (CAO), early vitellogenic oocyte (EVO), and late vitellogenic oocyte (LVO).

2.5.1.2. Liver proteomic. After 45 days of exposure, liver from 5 males and 5 females per condition were collected for the proteomics analysis. Protein extraction was made by homogenizing each liver with 300 μ l of lysis buffer (Tris-HCl pH = 7.5 supplemented with NaCl 150 mM, Triton x1 00 1 % and SDS 0.1 % to a final concentration of 50 mM) into tubes containing 0.5 mm zirconium oxide beads (Dutscher, Brumath, France) using a Bullet Blender Storm 24 (NextAdvance, New York, USA). Afterwards, samples were centrifuged (10 000 g, 5 min, 4 °C), supernatants were collected, and the protein quantification was performed using the Bradford method. Ten micrograms of protein extract from each sample was prepared using a modified Gelaided Sample Preparation protocol,

digested with trypsin/Lys-C overnight at 37 °C, and desalted using μ C18 Omix tips (Agilent). Peptides were separated on a NanoElute system with a reversed-phase column under a nanoflow gradient. Mass spectrometry was performed on a TIMS-TOF Pro in PASEF mode, excluding singly charged peptides. Peptide sequences were identified using Peaks X+ software with the UniProt *Danio rerio* database. Data were filtered at 1 % FDR and redundancy eliminated based on shared peptide sets. Post-translational modifications and digestion quality were assessed using Preview software. Relative protein abundance data were analysed in R (version 4.4.2) using a custom pipeline based on the Differential Enrichment Analysis of Proteomics (DEPs) package. Proteins with an excessive number of missing values were excluded from the analysis using a threshold of a maximum of one missing value per condition. Differential enrichment analysis was performed using linear models and empirical Bayes statistics, with cutoffs set at 0.05 for alpha and 1.5-fold change. Gene set enrichment analysis for Gene Ontology (GO) and KEGG pathways was conducted using the ClusterProfiler package, with a p-value cutoff of 0.05 and a q-value cutoff of 0.2.

2.5.1.3. Post reproduction phase endpoints

2.5.1.3.1. Reproduction assessment. The evaluation of reproductive endpoints was slightly adapted from OECD Test Guideline 229 (OECD, 2009). After 45 days of exposure to MPs, the reproductive capacity of the fish was assessed. Nine couples per condition with three couples per tank were maintained for 23 additional days under the same exposure condition. During reproduction, fish were fed *ad libitum* three times a day. Twice with commercial pellets (GEMMA Micro 300, Skretting, Netherlands) and supplemented with freshly hatched *Artemia* nauplii (*Artemia* Cysts 250.000 NPG, Ocean Nutrition GSL). Each morning, the fish from each tank were transferred to a spawning tank containing the same exposure solution (except from days 22 and 23) and equipped with a spawning trap. Freshly spawned eggs were collected one hour after spawning, rinsed, and transferred to Petri dishes filled with the reconstituted water. From day 1 to day 21 of the reproduction phase, the fecundity (number of eggs spawned per female per day) and the number of eggs successfully fertilized was recorded. Larvae obtained on days 22 and 23 of the reproduction phase were purposely used for the assessment of transgenerational effects of environmental microplastics (results not shown in this study). After the 23 days of reproduction, fish were euthanized using buffered MS-222 (Tricaine methane sulfonate, C₁₀H₁₅NO₅S; CAS 886–86–2) at a concentration of 250 mg/L (500 mg/L NaHCO₃). The liver and gonads were then sampled and stored at –80 °C for biomolecular analysis.

2.5.1.4. Vitellogenin (VTG) in liver. After the reproduction phase, 6 livers from females per condition were sampled for the VTG levels. Each sample was individually homogenized by adding 300 μ l of the homogenization buffer (50 mM Tris-HCl pH 7.4; 1 % Protease inhibitor cocktail [P8340, Sigma, USA]) into tubes containing 0.5 mm zirconium oxide beads (Dutscher, Brumath, France) using a Bullet Blender Storm 24 (NextAdvance, New York, USA). Thereafter, the homogenised samples were centrifuged (30 min, 20,000 g, 4 °C) and the supernatants were collected. The protein quantification was performed using the Bradford method. VTG concentrations were determined using a commercially available zebra- fish VTG enzyme-linked immunosorbent assay (ELISA) kit (V01008402; Biosense Laboratories, Norway) according to the manufacturer's instructions. Samples were analysed in duplicate, and 2 dilutions 1/100 and 1/10,000 and the absorbance was measured at 492 nm using a FLUOstar Omega (BMG LABTECH, Germany). The coefficient of variation (<10 %) and the correlation coefficient (>0.99 %) were checked.

2.5.1.5. MDA content in gonads. After the reproduction phase, 12 gonads (6 males, 6 females) per condition were sampled for the analysis of MDA content. Analysis was performed using the commercially available

Lipid peroxidation (MDA) Assay Kit (MAK085, Sigma-Aldrich®) according to the manufacturer's instructions.

2.6. Statistical analysis

Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene test, respectively. When the data followed parametric distribution, ANOVA was performed, followed by post-hoc comparisons using Tukey's test. For non-parametric distributions, the Kruskal-Wallis test was applied, followed by Dunn's test with Bonferroni-Holm (BH) adjustment. All statistical analyses were performed using R (version 4.4.2) with the significance level set at $\alpha = 0.05$.

3. Results

3.1. Mesoplastics and microplastics characteristics

The SEM images of all sampled particles from the Ikopa River (Fig. 1) reveal changes in surface properties, such as surface cracks, deep cracking and fractures, increased roughness, crystallinity, and colonization by microorganisms. After the cryomilling, the SEM-EDS spectral results showed the presence of different elements such as carbon, oxygen, magnesium, aluminium, silicon, sulfur, chlorine, potassium, calcium, titanium, iron (supplementary data Fig. S4).

Overall, 279 plastic particles having a size ranging from 500 μm to 10 mm were sampled from the Ikopa River (supplementary data Fig. S1). Eighteen polymers were identified, and the most dominant one was polyethylene (35.5 %), followed by polystyrene (29.9 %), poly(vinyl

chloride) (8.6 %) and high-density polyethylene with 5.4 % (Fig. 2A). In the prepared stock solution, 12 polymers were recovered from the Ikopa River mesoplastics. The most dominant polymer was polypropylene accounting for 31.4 %, followed by poly(ethylene-co-vinyl acetate) (PEVA) (21.1 %) while before cryomilling it was only 0.4 %, polyethylene (12.4 %), high density polyethylene (11.0 %) and poly(vinyl chloride) with 4.0 %. Polystyrene was no longer dominant in the stock solution with 2.3 % (Fig. 2A).

After the 45 days of exposure, a dose- and sex-dependent accumulation of microplastics were observed in the gonads of zebrafish. A significantly higher accumulation ($p < 0.05$) was observed in the males exposed at the concentration of 1000 $\mu\text{g/l}$ accounting for 117.88 ± 102.65 MP particles/mg of tissue (mean $\pm$ sd, $n = 4$). The lowest concentration was observed in the female control group with 1.0 ± 2.0 MP particles/mg of tissue (mean $\pm$ sd, $n = 4$) (Fig. 3). Eleven polymers were identified in gonads, with polyethylene being the most dominant (26.8 %), followed by poly(vinyl chloride) (21.1 %), poly(ethylene-co-vinyl acetate) 16.9 % and polystyrene (8.5 %). Polypropylene was the least common polymer found in gonads with 1.4 % (Fig. 2A). Concerning the size distribution, after cryomilling and sieving through the 50 μm stainless steel sieve, the dominant size was around 5 to 15 μm . No obvious differences were observed between polymers on both stages (Stock solution and gonads). (Fig. 2B).

3.2. POPs sorbed on MPs

The measured concentration of the six indicator PCBs and the seven PBDEs are shown in supplementary data Fig. S5. The level of the selected

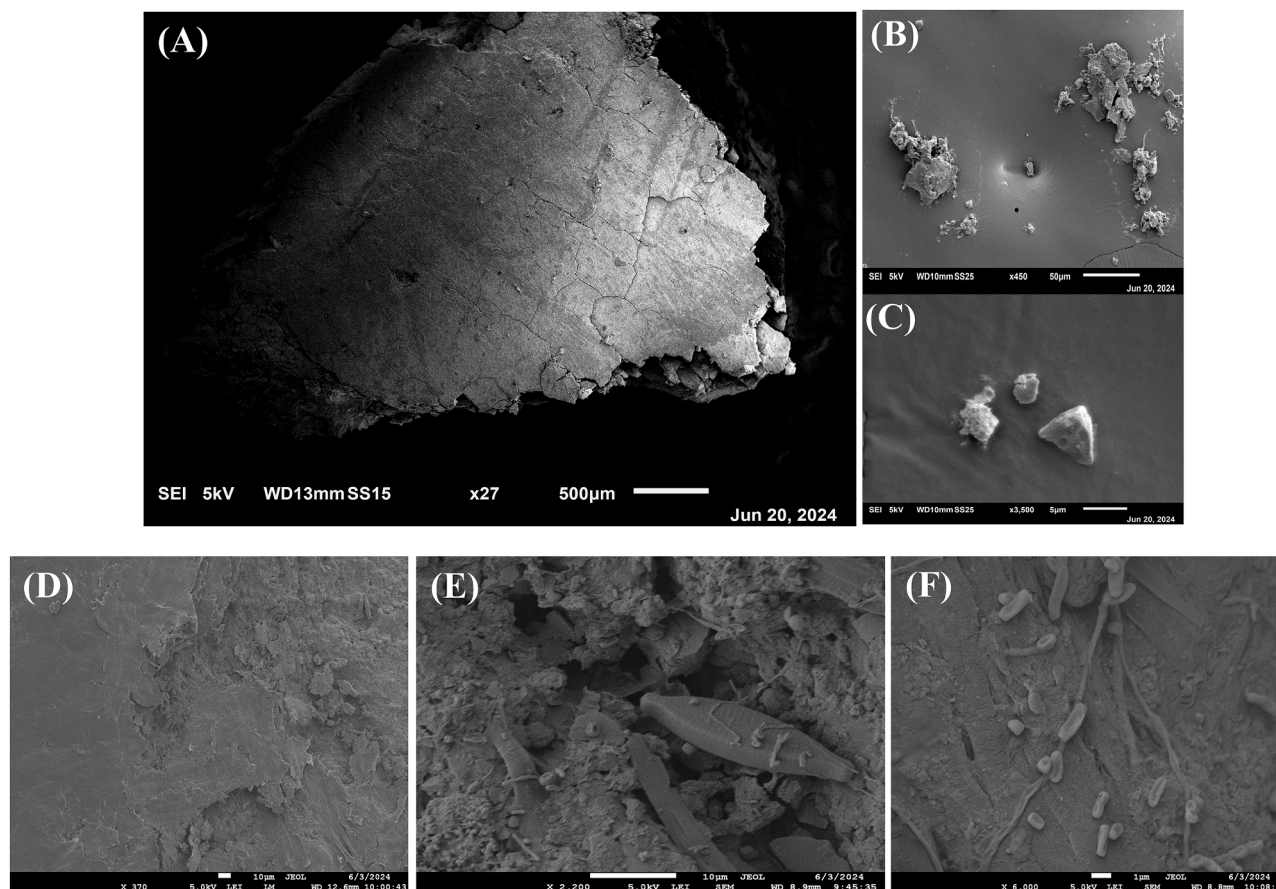


Fig. 1. (A) Scanning Electron Microscope (SEM) image of polyethylene, chlorinated (Cl 25 % by wt.), sampled in the Ikopa River-Madagascar downstream of the city of Antananarivo, using 500 μm plankton net at a depth of 0–50 cm. (B) and (C) SEM images of the stock solution after cryomilling. 1 mg of microplastics particles were resuspended in 10 ml of filtered water. Then, the solution was sonicated during 20 min to ensure the microplastics dispersion. (D) SEM image of weathered surface of environmental microplastic. (E) and (F) SEM images of microorganisms colonizing the surface of environmental microplastics.

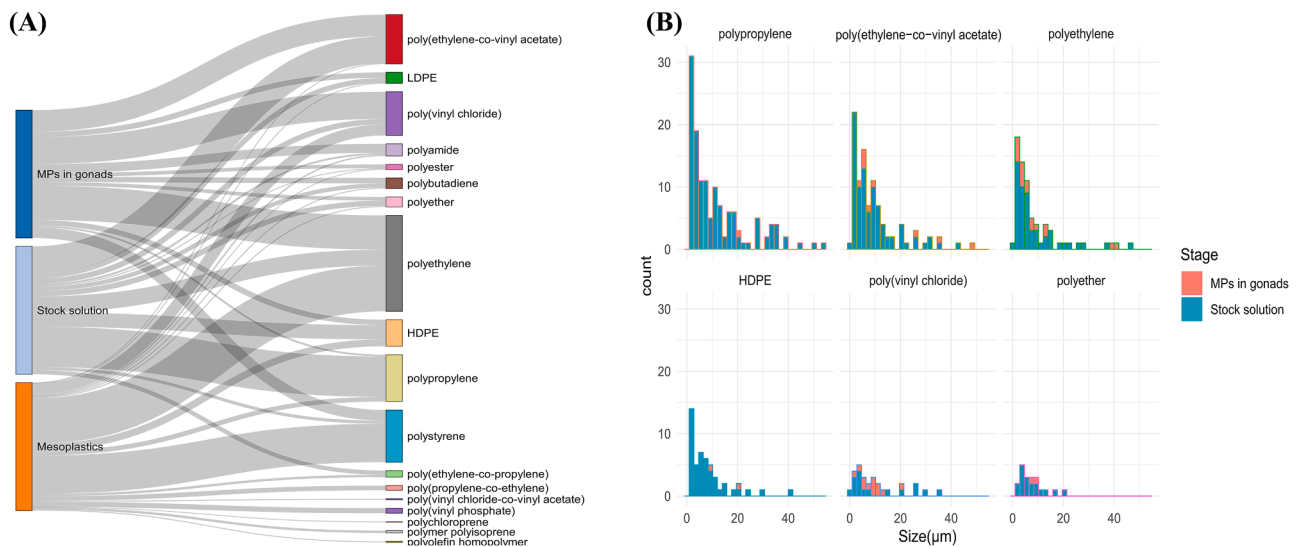


Fig. 2. (A) Sankey diagram representing the proportion of polymers sampled in Ikopa River (Antananarivo – Madagascar) (Mesoplastics) versus polymers characterized in the stock solution and those found in the zebrafish gonads after 45 days of exposure. Overall, 18 polymers were identified. (B) Size distribution of the 6 main polymers found in gonads and those characterized in the stock solution. MPs sizes were determined using the particles automatic detection setting of the Raman imaging Microscope (DXR3xi thermos scientific) supported by the OMNICxi software.

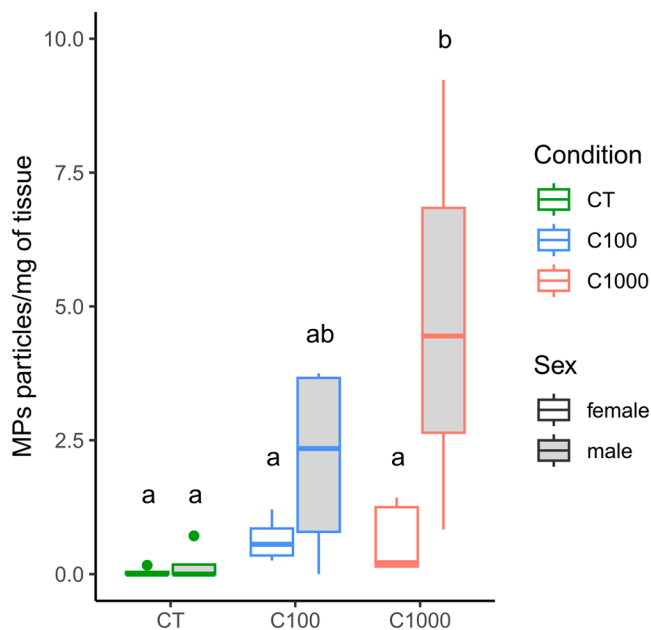


Fig. 3. Boxplot of microplastics found in gonads after 45 days of exposure reported in MPs particles per milligram of tissue. 8 gonads (4 males, 4 females) per condition were sampled to analyse the MPs in gonads. Letters above each boxplot indicate the significant difference between groups after the Anova on the generalize linear model (glm) taking as variable MPs particle per milligram of tissue and as factors the sex (male and female) and the condition (CT = 0 μg/L, C100 = 100 μg/L, C1000 = 1000 μg/L), followed by a Tukey test. $p < 0.05$ was considered as statistically different.

PCBs was one to two orders of magnitude higher than the corresponding PBDEs. All the targeted congeners of PCBs and PBDEs were detected, except for PBDE congeners 28 and 100. Furthermore, the chromatograms of the PCBs indicated the presence of many congeners other than those originally targeted. For example, the extracted ion chromatograms of the tetra- and penta-chlorinated biphenyls were characterised by a large number of peaks in addition to those corresponding to the target PCBs 52 and 101, respectively (Supplementary data Fig. S6). While most

of these additional peaks were less intense than the target PCBs, some were of similar or higher intensity. Each additional peak was checked for mass accuracy, isotopic pattern and collision cross section to confirm that the signals were indeed produced by PCBs. In this way, 14 additional TCBS and 16 PeCBs were tentatively identified. In total, 69 additional PCBs of various chlorination degree were tentatively identified (Supplementary data Table S1).

3.3. Gonad histology

Even though microplastics were found in gonads, no obvious histological alterations were observed (Fig. 4A). The percentage of perinuclear oocytes was slightly different, but not statistically significant, between groups, with an average of $74.9 \pm 6.5\%$ (mean $\pm$ sd, $n = 8$), $74.4 \pm 7.2\%$ (mean $\pm$ sd, $n = 7$) and $73.4 \pm 6.5\%$ (mean $\pm$ sd, $n = 9$) for CT, C100 and C1000 respectively. Nonetheless, the rate of the cortical alveolar oocytes was significantly lower ($p < 0.05$) in the control group with average of $10.0 \pm 2.3\%$ (mean $\pm$ sd, $n = 8$) compared to the treated conditions with an average of $17.1 \pm 7.0\%$ (mean $\pm$ sd, $n = 7$) and $17.6 \pm 4.8\%$ (mean $\pm$ sd, $n = 9$), for C100 and C1000 respectively. In the same trend, the percentage of the early vitellogenic oocytes was significantly higher ($p < 0.05$) in the control group ($9.5 \pm 2.3\%$, mean $\pm$ sd, $n = 8$) compared to the group exposed to 1000 μg/L ($4.8 \pm 7.2\%$, mean $\pm$ sd, $n = 9$). No significant difference was observed in the late vitellogenic oocyte stage. However, the highest rate was observed in the control group reaching an average of $5.6 \pm 3.1\%$ (mean $\pm$ sd, $n = 8$) and the lowest was observed in the group treated with 100 μg/L with an average of $2.9 \pm 1.2\%$ (mean $\pm$ sd, $n = 7$). (Fig. 4B). Male gonads were also sampled for histological analysis (supplementary data Fig. S7); however, because of the small size of the samples, histological section analysis of male gonads was not of sufficient quality. Therefore, no statistical analysis was performed due to the lack of replicates.

3.4. Liver proteomic

A total of 6806 proteins were reported in the Label-free quantification analysis. Fig. 5A illustrates the relative abundance of the proteins among conditions. Hierarchical cluster analysis showed that protein regulation profiles were similar between sex despite the concentration. The number of DEPs in each treatment group is shown in Fig. 5B. The highest numbers of DEPs were recorded in males exposed to the

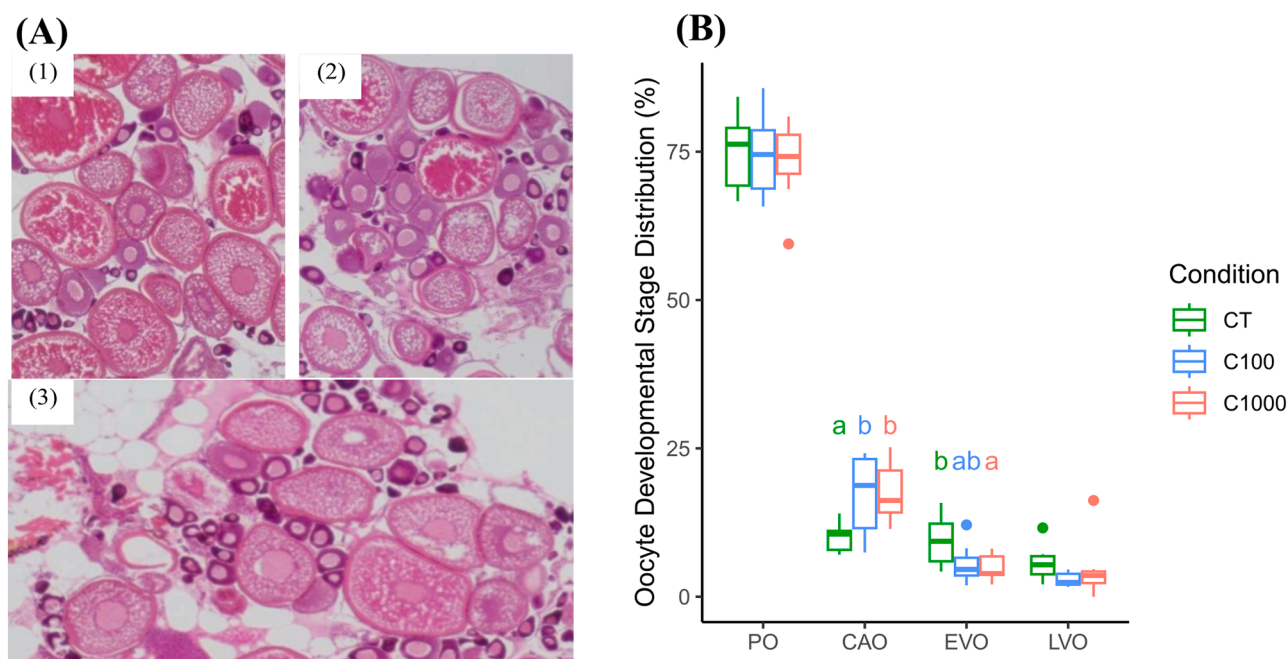


Fig. 4. Histological analysis of ovaries exposed to MPs for 45 days. (A)(1) Representative histological section of ovaries from the control group; (2) Representative histological section of ovaries exposed to 100 µg/L MPs; (3) Representative histological section of ovaries exposed to 1000 µg/L MPs. (B) Boxplot representing the percentage of oocytes at different developmental stages, including peri-nucleolar oocytes (PO), cortical alveolar oocytes (COA), early vitellogenic oocytes (EVO), and late vitellogenic oocytes (LVO). 8 females per condition were sampled to analyse the gonads histology. Letters above each boxplot indicate the significant difference between groups after the Anova on the generalize linear model (glm) taking as variable the percentage of oocytes in each stage and as factors the condition (CT = 0 µg/L, C100 = 100µg/L, C1000 = 1000 µg/L), followed by a Tukey test. $p < 0.05$ was considered as statistically different.

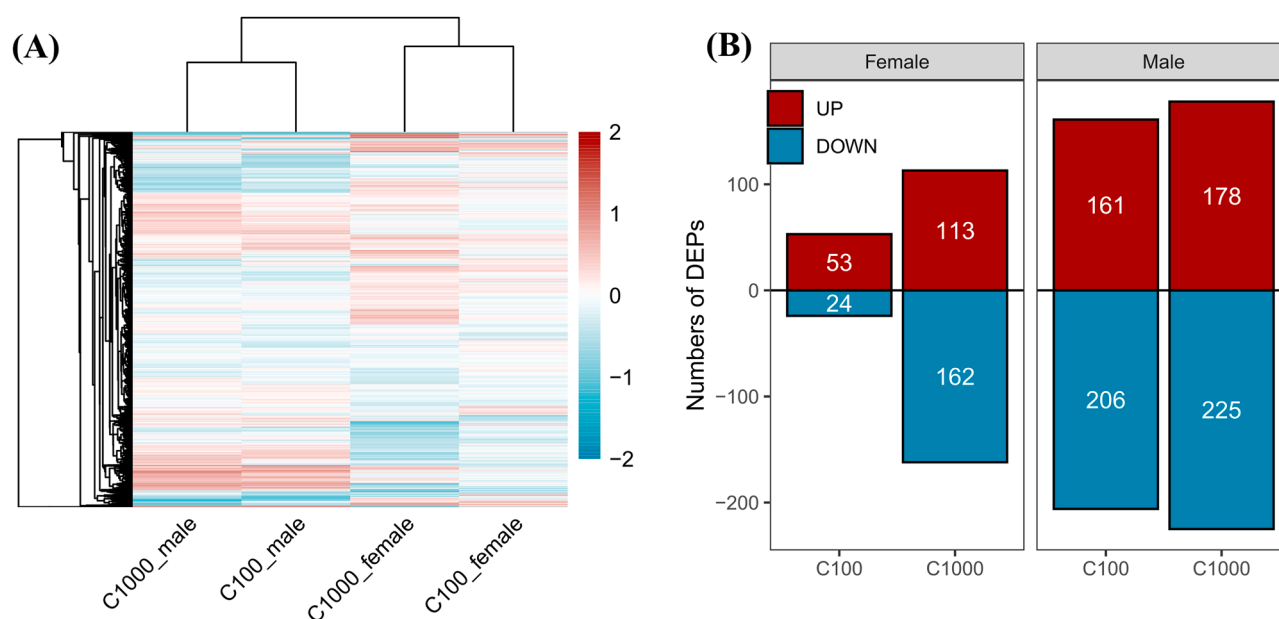


Fig. 5. (A) Heatmap showing normalized protein regulation profiles. (B) DEPs (Differentially Expressed Proteins) in zebrafish *Danio rerio* liver (male and female) exposed to environmental microplastics for 45 days at a concentration C100 = 100 µg/L and C1000 = 1000 µg/L.

concentration 1000 µg/L (C1000_male) with 255 downregulated and 178 upregulated proteins. The lowest numbers of DEPs were recorded in females exposed to the concentration 100 µg/L with 24 downregulated and 53 upregulated proteins.

Functional analysis employing the GO molecular function knowledgebase (Fig. 6A) showed that exposure to microplastics caused dysregulation of proteins participating in oxidoreductase activity, oxidoreductase activity-acting on CH—OH group of donor, transferase

activity-transferring nitrogenous group, transaminase activity in female exposed to 1000 µg/L and male exposed to 100 µg/L and 1000 µg/L. Furthermore, proteins participating in creatine kinase activity, phosphotransferase activity-nitrogenous group as acceptor were significantly dysregulated in the group male exposed to 100 µg/L and 1000 µg/L and ATP-dependent protein folding chaperone and protein folding chaperone were modulated only in male exposed to a concentration of 1000 µg/L. Moreover, Chord diagram (Fig. 6B) attributing DEPs shared by at

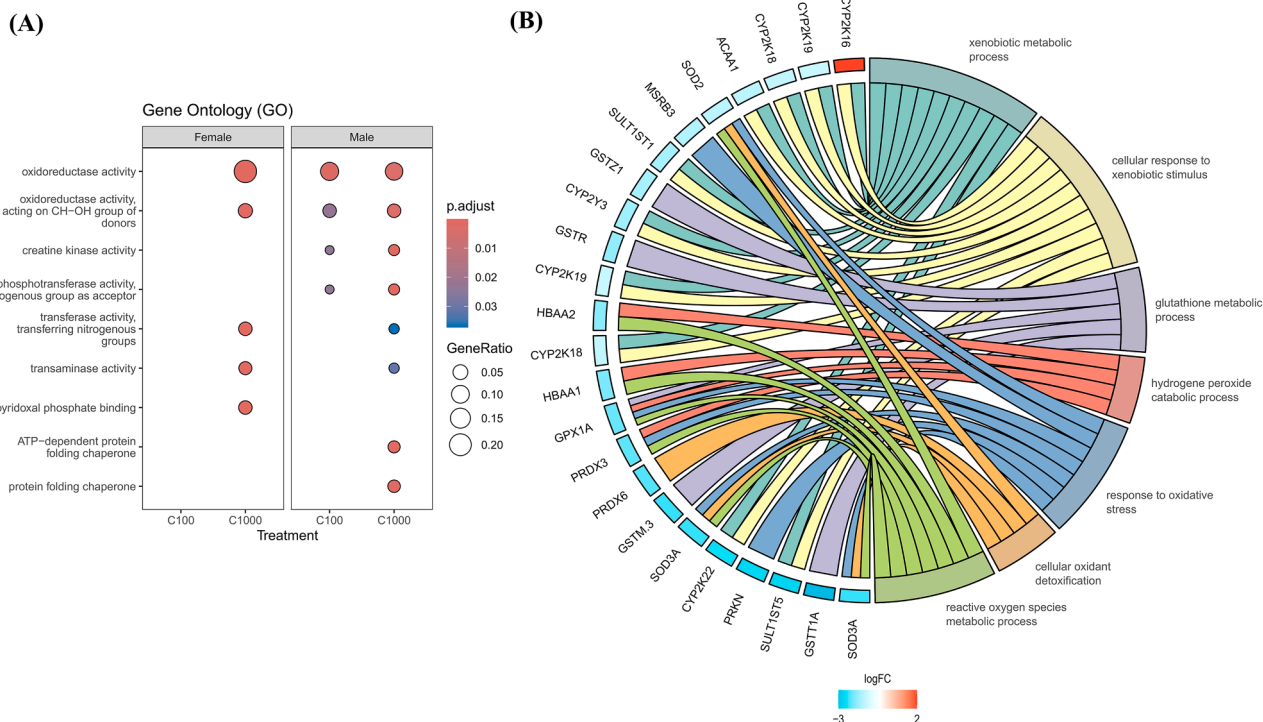


Fig. 6. (A) Dotplot of the Gene Set Enrichment Analysis for the top 9 GO (Gene Ontology) molecular function terms. From blue to red indicate the high significance of dysregulated proteins and the size of the dot indicates the gene ratio. Comparison was made between groups; taking as factor the sex (male and female) and microplastic concentrations (C100 exposed to 100 $\mu\text{g/L}$; C1000 exposed to 1000 $\mu\text{g/L}$). (B) Chord diagram attributing DEPs (Differentially Expressed Proteins) shared by at least two treatment groups to the GO biological process for response to xenobiotic stimulus, glutathione metabolic process and stress oxidant response. Blue blocks indicate proteins that were significantly downregulated and red block indicate proteins that were significantly upregulated.

least two treatment groups to the GO biological process terms showed downregulated protein participating in the response to xenobiotic stimulus, glutathione metabolic process and stress oxidant response.

Functional analysis by means of the KEGG pathway using Pathview showed that microplastics upregulated several proteins, components of the electron transport chain complexes (I, III, IV or V) (Fig. 7). In addition, functional analysis also showed dysregulated proteins involved in PPAR (peroxisome proliferator-activated receptor) signaling pathway (supplementary data Fig. S8), namely FATP (Fatty Acid Transport Protein, SLC27 family), ACS (Acyl-CoA Synthetase), SCP-X (Sterol Carrier Protein-X / SCP2), Perilipin which were upregulated, and FABP (Fatty Acid-Binding Protein), ME1 (Malic Enzyme 1), FABP1 (Liver-Type Fatty Acid-Binding Protein), ILK (Integrin-Linked Kinase) which were downregulated. Furthermore, proteins involved in apoptosis pathway were also dysregulated (supplementary data Fig. S9), such as FADD (Fas-Associated Death Domain Protein), Cathepsin (Lysosomal Proteases), CASP6 (Caspase-6), ARTS (Apoptosis-Related Protein in the TGF- β Pathway) which were downregulated and eIF2 α (eukaryotic Initiation Factor 2 α) and α -tubulin (microtubule component) which were upregulated. Finally, in male, proteins involved in necroptosis (supplementary data Fig. S10) were also dysregulated, namely: FADD, JAKs (Janus Kinases), H2AX (Histone H2AX, phosphorylated as γ H2AX), PYGL (Glycogen Phosphorylase L), ASC (Apoptosis-Associated Speck-like Protein), VDAC (Voltage-Dependent Anion Channel) which were downregulated and ANT (Adenine Nucleotide Translocase) which was upregulated.

3.5. Reproduction endpoints

During the 21-days of reproduction, there was no mortality or abnormal behavior observed in any of the treatment groups. The total number of eggs spawned was 2425, 1381 and 1944 for CT; C100 and C1000 respectively (Fig. 8A). A significant difference was observed in

terms of fecundity (the number of eggs spawned per female per day), as those in C100 were the lowest with an average of 21.9 ± 12.9 eggs per female per day (mean $\pm$ sd, $n = 63$) compared to the control group with an average of 38.5 ± 23.2 eggs per female per day (mean $\pm$ sd, $n = 63$) (Fig. 8B) ($p < 0.001$). A dose dependent effect was also observed in terms of fertilisation success. Fish exposed to MPs showed a significantly lower fertilisation rate compared to the control group ($p < 0.001$). The average fertilization rates were $74.1 \pm 29.7\%$, $68.9 \pm 23.8\%$, and $47.4 \pm 29.2\%$ for CT; C100 and C1000 respectively (mean $\pm$ sd, $n = 63$) (Fig. 8C).

3.6. Vitellogenin liver (post-reproduction)

The highest concentration of VTG in liver was observed in female C100 (treated with a concentration of 100 $\mu\text{g/L}$), with an average of 6088 ± 864 ng/mg Prot (mean $\pm$ sd, $n = 6$), and the lowest concentration was reported in the control group, with an average of 2127 ± 1581 ng/mg Prot (mean $\pm$ sd, $n = 6$). Nonetheless, the difference was not statistically significant (supplementary data Fig. S11A).

3.7. MDA content in gonads

No significant differences in terms of MDA content were observed in gonads between the treated groups and the control. Somehow, the highest observed concentration was in male exposed to 1000 $\mu\text{g/L}$ with an average of 4.36 ± 4.69 nmole/mg Prot (mean $\pm$ sd, $n = 6$) and the lowest concentration was found in female exposed to 100 $\mu\text{g/L}$ with an average of 0.93 ± 0.78 nmole/mg Prot (mean $\pm$ sd, $n = 6$) (supplementary data Fig. S11B).

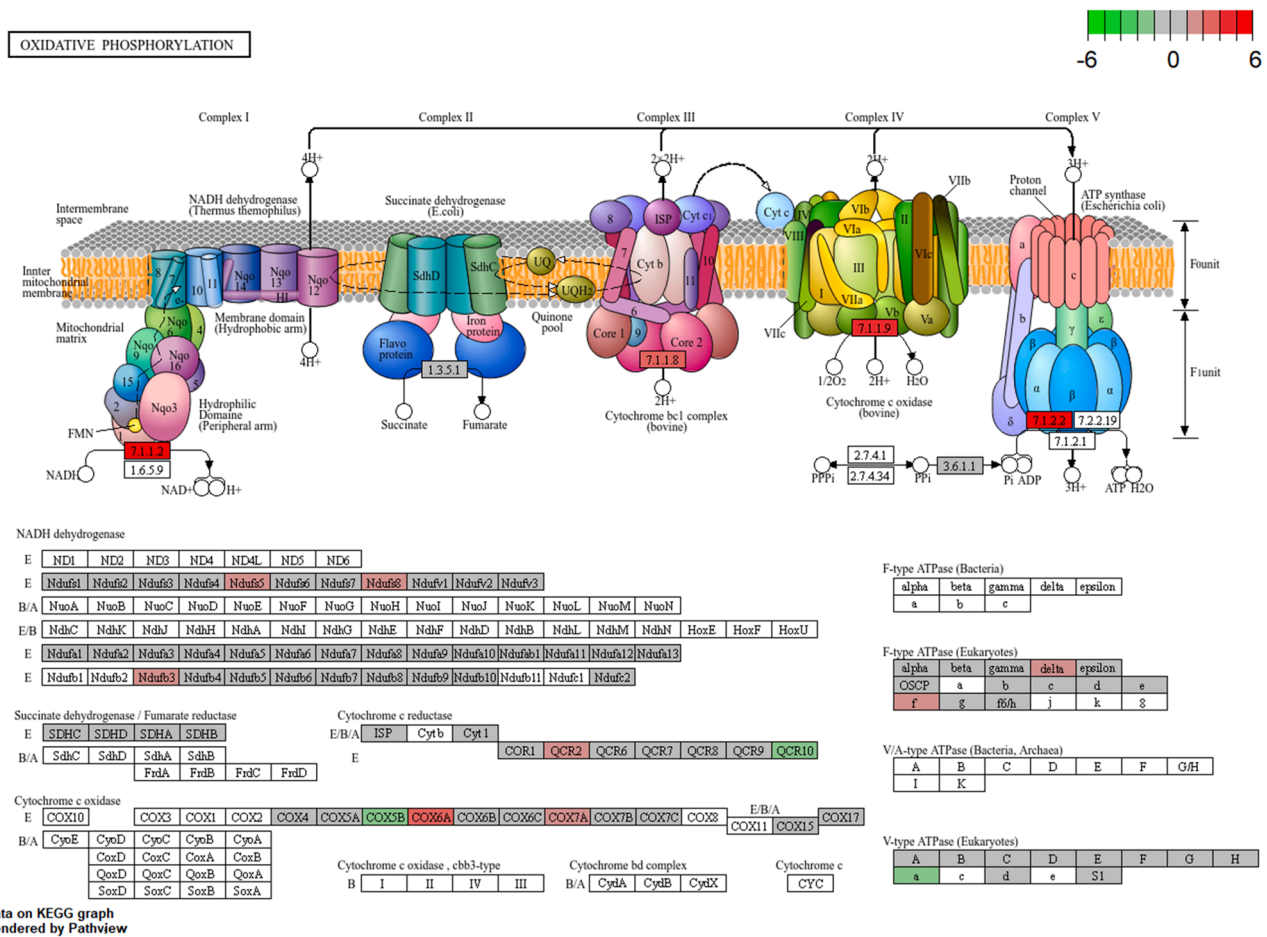


Fig. 7. Organism-specific KEGG pathway dre00190 – Oxidative phosphorylation. Red boxes indicate upregulated DAPs in the liver of zebrafish (*Danio rerio*) exposed to environmental microplastics at a concentration of 100 µg/L and 1000 µg/L. 7.1.1.2: NADH dehydrogenase [ubiquinone] iron-sulfur proteins (NDUFS3, NDUFS4, NDUFS5, NDUFS6), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunits (NDUFA4A, NDUFA7, NDUFA8, NDUFA12), and NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunits (NDUFB7, NDUFB9); 7.1.1.8: cytochrome b-c1 complex subunit 7 (UQCQB); 7.1.1.9: cytochrome c oxidase subunits (COX5B2, COX6B2, COX6C, COX7A2L); 7.1.2.2: V-type proton ATPase subunit E1 (ATP6V1E1B).

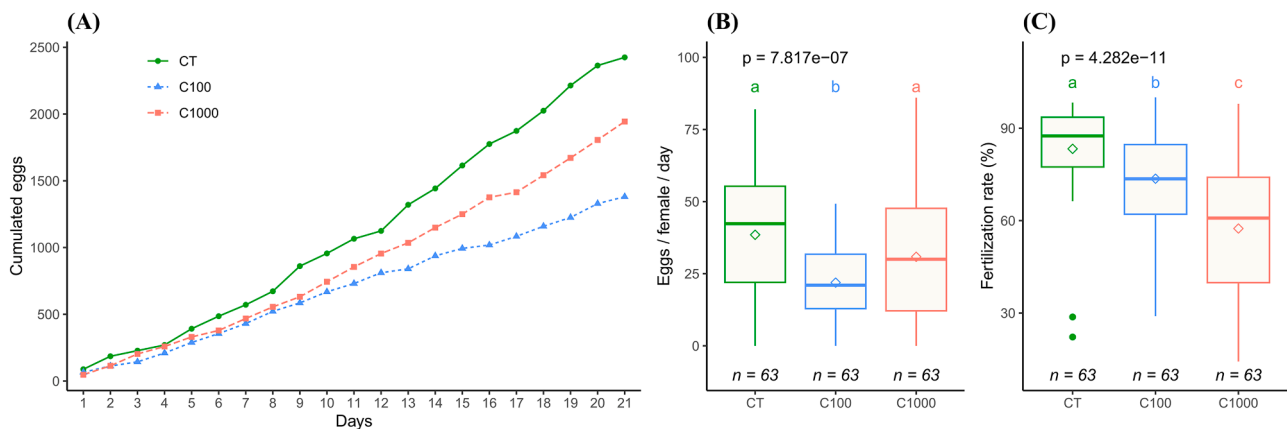


Fig. 8. (A) Cumulative eggs spawn per condition during the 21 days reproductive assay; (B) Female fecundity (eggs/female/day); and (C) Daily fertility. Significant differences between conditions are indicated by different letters (abc), while overall differences are shown with p-values ($p < 0.05$).

4. Discussion

4.1. Environmental microplastic characteristics from IKOPA river to the zebrafish gonads

Rozman and Kal (2022) and Cui et al. (2024) highlighted that studies

on microplastic toxicity continue to face challenges due to their limited ecological relevance. By using environmental MPs sampled from the downstream of an urban river as test material, our results provide additional insightful data to fill this gap. From Ikopa river to the gonads, three stages of microplastic characterization were set: the Mesoplastics stage which referred to the meso- and microplastics sampled from the

Ikopa river using a plankton net with 500 μm of mesh size, the stock solution stage which referred to the microplastic stock solution after the cryomilling process used as test material during the exposure and the MPs in gonads stage which referred to the microplastics found in the gonads of zebrafish male and female after 45 days of exposure at a concentration of 0, 100 and 1000 $\mu\text{g/L}$.

Before the cryomilling, polyethylene followed by polystyrene were the most dominant particles, which goes along with what is commonly observed in river using identical sampling techniques (Xiong et al., 2019). In the stock solution, the dominant size observed ranged between 5 and 20 μm , which is a typical size distribution reported in the literature for plastic cryomilling (Gardon et al., 2022; McColley et al., 2023). Nonetheless, an obvious shift of proportion was observed after the cryomilling, as the polypropylene became the most dominant accounting for 31.44 % followed by poly(ethylene-co-vinyl acetate).

Cryomilling is proposed by McColley et al. (2023) as a valuable method of producing MPs which exhibited characteristics similar to those found in the environment. Importantly, cryomilling does not significantly alter the surface particles. Nonetheless, in cryomilling, the size distribution of MPs particles really depends on the number of processed cycles, the more cycle we apply, the higher is the proportion of particles under 20 μm (McColley et al., 2023). Furthermore, the initial characteristics of each particle might also influence the final size distribution after the process, as aged polymers present higher surface crystallinity which can affect the way they break down (Gardon et al., 2022).

Somehow, it is worth noting the significant increase of PEVA proportion in the stock solution. PEVA has been already reported as a dominant polymer in the Ikopa river at the downstream of the city by Rabezanahary et al. (2024), by sampling with a pump associated with superposed stainless-steel sieves with a size cut off of 50 μm at a depth of 0 to 20 cm. In this study, this small percentage of PEVA in the mesoplastics stage (sampled with 500 μm plankton net) confirmed the size cut off influence on the polymer distribution (Biginagwa et al., 2016). Interestingly, the skyrocketed proportion of PEVA found in the stock solution after the cryomilling might be associated with the presence of polyethylene, HDPE or LDPE associated at high proportion (90/10) with PEVA. This is a well-known technique for improving the propriety of plastics (Faker et al., 2008). The PEVA polymer was not detected through the ATR-FTIR analysis due to this low proportion and also spectra overlapping peaks (Clough et al., 2024). However, the cryomilling process might cause their release in smaller-sized particles.

The scanning electron microscope image of particles sampled in the Ikopa River (Fig. 1) clearly confirmed the change in surface properties of the sampled plastics. Obviously, the change is due to an increased crystallinity after a long exposure to UV light (Quigley et al., 2024), leading to the presence of cracks and surface roughness, which is the initiation of particle degradation. In addition, deep cracking and fractures observed in this study (Fig. 1A) reveals the primary degradation of environmental microplastics as reported by Wang et al. (2017), resulting from an oxidative weathering of microplastics. The surface roughness facilitates the adsorption of secondary contaminants, thereby increasing the environmental toxicity of these particles. In other words, the degradation of microplastics leads to an increase in surface area, enhancing their ability to act as vectors for chemicals and microorganisms (Patchaiyappan et al., 2021). Study reported the presence of sorbed pollutants including Brominated flame retardant as tribromophenol ($\text{C}_6\text{H}_3\text{Br}_3\text{O}$) and its metabolite tribromoanisole ($\text{C}_7\text{H}_5\text{Br}_3\text{O}$) on environmental microplastics (Cormier et al., 2021), which goes in line with our finding as PCBs and PBDEs congeners were also detected on the environmentally derived microplastics used in this study. Furthermore, elements detected through SEM-EDS, such as aluminium and calcium, indicate the presence of complex additives, as these elements are commonly used in various hydrocarbon polymers to reduce oxidation processes (Patchaiyappan et al., 2021). Titanium peak is also an indicator of titanium oxide pigment on microplastics (Wang et al., 2017).

Overall, this study highlights the uniqueness of environmental microplastics, as in addition to polymer variability, mixtures of sorbed pollutants and additives can be observed, which may exacerbate their potential toxicity.

After 45 days of exposure, a concentration and sex dependent accumulation of MPs in gonads was observed, specifically males exposed to the highest concentration (1000 $\mu\text{g/L}$) presented a significantly higher concentration of MPs in their gonads. Studies have confirmed the accumulation of MPs in gonads, by the way of illustration, Xia et al. (2023) observed the presence of PE-MPs with a size ranging from 8–15 μm in gonad male and female of large-scale loach (*Paramisgurnus dabryanus*) after 30 days of waterborne exposure at a concentration of 1 and 10 mg/L. Qiang and Cheng (2021) have also stated that a waterborne exposure with 5 μm polystyrene MPs during 21 days at a concentration of 100 and 1000 $\mu\text{g/L}$, impaired gonads of zebrafish (*Danio rerio*) male and female, assumingly the cause might be associated to an internal accumulation. Fish absorb MPs through their gills, skin and mouth. Afterwards, MPs are accumulated in the gastro-intestinal tract (GIT) (Rabezanahary et al., 2024; Yi et al., 2024). In zebrafish, microplastics can be quickly accumulated in gills and gut than in other parts (Chen et al., 2017). Furthermore, the size is the driven factor of microplastic accumulation, because smaller particles can be also accumulated in the lymphatic or circulatory system by the mouth-gastrointestinal tract translocation and/or directly from peripheral vessels via gill/transdermal absorption (Bhagat et al., 2020) and eventually dispersing throughout the body (Kim et al., 2021). In adult zebrafish, 5 μm MPs are observed in the liver (Lu et al., 2016; Qiao et al., 2019), notably the size that can break through the circulatory system which is consistent with the size reported in the gonad in this study. The uptake and accumulation of microplastics are the primary source of toxicity in the organism.

4.2. Microplastics effects on reproductive capacity

The main objective of this study was to evaluate the reproductive toxicity of environmental derived MPs in zebrafish following chronic exposure. Different levels of biomarkers were assessed, such as biomolecular and biochemical responses at the sub-organismal level using enzymatic activity analysis and proteomic analysis as well as reproductive performance and histological analysis at the individual level.

Based on number and profile of DEPs in proteomic analysis, exposure to environmental MPs seems to cause sex and dose-dependent effects, with male groups showing exacerbating effects compared to female. Stress oxidant appears to be the main toxic effect of microplastics (Hu and Palić, 2020; Z. Li et al., 2022). The molecular initiating event of the oxidative stress in the formation of ROS (Reactive Oxygen Species) which have high potential to engage in different reactions, including oxidative chemical reactions (Hu and Palić, 2020). Interestingly, as reported by Pannetier et al. (2020), weathered MPs through photo-oxidation and UV light can generate free radical on their surface, which might be one of the reasons of significant increased ROS after the cellular contact with aged MPs. In our study, apart from the free radicals formed on the particle surface, the pollutants sorbed onto the MP surface, such as the PCB and PBDE congeners (supplementary data Fig. S5), might act a potentiating agent for ROS generation (Varshney et al., 2024). This is supported by findings that PCBs can leach from microplastic under simulated gut fluid conditions (Mohamed Nor and Koelmans, 2019).

The oxidative stress takes place when there is an imbalance between the production of ROS and antioxidant-based neutralization (Pisoschi and Pop, 2015). Following microplastic exposure, the activities of antioxidant enzymes increased during short-term exposure but significantly declined after a chronic exposure (Z. Li et al., 2022), this observation goes in line with our observation as all biological process linked with antioxidant defense were downregulated after the 45 days of exposure (Fig. 6B).

Since the antioxidant enzymes being shut down, the increasing ROS

level is the downfall cascade of oxidative damage (Hu and Palić, 2020). Ding et al. (2024) reported that accumulated ROS caused by aged microplastics affected the mitochondrial function by dysregulating the mitochondrial electron transport chain, complex I gene (*ndufs1*), complex II gene (*Sdha*), complex III gene (*uqcrc1*), complex IV gene (*cox41l*) and complex V gene (*atp5f1c*) which is rather consistent with our findings (Fig. 7). On one hand, mitochondria produce ATP for cells which is the first source of energy. Therefore, mitochondrial dysfunction is linked to energy metabolism imbalance (Hu and Palić, 2020; Morozov and Yurchenko, 2024). In the present study, energy metabolism dysfunction was observed in the glycolysis pathway and PPAR signalling pathway (supplementary data Fig. S7 and S8). On the other hand, mitochondrial dysfunction is a key driver of apoptosis. Genes related to apoptosis mediator (*bcl-2*, *Apaf-1*, *caspase 3,9*, *bax*, *p53*) were overexpressed in a concentration dependent manner after an aged polystyrene exposure in zebrafish larvae, suggesting the induction of apoptosis in zebrafish larvae (Ding et al., 2024; Wang et al., 2024). In our study, apoptosis pathway was also dysregulated. However, instead of an apoptosis induction, a suppression of apoptotic signalling was observed, as proteins such as FADD, Cathepsin, CAPS6, ARTS were downregulated (supplementary data Fig. S9). FADD is crucial for the extrinsic apoptotic pathway (Mai et al., 2023), its downregulation means reduced activation of caspase-8, leading to impaired death receptor induced apoptosis. To the best of our knowledge, microplastic exposure consistently tends to trigger the apoptosis signalling pathway (Wang et al., 2024). Even aged MP (PE 100–180 µm in size), as reported by Michailidou et al. (2024), in foodborne exposure at a concentration of 1 mg/g of dry food over a 15-day period induced such effects in European perch (*Perca fluviatilis*). Cases of apoptosis inhibition may occur during an exposure to a tumour potentiating pollutant, such as non-dioxin like PCBs (Bohnenberger et al., 2003), via the suppression of cell death signalling pathway (p53) (Mohamed Helal, Sara Ghanem, 2022). In the present study, as the majority of PCBs congeners sorbed in the environmental MPs are non-dioxin like PCBs (Fig. S5), apoptosis inhibition might be associated with their bioaccumulation in liver. Effort had been made to mimic the toxicity of microplastic associated with PCBs, as demonstrated by Hou et al. (2024), in which Pacific oyster (*Crassostrea gigas*) were exposed to 50 µg/L (30 µm porous MPs) combined with 10 or 100 ng/L PCBs for 28 days. This exposure led to the suppression of immune-related genes (NF-κB, TNF-α, TLR-6) while the expression of apoptosis-related genes (BAX and Caspase-3) were significantly increased. The divergence between our results and those reported in literature underscores the importance of using environmental microplastics in toxicity assessments. Special attention should be given to the potential contaminants sorbed onto MPs, as these may gradually influence their overall toxicity.

In addition, the Gene Set Enrichment Analysis for the GO molecular function terms showed a dysregulation of protein participating in creatine kinase activity in male group exposed to MPs (Fig. 6A). Creatine kinase is an enzyme crucial for energy metabolism, its dysregulation in liver is a biomarker of hepatotoxicity (Jia et al., 2020). In our study, dysregulated creatine kinase activity in male really confirmed the sex-dependent effect of microplastic exposure, which was also observed by Xia et al. (2023) in Large scale loach (*Paramisgurnus dabryanus*) after 30 days of exposure to rough surface polyethylene (8–15 µm mean diameter). Our data seems to assert that environmental microplastic toxicity is strongly linked with energy metabolism dysregulation caused by oxidative damage in the liver.

Otherwise, no histopathological lesion was observed in gonad female after the 45 days of exposure, even though microplastic particles were confirmed to be present in gonads. Our finding goes in line with the observation of Qiang and Cheng (2021) on zebrafish adult when exposed to 1 µm polystyrene microspheres for 21 days at concentrations of 0–10–100–1000 µg/L. In Marine medaka (*Oryzias melastigma*), Wang et al. (2019) did confirm tissue damages in gills, liver and testis with 10 µm polystyrene exposure but, in ovaries, only a slight delayed oocyte

maturation was observed which was also confirmed in our study (Fig. 4).

Furthermore, after the 21 days of reproduction trial, vitellogenin analysis in female liver and MDA content analysis in gonads did not show any significant difference between the exposed groups and the control. On one hand, vitellogenin is a sensitive biomarker for endocrine disrupting chemicals (EDCs) (Tran et al., 2019), its dysregulation is a key driven factor of reproductive toxicity (Baekelandt et al., 2023). Our data goes on counter to the endocrine disrupting capacity of MPs. Nevertheless, studies confirmed that microplastics can act as a transporter of EDCs in organism (Ullah et al., 2023). On other hand, MDA content is the biomarker of lipid peroxidation in tissue, which is a self-sustaining chain reaction induced by excessive ROS production resulting from an oxidative damage (Nam, 2011). Somehow, lipid peroxidation induced by microplastic exposure seems to be controversial in the literature, as some authors did not observe any change in MDA level after a microplastics exposure (Hu and Palić, 2020).

Despite any changes observed in vitellogenin and MDA content, at the individual level, our study confirmed the reproductive toxicity of MPs, as fertility and fecundity were significantly lower in the group exposed to MPs compared to the control. Several authors go in line with our observations, for example: Qiang and Cheng (2021), assumingly stated the reproductive toxicity of microplastics as increased level of apoptotic cells were observed in testis of zebrafish adult, after a 1 µm polystyrene exposure for 21 days. Cormier et al. (2022) exposed adult zebrafish to a food supplemented with environmental microplastics (10 mg/g ww of particles having a size ranging from 53 to 100 µm) sampled from the sand surface of two beaches: Capesterre in Marie-Galante Island (MG), and Petit Bourg (PB) in Guadeloupe Basse-Terre Island. The authors reported that microplastics from PB really altered the reproductive capacity, assuming that the toxicity might be due to bioenergetic disruptions as no oxidative stress were observed. However, in our study, reproductive toxicity might more likely be linked to the male group as not only they accumulated higher amount of MPs in gonads but also seems to be more sensitive to the oxidative damage and metabolism disruption. Hepatotoxicity may contribute to reproductive failure through several mechanisms, including altered steroid metabolism (Z. Li et al., 2022), energy reallocation (Sokolova et al., 2012), and mitochondrial dysfunction leading to energy deficiency (Pan et al., 2018). Microplastic accumulation in the gonads may also impair sperm quality via oxidative stress (Gao et al., 2023).

5. Conclusions

This study demonstrates the reproductive toxicity of environmentally derived microplastics collected from a river in a densely populated area. These microplastics, composed of diverse polymers, additives, and sorbed pollutants, underwent proportional shifts in term of polymer composition from the environment to zebrafish gonads, with polymer properties influencing their distribution (e.g., PEVA predominance in specific size ranges). Waterborne exposure significantly reduced zebrafish fecundity and fertility, with males showing higher sensitivity than females, likely due to oxidative stress, metabolic disruption, and greater microplastic accumulation in their gonads. Notably, apoptosis inhibition rarely reported in microplastic exposure was observed during this study. This key event might be linked to the bioaccumulation of sorbed pollutants identified on microplastics, underscoring the importance of using environmentally derived microplastics as test material. Due to the constraints in the amount of collected microplastics, this study was only focused on the onset of sexual maturation which is already a critical but narrow developmental window. Future studies should extend exposures from fertilization to sexual maturity to better reflect real-world scenarios. Moreover, research should investigate the dynamics of microplastic accumulation and depuration across developmental stages, as well as the combined effects of sorbed pollutants, to provide deeper insight into the mechanisms driving reproductive toxicity.

Environmental implications

Using environmentally derived microplastics as test material *in vivo* experiments represents a significant step forward in improving the representativeness of microplastic toxicity assessment studies. As previously emphasized by several authors and further confirmed in this study, the complex mixture of polymer types, microplastic sizes, additives, and sorbed pollutants underscores the uniqueness of environmental microplastics. By linking comprehensive chemical profiling to reproductive toxicity in zebrafish, the research demonstrates the critical ecological risks posed by realistic environmental MPs. These findings emphasise the urgent need to incorporate a thorough chemical analysis of environmental MPs into ecological risk assessments. Their complex interactions and toxicological impacts pose a significant threat to aquatic biodiversity and the sustainability of ecosystems.

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CRediT authorship contribution statement

Andry Ny Aina Rabezanahary: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Baptiste Ducarme:** Investigation, Formal analysis, Data curation. **Andriamalala Rajoelisoa:** Methodology. **Yves Jean Michel Mong:** Supervision, Resources. **Aala Azari:** Methodology. **Manosij Ghosh:** Methodology. **Peter H.M. Hoet:** Supervision, Resources. **Hugo B. Muller:** Methodology. **Gauthier Eppe:** Methodology. **Benoît Bernay:** Methodology, Investigation. **Ranjana Hanitra Randrianarivo:** Validation, Supervision. **Patrick Kestemont:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Omayma Missawi:** Writing – review & editing, Investigation, Conceptualization. **Valérie Cornet:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Patrick Kestemont reports financial support was provided by Fund for Scientific Research. Andry Ny Aina Rabezanahary reports financial support was provided by Academy of Research and Higher Education. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.hazadv.2025.100923](https://doi.org/10.1016/j.hazadv.2025.100923).

Data availability

Data will be made available on request.

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