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Effects of polystyrene nano- and microplastics and of microplastics with sorbed polycyclic aromatic hydrocarbons in adult zebrafish

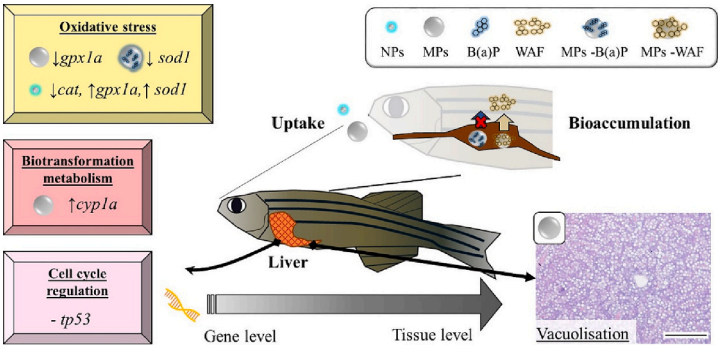
Ignacio Martínez-Álvarez [a,b], Karyn Le Menach [b], Miren P. Cajaraville [a], Hélène Budzinski [b], Amaia Orbea [a,*]

[a] CBET Research Group, Dept. of Zoology and Animal Cell Biology; Research Centre for Experimental Marine Biology and Biotechnology PiE and Science and Technology Faculty, University of the Basque Country (UPV/EHU). Sarriena z/g, E-48940 Leioa, Basque Country, Spain
[b] University of Bordeaux, EPOC-LPTC, UMR 5805 CNRS, F-33405 Talence Cedex, France

HIGHLIGHTS

- Zebrafish were exposed to PS MPs with sorbed B(*a*)P or a realistic mixture of oil PAHs.
- Contaminated MPs did not cause PAHs accumulation in zebrafish.
- MPs and NPs caused differential effects to adult zebrafish.
- Exposure to MPs upregulated *cyp1a* and *gstp1*in zebrafish liver.
- Exposure to NPs regulated oxidative stress related genes in liver.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of nanoplastics (NPs) and microplastics (MPs) in the environment is recognised as a global-scale problem. Due to their hydrophobic nature and large specific surface, NPs and MPs can adsorb other contaminants, as polycyclic aromatic hydrocarbons (PAHs), and modulate their bioavailability and hazard. Adult zebrafish were exposed for 3 and 21 days to: (1) 0.07 mg/L NPs (50 nm), (2) 0.05 mg/L MPs (4.5 μm), (3) MPs with sorbed oil compounds of the water accommodated fraction (WAF) of a naphthenic crude oil (MPs-WAF), (4) MPs with sorbed benzo(*a*)pyrene (MPs-B(*a*)P), (5) 5 % WAF and (6) 21 μg/L B(*a*)P. Electrodense particles resembling NPs were seen in the intestine lumen close to microvilli. MPs were abundantly found in the intestine lumen, but not internalised into the tissues. After 21 days, NPs caused a significant downregulation of *cat*, and upregulation of *gpx1a* and *sod1*, while MPs upregulated *cyp1a* and increased the prevalence of liver vacuolisation. No histopathological alteration was observed in gills. In this study, contaminated MPs did not increase PAH levels in zebrafish but results highlight the potential differential impact of plastic particles depending on their size, making it necessary to urgently address the ecotoxicological impact of real environmental NPs and MPs.

* Corresponding author.
E-mail address: amaia.orbea@ehu.eus (A. Orbea).

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1. Introduction

Plastic debris has been identified as a source of contamination of high concern in aquatic ecosystems (Villarubia-Gómez et al., 2018). The main focus of research, in terms of debris amount, has been put on the marine ecosystems (Pham et al., 2014; Werner et al., 2016), outshining freshwater ecosystems which have been mainly considered only as pathways to the marine ecosystems (Schmidt et al., 2017) and not as a plastic debris storage compartment. Within debris, a large number of items are microplastics (MPs, <5 mm) (Barnes et al., 2009; Sadri and Thompson, 2014), while the smallest particles, nanoplastics (NPs, <1000 nm), are overall largely unquantified due to the lack of reliable quantification techniques. Nevertheless, NPs are expected to appear in similar quantities as MPs (Gigault et al., 2016). According to the analyses carried out by Materić et al. (2022) in the Dutch Wadden Sea, polystyrene (PS) NPs reach a concentration of 4.2 ± 2.4 μg/L. Due to the manufacture of MPs and NPs (primary plastics) and the breaking down of larger plastic to MPs and NPs (secondary plastics), the number of MPs and NPs present in aquatic ecosystems will continue to increase in the next years (Besseling et al., 2019; Walker and Fequet, 2023). Therefore, it becomes necessary to analyse the potential impact of the presence of MPs and NPs on aquatic organisms.

Fish are target species for MP and NP ingestion because of the similar size of plastic particles and their preys, such as planktonic species. Moreover, plankton can also accumulate plastic particles, increasing the risk for fish to ingest and accumulate MPs and NPs through a biomagnification process (Saley et al., 2019; Elizalde-Velázquez et al., 2020). Ingestion of noticeable quantities of MPs (1–20 items/individual) has been reported in freshwater fish inhabiting areas with high population pressure (Jabeen et al., 2017; Wang et al., 2020), with polyethylene (PE), polypropylene (PP) and PS being the three most abundant polymer types found in freshwater fish (Egessa et al., 2020). Among them, PS shows an intermediate density (0.96–1.05 g/cm^3), similar to that of water, which allows this polymer to be distributed on surface waters or to sink to bottom waters and sediments. Hazard of PS MPs and NPs to aquatic and terrestrial organisms has been well documented, especially for aquatic invertebrates as reviewed by Qiao et al. (2022). Nevertheless, the need of further studies focused on environmentally relevant concentrations and combined exposures with other pollutants is being increasingly highlighted (Qiao et al., 2022; Shi et al., 2024).

Zebrafish (*Danio rerio*) is a common experimental freshwater species used in toxicology to assess the impact of MPs and NPs alone and in combination with other environmental pollutants (Chen et al., 2017a; Jin et al., 2018; Lei et al., 2018; Lu et al., 2018; Zhang et al., 2023; Jian et al., 2024; Pamanji et al., 2024; Rong et al., 2024; Xu et al., 2024; Zhang et al., 2024; Zhao et al., 2024). Thus, it has been proposed as an emerging model for research on MP and NP effects (Bhagat et al., 2020). MPs and NPs of different size (0.1–90 μm), composition (PS, PP, PE, polyvinyl-chloride (PVC)) and concentrations (1–10,000 μg/L) have been evaluated in zebrafish. Results showed a great variety of biological effects. Gut inflammation and changes in genes related to the immune system in the gills, driven by MP concentration and size have been reported (Lei et al., 2018; Gu et al., 2020; Umamaheswari et al., 2021). Oxidative stress has also been observed in zebrafish after exposure to PS NPs (Sarasamma et al., 2020; Shi et al., 2024). Increased reactive oxygen species (ROS) levels in zebrafish muscle along with a neurotoxic effect in brain (decreased acetylcholinesterase activity) has been reported in zebrafish exposed for 7 days to 1.5 mg/L of 70 nm PS NPs (Sarasamma et al., 2020). The ability of MPs and NPs to disturb the lipid and energy metabolism (Sarasamma et al., 2020) and dysbiosis of gut microbiota are being increasingly reported (Li et al., 2023). In addition to size, other features of the MPs, such as shape and additive content, can also modulate their effects on aquatic organisms (Teuten et al., 2007; Ašmonaitė et al., 2018).

One of the most concerning characteristic of MPs and NPs is their capability to sorb organic pollutants due to their hydrophobicity, porosity and large surface area to volume ratio (Wang et al., 2019b; Wirnkor et al., 2019; Cássio et al., 2022; Martínez-Álvarez et al., 2022a). MPs can reach to sites with high contamination pressure, such as harbours, where crude oil pollution is common, or water waste treatment plants (Xu et al., 2019), where the plastic could sorb contaminants and return to aquatic ecosystems through effluents. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants in the aquatic ecosystems and, due to their high hydrophobicity, can be sorbed to MPs (Rochman et al., 2013; Bouhroum et al., 2019; Martínez-Álvarez et al., 2022a). PAHs sorbed to MPs could produce toxicological effects on aquatic organisms after ingestion, as sorbed pollutants could be released within the organism (Bhagat et al., 2021). Some studies have already addressed the transfer of PAHs from contaminated MPs to adult fish (Batel et al., 2016; Karami et al., 2016; Ašmonaitė et al., 2018; Batel et al., 2018; Bussolaro et al., 2019; Tarasco et al., 2022) and have reported diverse effects of MPs or NPs and associated PAHs on different fish species, such as rainbow trout (Ašmonaitė et al., 2018; Bussolaro et al., 2019), zebrafish (Batel et al., 2018; Tarasco et al., 2022) and African catfish (Karami et al., 2016), showing alterations in genotoxicity and potential reduction in PAHs toxicity when exposed to B(*a*)P and phenanthrene. Additionally, 100–140 μm contaminated plastic pellets could induce hepatic stress in rainbow trout without affecting fillet quality after 28 days of exposure (Ašmonaitė et al., 2018), while in zebrafish, PE MPs facilitated the transport of pollutants like B(*a*)P, particularly through direct contact with gills after 24 h of exposure (Batel et al., 2018).

The aim of the present study was to determine the toxicity of PS NPs and MPs and of MPs with sorbed PAHs to adult zebrafish. We hypothesised that exposure to MPs in combination with PAHs will lead to PAH transfer and, thus, bioaccumulation in fish tissues. This, in turn, could lead to alterations in affect biotransformation metabolism and oxidative stress, which could result into histopathological alterations in the gills and liver. Higher impact would be expected for MPs with sorbed PAHs than for pristine MPs. In addition, the size of PS particles (NPs versus MPs) could determine their mechanism of toxicity. Therefore, the specific objectives were: (1) to assess the sublethal toxicity of 50 nm PS NPs and of 4.5 μm PS MPs alone and with sorbed B(*a*)P, as a model pyrolytic PAH, or with sorbed petrogenic PAHs from an environmentally relevant mixture, namely, the water accommodated fraction (WAF) of a naphthenic North Sea crude oil; (2) to assess the potential transfer and bioaccumulation of PAHs from 4.5 μm PS MPs to adult zebrafish.

2. Materials and methods

2.1. Plastic particles and chemicals

50 nm fluorescent Fluoresbrite® carboxylate polystyrene NPs (excitation/emission wavelengths of 360/407 nm) and 4.5 μm polystyrene MPs in aqueous suspensions were purchased from Polysciences, Inc. (Warrington, PA, USA). The concentration of the commercial stocks was 2.5 % (3.64 × 10^{14} particles/mL for 50 nm NPs and 4.99 × 10^8 particles/mL for 4.5 μm MPs). According to the manufacturer's information, particles presented a glass transition temperature of 95 °C, density: 1.05 g/cm^3, and a refractive index at 589 nm of 1.59. Behaviour of these particles with and without sorbed benzo(*a*)pyrene (B(*a*)P) was previously analysed by dynamic light scattering and results are reported in Katsumiti et al. (2021). B(*a*)P ($C_{20}H_{12}$, purity ≥96 %) and dimethyl sulfoxide (DMSO, purity ≥96 %) were purchased from Sigma-Aldrich. The naphthenic North Sea (NNS) crude oil was provided by Driftslaboratoriet Mongstad, Equinor (former Statoil). The oil was a very light naphthenic crude oil, with density of 0.845 g/cm^3 at 15 °C and pour point at −15 °C, rich in branched and cyclic saturated hydrocarbons, little wax content, poor thermal and oxidative stability and high octane content (Statoil, 2011). Deuterated PAHs (naphthalene d_8, acenaphthylene d_{10}, acenaphthene d_{10}, fluorene d_{10}, anthracene d_{10}, phenanthrene d_{10}, fluoranthene d_{10}, pyrene d_{10}, benzo(*a*)anthracene d_{12},

chrysene d_{12}, benzo(*e*)pyrene d_{12}, B(a)P d_{12}, perylene d_{12}, benzo(*b*) fluoranthene d_{12}, benzo(*k*)fluoranthene d_{12}, indeno(1,2,3-*cd*)pyrene d_{12}, benzo(*ghi*)perylene d_{12} and dibenz(*a*,*h*)anthracene d_{14}) used for chemical analyses were also purchased from Sigma-Aldrich.

2.2. Preparation of PAH solutions and MPs with sorbed PAHs

The WAF was prepared based on Singer et al. (2000). A 5 L glass bottle was filled with 4 L of conditioned water (600 μS/cm, 7–7.5 pH) and 20 g of NNS oil. The bottle was wrapped with aluminium foil and placed in a magnetic stirrer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 800 rpm and 21 °C. After 40 h, the aqueous phase was collected in a glass bottle avoiding taking oil droplets. MPs with sorbed PAHs were prepared according to the protocol described by Martínez-Álvarez et al. (2022a). B(*a*)P was firstly dissolved in 100 % DMSO at a concentration of 10 g/L. This solution was diluted in pure DMSO to obtain a B(*a*)P stock of 1 g/L. Then, a 1:10000 dilution was made in MilliQ water to obtain a B(*a*)P solution of 100 μg/L (0.01 % DMSO (*v*/v)) which was used to contaminate MPs. For MP contamination, 1.75 mg of 4.5 μm MPs were incubated in a glass bottle containing 35 mL of 100 % WAF or 35 mL of 100 μg/L B(*a*)P in order to maintain the plastic mass to incubation volume ratio used in Martínez-Álvarez et al. (2022a). MP suspensions were wrapped with aluminium foil and shaken at 300 rpm for 24 h at 20 °C. Then, samples were filtered through a polyethersulfone filter (0.45 μm filter pore, Sarstedt AG & Co., Nümbrecht, Germany) and washed two times with 10 mL of MilliQ water. MPs were recovered from the filter using 10 mL of conditioned water. These MPs-B(*a*)P and MPs-WAF suspensions were added to the exposure water tanks (35 L) to reach the desired MP concentration (0.05 mg/L). This process was repeated every three days to prepare freshly contaminated MPs for each redosing.

2.3. Waterborne exposure of adult zebrafish

The wild type zebrafish stock was maintained in a temperature-controlled room at 28 °C with a 14-h light/10-h dark cycle in 100 L tanks provided with mechanic and biological filters following standard protocols for zebrafish culture. Conditioned water (600 μS/cm, 7–7.5 pH) was prepared from deionised water and commercial salt (SERA, Heinsberg, Germany). Fish were fed with Vipagran Baby Nature (Sera) and brine shrimp larvae (Artemia Koral GmbH, Nürnberg, Germany) twice per day. The experimental procedure described herein was approved by the Ethics Committee in Animal Experimentation of the UPV/EHU (M20/2017/152) according to the current regulations. Adult fish of >7 months old were placed in 35 L aquaria with conditioned water. Fish were exposed to NPs, MPs, MPs-WAF, WAF, MPs-B(*a*)P and B(*a*)P for 3 or 21 days. In the case of 50 nm NPs, fish were exposed to ~0.07 mg/L equivalent to 10^{12} particles/L. In the treatments containing 4.5 μm MPs (MPs, MPs-WAF and MPs-B(*a*)P), fish were exposed to ~0.05 mg/L equivalent to 10^{6} particles/L. A group of fish was exposed to 5 % WAF, a WAF dilution containing an equivalent PAH amount to that which was sorbed to MPs. Another group of fish was exposed to 21 μg B(*a*)P/L, which was equivalent to the B(*a*)P which was sorbed to MPs. The WAF dilution and the B(*a*)P concentration were selected based on the study of PAH sorption onto PS MPs described in Martínez-Álvarez et al. (2022a) and on Chen et al. (2017b). Finally, an unexposed control group was run in parallel in identical experimental conditions. Every 3 days, 2/3 of the water volume of each tank was renewed to re-establish contaminant concentrations (Esteban-Sánchez et al., 2021) and to maintain water parameters without the need of biological filters. Over the first three days of the experiment, fish were distributed in two aquaria per experimental group to maintain a fish density of 1–2 fish/L. During the experimental period, fish were fed with live brine shrimp larvae twice per day. No mortality was reported during experiments. Fish samples were collected after 3 and 21 days of exposure after euthanasia by overdose of anaesthetic (200 mg/L benzocaine).

2.4. Subcellular localisation of NPs: Transmission electron microscopy (TEM) analysis

Gills, liver and intestine from five control fish and five fish exposed to NPs for 21 days were dissected and fixed for 1 h at 4 °C in sodium cacodylate (Sigma-Aldrich) buffer 0.1 M, pH 7.2, containing 2.5 % glutaraldehyde (Panreac, Barcelona, Spain). Zebrafish tissue samples were treated and processed as described in Lacave et al. (2018). Ten ultrathin sections of 50 nm in thickness were cut using a Reichert Ultracut S ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were picked up in 150 mesh copper grids, contrasted with 1 % uranyl acetate (Fluka, Steinheim, Germany) for 3 min and with 0.3 % lead citrate (Fluka) for 4 min and, finally, examined and photographed using a Jeol JEM-1230 transmission electron microscope (Tokyo, Japan) at 60 kV. Additionally, the NP stock suspension was also observed at TEM. For that, one drop of the suspension (3.64 10^{14} particles/mL or 25 g/L) was placed over a 150 mesh copper grid previously covered with Formvar® (Sigma Aldrich) and dried at 35 °C.

2.5. Histological processing

Visceral mass and gills were dissected from 10 female fish per experimental group after 3 and 21 days of exposure. Samples were processed for histological analysis using N-butyl alcohol (Stiles, 1934) instead of other organic solvents to prevent the degradation of the PS MPs during standard histological tissue processing. Tissues were placed in histological cassettes and immersed in neutral buffered formalin (4 % formaldehyde) overnight at 4 °C. Then, samples were dehydrated in ascending ethanol/N-butyl solutions (González-Soto et al., 2019) and embedded in paraffin wax. 5 μm sections were cut in a RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). Afterwards, sections were dewaxed utilising N-butyl alcohol and stained with haematoxylin/eosin (H/E) in an Auto Stainer XL (Leica Microsystems) and manually mounted in glycerol (Sigma-Aldrich). Tissue preparations were examined and photographed under a BX51 light microscope (Olympus, Tokyo, Japan). For MP quantification in the lumen of zebrafish intestine, four sections per individual were used and the quantity of particles found in those four sections were summed.

2.6. Measurement of PAH concentration in water and fish tissue

In the treatments containing PAHs (MPs-WAF, WAF, MPs-B(*a*)P and B(*a*)P) and in the control group, water samples were taken in triplicate in glass vials at day 0, 9 and 18 (1st, 4th and 7th dose) 30 min after dosing and at day 3, 12 and 21 just before water renewal. PAH quantification in water was performed by gas chromatography and mass spectrometry (GC/MS) after solid phase micro extraction (SPME). SPME consisted of a heating process at 40 °C with a 35 min stirring period at 250 rpm of the polydimethylsiloxane (100 μm PDMS) fibre (Supelco, Sigma-Aldrich, South Africa). After extraction, the fibre was thermally desorbed into the GC/MS system (Agilent GC 7890 A/Agilent MSD 5975C, Agilent Technology, California) for 10 min at 280 °C.

At 3 and 21 days of exposure, 20 fish per experimental group (control, MPs-WAF, WAF, MPs-B(*a*)P and B(*a*)P) were collected and grouped in 4 pools of 5 fish of similar weight (≈1 g wet weight). Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were freeze dried (Power Dry LL3000, ThermoFisher Scientific, Vantaa, Finland) before being grinded in an IKA tube mill (ThermoFisher Scientific). PAHs were extracted from 0.2 g of dry weight samples by microwave-assisted extraction (Start E, Milestone, Italy) with dichloromethane (5 min at 900 W and 5 min at 500 W 70 °C). After extraction, dichloromethane was reconcentrated to 500 μL using a Vacuum Evaporation System (Rapidvap Labconco, Kansas City, USA). The organic extracts followed a purification step through alumina and silica micro-columns to remove macromolecules and polar molecules to avoid interference with PAH quantification. First, the extracts were

passed through alumina column by dichloromethane elution. The extract was reconcentrated with gas nitrogen and then samples were passed through a silica column. The aliphatic fraction was eluted with pentane and discarded, followed by PAH fraction elution with a first elution using a mix of pentane/dichloromethane (65/35, *v*/v) and second elution using dichloromethane. The final extracts were reconcentrated again with gas nitrogen in 150 μL isooctane and analysed by GC–MS.

2.7. Analysis of gene transcription levels

The liver of 15 male zebrafish per experimental group were dissected, placed in cryovials, frozen in liquid nitrogen and stored at −80 °C. Analysis of the transcription levels of target genes was done in pools of three livers resulting in five biological replicates per experimental group and exposure time. The analysed genes were: cytochrome P450 1A1 (*cyp1a*, ID: Dr03112441_m1*)* and glutathione S-transferase pi 1 (*gstp1*, ID: Dr03118992_g1) as genes related with biotransformation metabolism of organic compounds; catalase (*cat*, ID: Dr03099094_m1*)*, superoxide dismutase 1 (*sod1*, ID: Dr03074068_g1*)* and glutathione peroxidase 1a (*gpx1a, ID:* Dr03071768_m1*)* as genes related with oxidative stress; and tumour protein 53 (*tp53*, ID: Dr03112086_m1) as a gene related with cell cycle regulation. Ribosomal protein S18 (*rps18*, ID: Dr03144509_ml) was used as a housekeeping gene. Taqman® probes were purchased from ThermoFisher Scientific. RNA extraction was carried out by homogenisation of the sample in cold TRIzol® using an electric disperser. RNA was measured for integrity and purity before cDNA synthesis. 3 μg of total RNA were retrotranscribed using the Affinity Script Multiple Temperature cDNA synthesis Kit (Agilent Technologies) following manufacturer's instructions in a 2720 Thermal Cycler (ThermoFisher Scientific). qPCRs were run in 7300 Applied Biosystems thermocycler (ThermoFisher Scientific). A dilution 1:5 of cDNA was done for each target gene. A final volume of 2.5 μL cDNA sample and 22.5 μL mix TaqMan® reaction (12.5 μL master mix +1.25 μl primer probe +8.75 μL water RNase free) was used. Relative transcription levels were calculated based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the mean value of the control group at each corresponding exposure time as calibrator and *rps18* transcription levels as a reference gene. *rps18* transcription levels presented a coefficient of variation of 3.46 % among all the samples. Results of transcription levels are represented as $RQ = \log_2 (2^{-\Delta\Delta CT})$.

2.8. Data analysis and statistics

Data were tested for normality (Kolmogorov-Smirnov's test) and homogeneity of variance (Bartlett's test) using the GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, California, USA). The interaction between MPs and PAHs was analysed using a two-way ANOVA in R package (R Foundation for Statistical Computing, Vienna, Austria). Data that did not follow a Normal distribution were ranked-based transformed before conducting the test. In order to analyse specific effect of each variable and statistical differences among treatments, one-way ANOVA followed by the Tukey's post-hoc test was applied for data following a Normal distribution and Kruskal-Wallis's test followed by the Dunn's post-hoc test for non-parametric data using GraphPad Prism. For number of particles per fish, Mann-Whitney's *U* test was applied. For prevalence of histopathological alterations, Fisher's exact test was used. In all cases, significance level was established at $p < 0.05$.

3. Results

3.1. Localisation of plastic particles

50 nm PS NPs were visualised at the TEM as round electrodense particles (Fig. 1A). Presence of small electrodense structures resembling NPs according to the appearance shown in Fig. 1A were localised among microvilli located in the apical zone of the intestine cells (Fig. 1B). Inside cells, vesicles that could contain NPs internalised by endocytic processes were not observed and free particles in the cytosol could not be distinguished from other cell structures. In the secondary lamella of the gills and in liver of fish exposed to NPs, the presence of round structures with similar size to the commercial PS NPs were observed inside the tissues but they could not be unequivocally distinguished from other cell structures.

Fluorescent PS NPs of 50 nm could not be localised in paraffin-embedded tissues due to high autofluorescence of the sample. Ingestion of 4.5 μm MPs was observed in all groups exposed to MPs (MPs, MPs-WAF, MPs-B(*a*)P) as can be seen in Fig. 2. Unexposed fish did not show presence of MPs. At light microscopy level, MPs were not seen inside the cells of the organs examined (intestine, liver, and gills), but abundant presence of MPs was detected in the lumen of the intestine at the two exposure times (Fig. 3) with high variability among individuals. The number of MPs found increased with exposure time. After 3 days, fish exposed to MPs-B(*a*)P presented 30.5 ± 37.37 particles/fish, followed by fish exposed to pristine MPs with 6.4 ± 16.574 particles/fish and finally by fish exposed to MPs-WAF with a presence of 0.44 ± 1.33 particles/fish. After 21 days, fish exposed to MPs-WAF showed the highest presence of particles with a value of 112.78 ± 222.32 particles/fish, being this amount significantly higher than that scored in the same exposure group at 3 days of exposure. Similar values were obtained in fish exposed to pristine MPs and to MPs-B(*a*)P with values of 45.5 ±

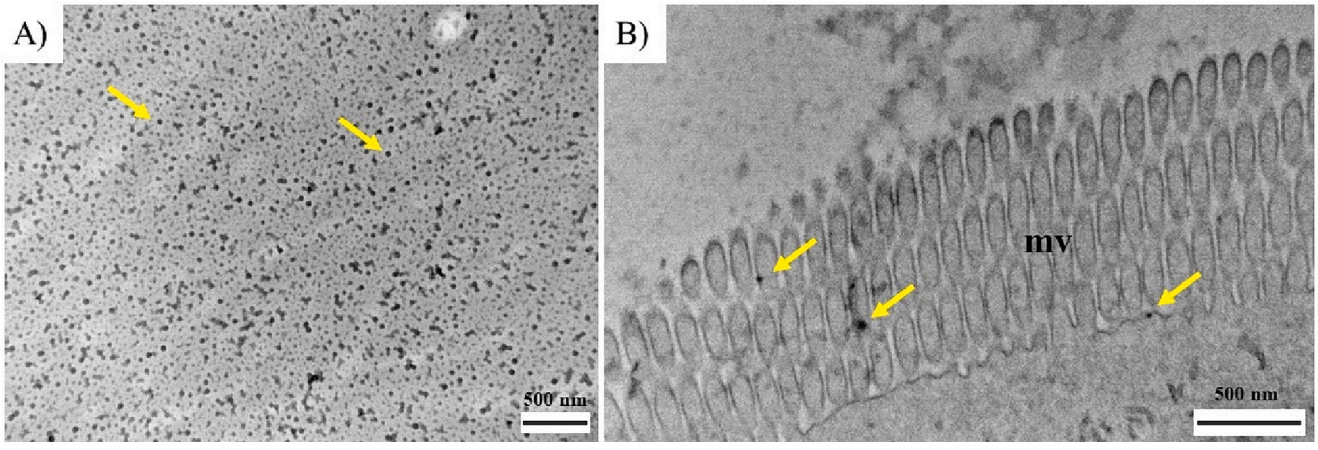

Fig. 1-. TEM micrographs showing A) 50 nm NPs; B) apical zone of the enterocytes of adult zebrafish after 21 days of exposure to 0.07 mg/L of NPs. The presence of electrodense structures resembling NPs (yellow arrows) was detected close to the microvilli (mv).

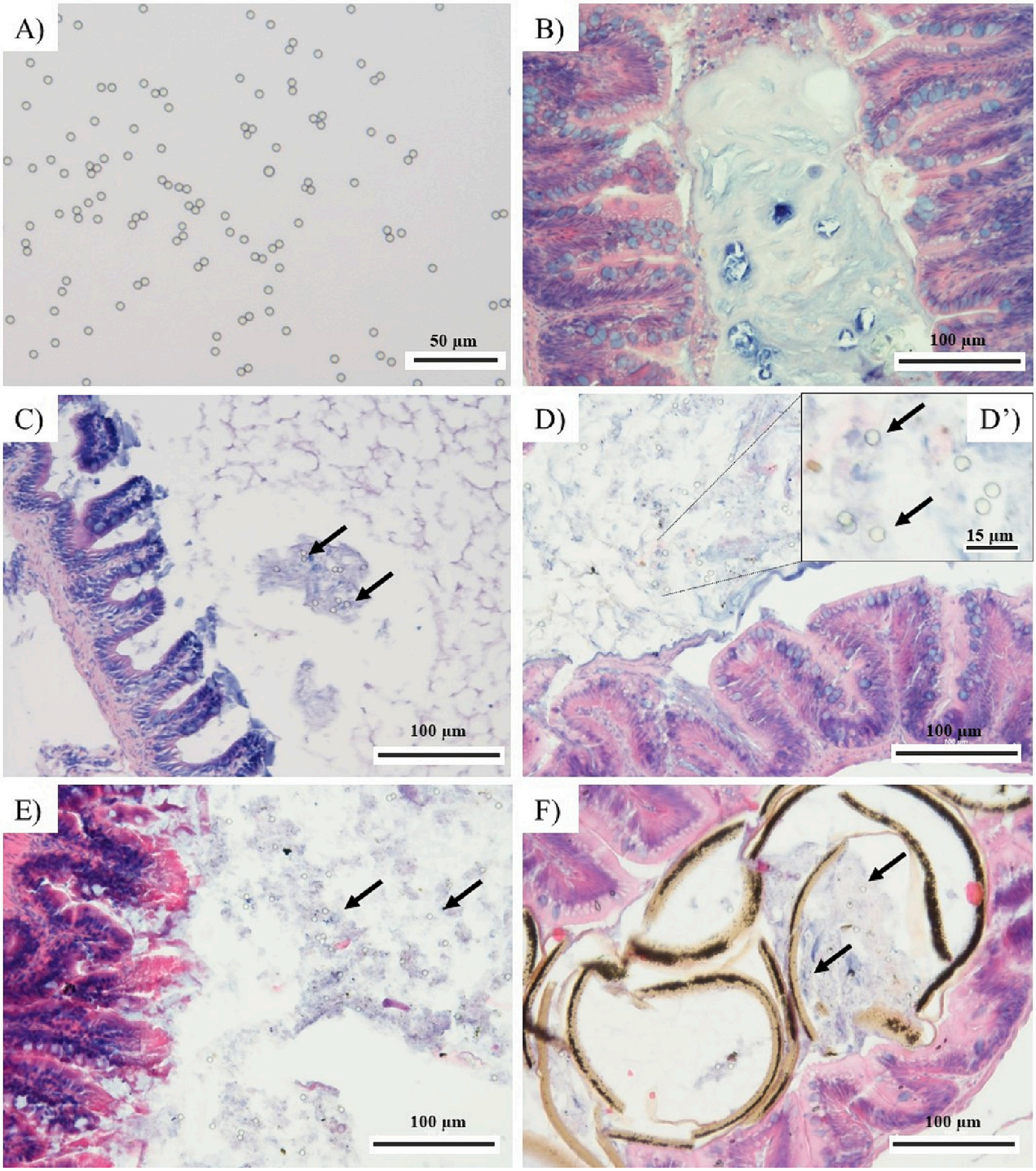

Fig. 2-. Micrographs of 4.5 µm MPs and of histological sections of zebrafish intestine. A) 4.5 µm MPs; B) control zebrafish at 3 days; C) zebrafish exposed to 4.5 µm pristine MPs for 3 days showing accumulation of MPs in the intestine lumen (black arrows); D) zebrafish exposed to 4.5 µm pristine MPs for 21 days; D′) MPs (black arrows) in the intestinal lumen observed at higher magnification; E) zebrafish exposed to MPs-WAF for 21 days, also showing abundant MPs (black arrows) in the lumen; F) zebrafish exposed to MPs-B(*a*)P for 21 days, MPs (black arrows) appear together with empty *Artemia* cysts in the lumen of the intestine.

63.92 and 38.3 ± 60.89 particles/fish, respectively. The number of particles scored in fish exposed to pristine MPs for 21 days was also significantly higher than after 3 days of exposure.

3.2. PAH concentration in water and fish tissues

PAH concentrations in the WAF stock, in the experimental tanks and the limits of quantification are given in Table 1. PAHs were not detected in the control tank, except for naphthalene, which appeared at low concentrations in only one of the three replicates collected at day 0 (0.03 µg/L) and day 3 just before water renewal (0.02 µg/L). In the tank dosed with 5 % WAF, the measured PAH concentrations were lower than the expected concentrations based on the measurements done in the WAF stock solution. For example, concentration of naphthalene was 6.56 ± 0.96 µg/L, 43 times lower than in the 100 % WAF stock (286.77 ± 37.49 µg/L). Similar concentration ratios were measured for fluorene

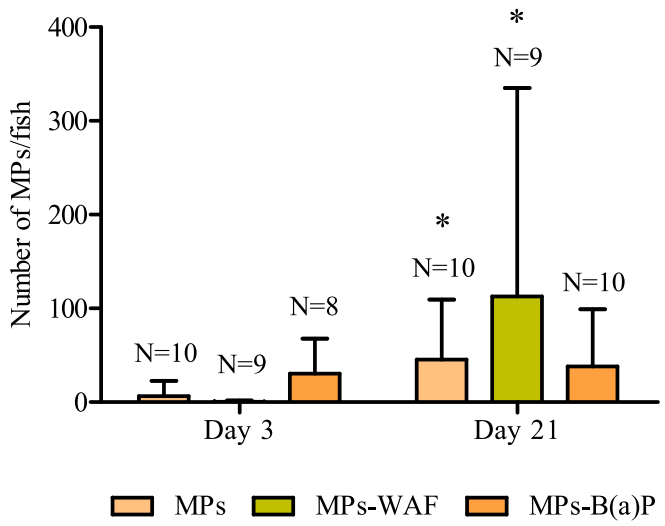

Fig. 3-. MPs found in the lumen of the intestine. Data are shown as means ± SD. Asterisks indicate statistically significant differences ($p < 0.05$) for the same treatment between exposure days according to Mann Whitney U test. N = individuals per experimental group. In some cases, N < 10 because the intestine tissue was not always present in the histological sections used for the analysis.

and phenanthrene, with a concentration of 0.12 ± 0.01 μg/L and 0.09 ± 0.02 μg/L, respectively, in the 5 % WAF tank 30 min after dosing, when the concentration in the 100 % stock was 4.66 ± 0.99 μg/L and 3.33 ± 1.48 μg/L. Three days after redosing, almost all the PAHs disappeared from the 5 % WAF exposure tank or were lower than the limits of quantification, except for naphthalene (0.33 ± 0.41 μg/L), fluorene (0.01 μg/L) and phenanthrene (0.02 μg/L).

In the tank containing MPs-WAF, only naphthalene was detected at a concentration of 0.063 ± 0.042 μg/L 30 min after dosing and 0.04 ± 0.03 μg/L after 3 days. In the tank dosed with B(*a*)P alone, the measured B(*a*)P concentration was 4.92 ± 3.16 μg/L after 30 min when the nominal exposure concentration was 21 μg/L. After 3 days, B(*a*)P concentration decreased up to 0.16 ± 0.12 μg/L. Finally, B(*a*)P was not detected in the tank dosed with MPs-B(*a*)P.

PAH accumulation after 3 and 21 days of exposure was analysed in all groups of fish exposed to PAHs alone or to MPs with sorbed PAHs as well as in control fish (Table 2). Unexposed fish showed a total PAH concentration of 68.65 ± 8.19 ng/g at 3 days and 64.01 ± 17.49 ng/g at 21 days. Similar concentrations were quantified in fish exposed to MPs-WAF, 78.67 ± 22.05 ng/g and 77.98 ± 25.65 ng/g at 3 and 21 days, respectively, showing no increased accumulation along exposure time. Furthermore, fluorene, with values of about 25 ng/g at 3 and 21 days, and phenanthrene, with values of 38.98 ± 3.04 ng/g at 3 days and 35.21 ± 4.40 ng/g at 21 days, showed the highest concentrations. Fish exposed to WAF showed increased PAH concentration up to 910.03 ± 203.69 ng/g and 736.53 ± 108.83 ng/g at 3 and 21 days, respectively. Naphthalene was the most concentrated compound at 3 days, but the concentration dropped from 502.88 ± 94.10 ng/g to 109.16 ± 41.84 ng/g at 21 days. On the contrary, the concentrations of other PAHs in whole fish tissues, such as acenaphthene, fluorene, phenanthrene, and anthracene, increased with exposure time. In fish exposed to MPs-B(*a*)P, B(*a*)P was quantifiable only in one replicate at 21 days of exposure (3 ng/g). For fish exposed to B(*a*)P alone, B(*a*)P concentration did not increase along exposure time with values of 40.25 ± 4.09 and 33.00 ± 3.5 μg/L at 3 and 21 days of exposure, respectively.

3.3. Transcription levels of target genes

The gene transcription levels in the liver of fish exposed to NPs and pristine MPs alone are shown in Fig. 4. Exposure to NPs for 3 or 21 days did not significantly alter the transcription level of genes related to biotransformation metabolism (*cyp1a* and *gstp1*) and cell cycle regulation (*tp53*). Exposure to MPs for 21 days resulted in a significant up-regulation of *cyp1a* and *gstp1* compared to control fish and to fish exposed to NPs, respectively. Regarding the genes coding for the antioxidant enzymes, exposure to NPs for 3 days did not provoke any effect, but at 21 days significant alteration of the transcription levels of the three genes was observed. Whereas *cat* was downregulated compared to control fish and fish exposed to MPs, *gpx1a* and *sod1* were upregulated. Treatment with MPs only provoked downregulation of *gpx1a* after 3 days of exposure, compared to the control group and to fish exposed to NPs.

The transcription levels of *cyp1a, gstp1* and *tp53* in the liver of fish exposed to MPs alone or with sorbed PAHs and to PAHs alone are shown in Fig. 5 and the results of the two-way ANOVA are given in Table 3. *Cyp1a* showed a significant up-regulation in fish exposed to MPs-WAF for 3 days compared to fish exposed to MPs, MPs-B(*a*)P and B(*a*)P. Exposure for 21 days to MPs with sorbed PAHs or to WAF alone provoked elevated levels of *cyp1a*, but these changes were not statistically

Table 1-
Mean (±S.D.) of PAH concentration (μg/L) in the WAF stock solution and in exposure media at 30 min and 3 days after dosing in three medium renewal cycles. Acenaphthyelne, anthracene, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*e*)pyrene, perylene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, indeno(1,2,3-*cd*)pyrene, benzo(*ghi*)perylene and dibenz(*a,h*)anthracene were not detected in any water samples.

	WAF	Control		MPs-WAF		WAF		MPs-B(*a*)P		B(*a*)P		
	Stock	30'	3 d	30'	3 d	30'	3 d	30'	3 d	30'	3 d	L.Q.
Naphthalene	286.77± 37.49	0.03*	0.02*	0.06± 0.04	0.04± 0.03	6.57± 0.96	0.33± 0.41	–	–	–	–	0.005
Acenaphthene	1.64± 0.82	n.d.	n.d.	n.d.	n.d.	0.038± 0.004	n.d.	–	–	–	–	0.004
Fluorene	4.66± 0.99	n.d.	n.d.	n.d.	n.d.	0.12± 0.01	0.01*	–	–	–	–	0.004
Anthracene	0.12± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	0.003
Phenanthrene	3.33± 1.48	n.d.	n.d.	n.d.	n.d.	0.09± 0.02	0.02*	–	–	–	–	0.003
Fluoranthene	0.04± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	0.005
Pyrene	0.04± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–	–	0.005
Benzo(*a*)pyrene	<L.Q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.92± 3.16	0.16± 0.12	0.006
ΣPAHs	296.60	0.03	0.02	0.06	0.04	6.80	0.36	–	–	–	–	

n.d.: not detected; *: values corresponding to a single replicate where PAHs were detected; –: not measured; L.Q.: limit of quantification.

Table 2-
Concentration of PAHs (ng/g dry weight) in adult zebrafish at 3 and 21 days of exposure. Values are expressed as mean ± S.D. of fourth replicates for 3 days and sixth replicates for 21 days. Concentration of benzo(*a*)anthracene, perylene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, indeno(1,2,3-*cd*)pyrene, benzo(*ghi*)perylene and dibenz(*a*,*h*)anthracene in all the quantified samples were not detected or under limit of quantification.

	Control		MPs-WAF		WAF		MPs-B(*a*)P		B(*a*)P		L.Q.
	3 d	21 d	3 d	21 d	3 d	21 d	3 d	21 d	3 d	21 d	
Naphthalene	7.49 ± 1.64	6.89 ± 1.98	9.33 ± 0.12	8.90 ± 0.78	502.88 ± 94.10	109.16 ± 41.84	-	-	-	-	9.50
Acenaphthylene	2.23 ± 0.26	<L.Q.	1.87 ± 0.84	1.92 ± 0.41	2.52 ± 0.67	1.92 ± 0.51	-	-	-	-	0.60
Acenaphthene	9.55 ± 1.20	11.14 ± 5.23	8.55 ± 4.74	11.19 ± 1.80	46.2 ± 16.73	52.80 ± 6.94	-	-	-	-	0.88
Fluorene	19.77 ± 2.77	21.51 ± 7.83	25.43 ± 4.67	25.06 ± 5.56	138.03 ± 103.47	260.61 ± 22.81	-	-	-	-	0.80
Phenanthrene	23.85 ± 6.02	21.55 ± 3.57	38.98 ± 3.04	35.21 ± 4.40	163.52 ± 42.98	293.63 ± 45.79	-	-	-	-	5.50
Anthracene	1.46 ± 0.75	0.89 ± 0.38	2.61 ± 0.33	1.57 ± 0.45	4.81 ± 3.25	16.29 ± 7.44	-	-	-	-	0.58
Fluoranthene	3.55 ± 3.55	1.61 ± 0.00	2.56 ± 0.28	2.1 ± 0.27	2.96 ± 0.75	2.47 ± 0.26	-	-	-	-	1.60
Pyrene	2.60 ± 1.47	2.11 ± 0.39	2.26 ± 0.50	2.33 ± 0.22	2.4 ± 0.11	2.82 ± 0.00	-	-	-	-	1.88
Chrysene + triphenylene	<L.Q.	<L.Q.	<L.Q.	<L.Q.	1.12*	<L.Q.	-	-	-	-	0.50
Benzo(*e*)pyrene	<L.Q.	<L.Q.	<L.Q.	<L.Q.	1.04*	<L.Q.	-	-	-	-	1.00
Benzo(*a*)pyrene	<L.Q.	<L.Q.	<L.Q.	<L.Q.	<L.Q.	<L.Q.	<L.Q.	3*	40.25 ± 4.09	33.00 ± 3.5	1.00
ΣPAHs	68.65 ± 8.19	64.01 ± 17.49	78.67 ± 22.05	77.98 ± 25.65	910.03 ± 203.69	736.53 ± 108.83	-	-	-	-	

L.Q.: limit of quantification; *: value corresponding to a single replicate where PAHs were detected; –: not measured.

significant with respect to control fish. However, *cyp1a* transcription level in fish exposed to WAF was significantly higher than in individuals exposed to B(*a*)P. The two-ANOVA revealed a significant interaction of the PAHs and MPs at this exposure time. Regarding *gstp1*, significant differences were observed only at 21 days. Fish exposed to MPs-WAF and WAF presented significantly higher transcription levels than fish exposed to B(*a*)P, whereas no differences were found among fish treated with MPs alone or with sorbed PAHs. Significant differences among treatments were not observed for *tp53* transcription levels at any exposure time but the two-ANOVA revealed that exposure to PAHs had a significant effect on data variability at 3 days of exposure.

Results of the transcription levels of genes coding for the antioxidant enzymes in liver of fish exposed to MPs alone or in combination with PAHs are shown in Fig. 6 and Table 3. Compared to control fish, only a significant downregulation was observed for *sod1* only in fish exposed to MPs-B(*a*)P for 3 days. This group showed the lowest mean transcription levels for the three genes, being significantly lower than for fish exposed to MP-WAF. Fish exposed to MPs-WAF also showed significantly higher *gpx1a* than fish exposed to MPs alone and the two-ANOVA indicated that exposure to PAHs had a significant effect on *gpx1a* variability at 3 days of exposure.

3.4. Histopathological alterations

In liver, different pathological conditions, such as vacuolisation, presence of megalocytosis, necrotic focus and eosinophilic focus were observed in exposed fish (Table 4). Control fish showed normal architecture of the liver (Fig. 7A), although two individuals at each sampling time showed vacuolisation. In general, treated fish presented, in general, higher prevalence of vacuolisation (Fig. 7B), this increase (up to 80 %) being statistically significant in the liver of fish exposed to MPs for 21 days (Fig. 7C) and noticeable (70 %) in the case of fish exposed to WAF for 3 days. At 21 days, vacuolisation prevalence was reduced to 44.4 % in fish exposed to WAF (Fig. 7E). Other histopathological conditions, such as megalocytosis and necrotic focus (Fig. 7D and F) appeared at low prevalence in fish exposed to NPs, MPs-WAF and WAF.

Overall, the prevalence of histopathological alterations in gills was low. Control fish showed normal architecture of the gills (Fig. 8A). Besides one individual showing inflammation (Fig. 8B), fish exposed to NPs also presented normal gill histology. Inflammation was also seen in few individuals exposed to MPs (Fig. 8C), but not in the other treatment groups. Aneurisms were found on one individual exposed to MPs for 21 days (Fig. 8D) and in one individual (out of four) exposed to B(*a*)P for 3 days. Finally, one individual exposed to MPs for 3 days presented hyperplasia.

4. Discussion

4.1. MPs and NPs localisation

Due to their small size, NPs can cross biological barriers, such as the intestinal barrier (Zhao et al., 2023). However, in the present study, ingestion and internalisation of fluorescent NPs could not be demonstrated in histological sections due to the high autofluorescence of the samples. At TEM, NPs could be seen as a well dispersed suspension of round particles, although some aggregates could be observed. Round electrodense particles resembling NPs were found in in the intestinal lumen close to microvilli. Accumulation of NPs in the intestinal voids hinders their complete elimination (Zhao et al., 2023). Inside cells, NPs could not be clearly identified; vesicles containing particles as those seen in the suspension were not found. In previous studies, NP ingestion has been observed in zebrafish embryos (Pitt et al., 2018; Martínez-Álvarez et al., 2022b) and in adult zebrafish (Skjolding et al., 2017). In vitro studies with rainbow trout intestinal cells have also demonstrated accumulation of fluorescent PS NPs (73 ± 18 nm) and their ability to prevent the translocation of PS-NPs to the basolateral compartment

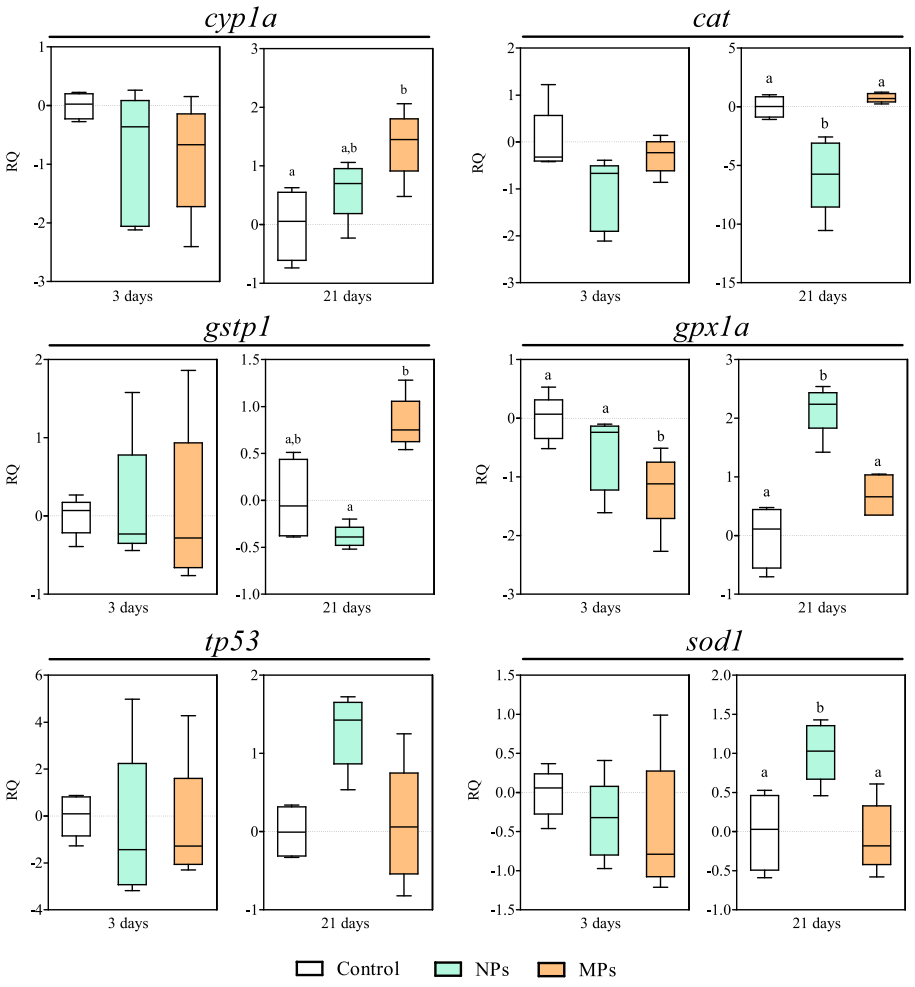

Fig. 4-. Relative quantification (RQ) of transcription levels of the biotransformation metabolism related genes *cyp1a* and *gstp1*, cell cycle related gene *tp53* and oxidative stress related genes *cat*, *gpx1a* and *sod1* in adult zebrafish liver after 3 and 21 days of exposure. Box-plots represent the percentage data value in between the 25th and the 75th percentile, median indicated by a line in the middle of the box. Whiskers are the data values in up to the 5th percentile and 95th percentile. Different letters indicate statistically significant differences ($p < 0.05$) within each exposure time according to the Kruskal-Wallis test followed by the post hoc Dunn's test for the non-parametric data sets (*gstp1, tp53, cat, gpx1*) and one-way ANOVA followed by the post hoc Tukey test for the parametric data sets (*cyp1a, sod1*).

(Geppert et al., 2016), but further in vivo studies are required to study the potential translocation (internalisation) to the tissues.

MPs were observed in the intestinal lumen of zebrafish exposed to all treatments containing MPs, but not inside cells or tissues. Several studies have reported the ingestion of MPs of different size by zebrafish (Karami et al., 2017; Lei et al., 2018; Qiao et al., 2019; Batel et al., 2020). PS MPs (5 μm) were numerously localised in the gut duct of zebrafish exposed for 21 days to 50 and 500 μg/L (Qiao et al., 2019). Similar results were obtained by Karami et al. (2017), who detected an abundant presence of low-density PE fragments (<18 μm) in the intestinal lumen of adult zebrafish after 10 days of exposure (50 and 500 μg/L). As observed in the present study, MPs are commonly found in the intestinal lumen of fish, but their ability to internalise into fish tissues is still being questioned (Batel et al., 2020).

4.2. MPs as vector of PAHs accumulation

To address whether MPs act as vectors for sorbed PAHs, zebrafish were exposed to MPs with sorbed PAHs and to the corresponding dissolved fraction of the PAHs. In the WAF tank, PAH concentration in water was lower than the corresponding dilution (nominal 5 %, measured 2.3 %) according to concentration measured in the 100 % WAF stock. WAF used in the study is mainly composed by low weight polycyclic aromatic hydrocarbons and represented by naphthalene as expected for a low energy WAF of a NNS crude oil (Perrichon et al., 2016; Esteban-Sánchez et al., 2021; Martínez-Álvarez et al., 2022a). In addition, the WAF stock used to contaminate the aquaria was prepared in advance to also be used to contaminate MPs for 24 h before dosage. The WAF stock was stored in the fridge overnight, which could also lead to some PAH loss. Lower concentration than the nominal concentration was also measured in the B(*a*)P exposure tank. This has also been reported in previous exposure experiments with freshwater fish (Zhao et al., 2013) and in sorption experiments of PAHs to MPs (Martínez-Álvarez et al., 2022a) and could be due to the low solubility of B(*a*)P in water (1.64 μg/L; May et al., 1983).

PAH concentration in exposure tanks was notably reduced after 3 days of dosing as reported previously (Esteban-Sánchez et al., 2021), which could be due to PAH uptake by zebrafish and to other processes like PAH adsorption to the tank walls, degradation and volatilisation, the latter especially in the case of the low molecular weight (MW) PAHs present in the WAF. In the case of B(*a*)P alone, 97 % of the B(*a*)P was lost after 3 days. Similar results were obtained by Costa et al. (2011), who reported a 96.97 % loss of B(*a*)P after 24 h of exposure of Nile tilapia. In the exposure tanks containing MPs with sorbed PAHs, PAH concentration in water was below the limit of quantification showing that probably sorbed PAHs were not release into the water or they did at very low concentrations below the detection limits due to their low water solubility and the PS capacity to sorb them (Martínez-Álvarez et al., 2022a).

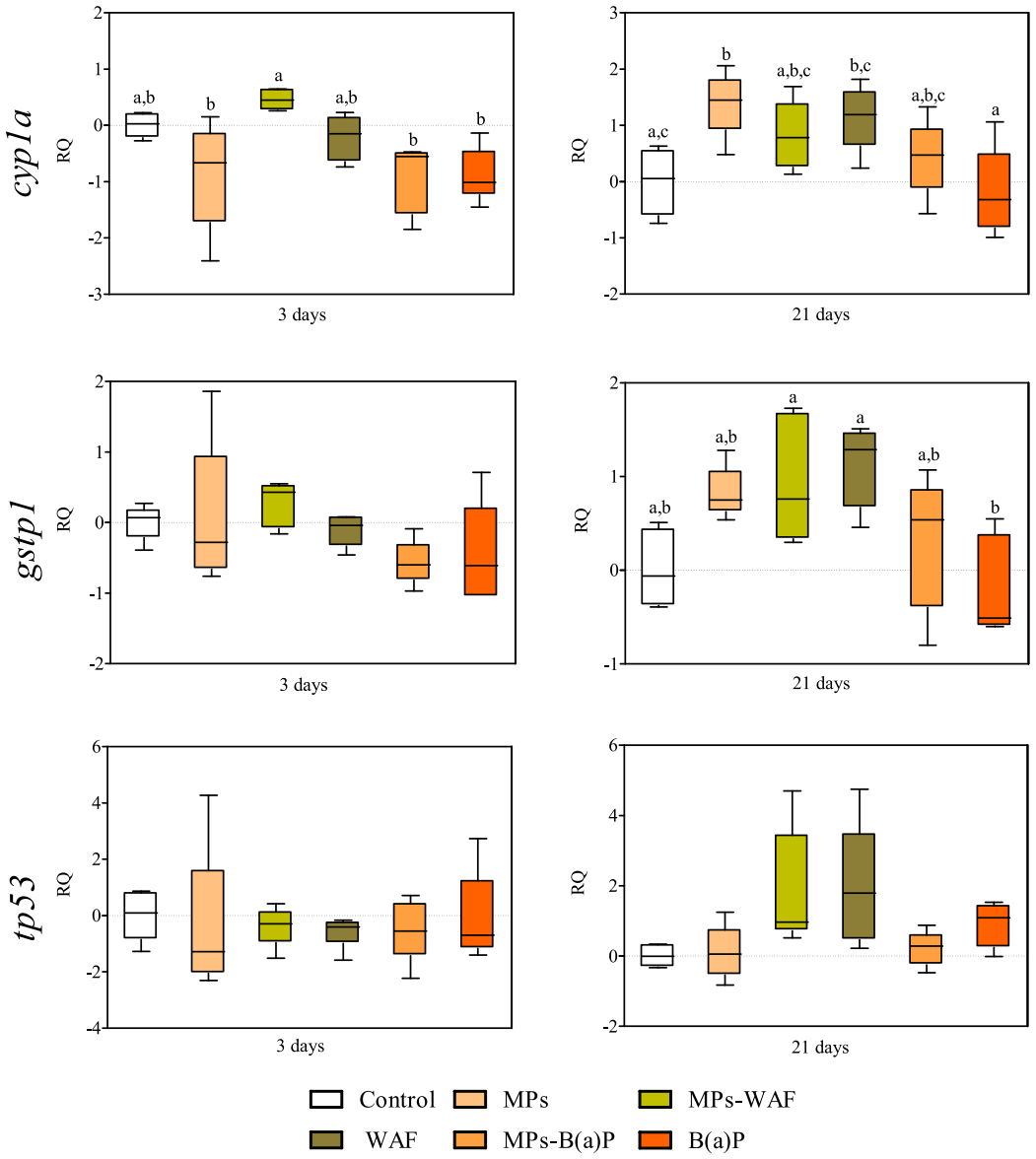

Fig. 5-. Relative quantification (RQ) of transcription levels of the biotransformation metabolism related genes *cyp1a* and *gstp1* and cell cycle related gene *tp53* in adult zebrafish liver after 3 and 21 days of exposure. Data representation as in Fig. 4. Different letters indicate statistically significant differences ($p < 0.05$) within each exposure time according to the Kruskal-Wallis test followed by the post hoc Dunn's test (*tp53*) and one-way ANOVA followed by the post hoc Tukey test (*cyp1a, gst1*).

As observed by Zuo et al. (2019), 250 μm PS MPs showed less desorption of phenanthrene than other polymers, like PE or PVC, indicating a stronger interaction between the PAH and PS.

Fish exposed to dissolved PAHs accumulated higher concentration of PAHs than fish exposed to MPs with sorbed PAHs. Only in fish exposed to WAF, an exposure time dependent increase in the concentration was observed. It is well known that PAHs are easily metabolised by fish (Livingstone, 1998), the metabolism of low MW PAHs (log $K_{ow} < 5$) being slower than the metabolism of high MW PAHs (log $K_{ow} > 5$) (Budzinski et al., 2004) due to their higher ability to induce the aryl hydrocarbon receptor. In addition, the higher solubility of low MW PAHs make them available through the gills-water barrier, while high MW PAHs are more prone to be assimilated by the digestive system (Jafarabadi et al., 2019). This would also explain the lower B(*a*)P accumulation compared to PAHs from WAF, even when the measured concentration of B(*a*)P during exposure was higher than for individual PAHs from WAF.

4.3. Expression of biotransformation metabolism, cell cycle regulation and oxidative stress-related genes

Cytochrome P450 1 A (*cyp1a*) and glutathione S-transferase pi (*gstp1*) play an important role in metabolic activation and detoxification of PAHs in fish liver. Compared to control fish, only fish exposed to MPs for 21 days showed a significant upregulation of *cyp1a* transcription levels, along with an increase of *gstp1* transcription levels. In red tilapia, Ding et al. (2018) observed that short-term exposure of up to 6 days to up to 100 μg/L of 100 nm PS MPs inhibited liver 7-ethoxyresorufin O-deethylase (EROD) activity, an enzyme coded by *cyp1a*, but at longer term (14 days) EROD induction was observed. A statistically significant upregulation of *gstp1* was also observed in the gills of zebrafish exposed to 100 nm PS MPs for 7 and 35 days (Umamaheswari et al., 2021). On the contrary, exposure to PS MPs resulted in inhibited hepatic GST enzyme activity in adult marine medaka across all tested concentrations (2, 20 and 200 μg/L) (Wang et al., 2019a) and in zebrafish after 21 days of exposure to 1 mg/L of UV-weathered MPs (Felix et al., 2023). In the

Table 3-
Summary of the two-way ANOVA showing the effects of the exposure to 4.5 μm MPs, to PAHs and their interaction on the transcription levels of the target genes. Asterisks indicate statistically significant differences ($p < 0.05$). Data on *tp53*, *cat* and *gpx1a* were ranked-based transformed before the ANOVA since the variance depended on mean values.

		PAHs		MPs		PAHs x MPs	
		F	*P*	F	*P*	F	*P*
cyp1a	3 d	0.090	0.767	0.043	0.837	4.210	0.051
	21 d	1.722	0.201	2.077	0.162	5.882	0.023*
gstp1	3 d	1.077	0.309	0.220	0.643	0.024	0.878
	21 d	0.097	0.758	2.009	0.169	1.389	0.250
tp53	3 d	0.049	0.826	0.698	0.411	2.638	0.116
	21 d	9.126	0.006*	0.605	0.444	0.644	0.430
cat	3 d	0.007	0.933	0.962	0.336	0.010	0.922
	21 d	0.575	0.455	0.041	0.841	2.679	0.114
gpx1a	3 d	0.184	0.672	0.836	0.369	3.447	0.075
	21 d	5.244	0.031*	1.520	0.229	2.359	0.137
sod1	3 d	0.102	0.752	0.585	0.451	0.763	0.390
	21 d	2.526	0.125	1.280	0.269	0.272	0.607

df (PAHs) = 1; df (MPs) = 1; df (PAHs vs MPs) = 1; df (residual) = 25.

present study, *cyp1a* and *gstp1* were not affected by NPs exposure, suggesting that the size of plastic could influence the pathways in which the organism is affected. The potential causes of increased CYP P450 activity as result of MPs exposure in fish are unknown, but other chemicals associated with the plastic polymers could contribute to the observed effects.

Regarding interactive effects between pollutants, the two-way ANOVA revealed a significant interaction between contaminants for *cyp1a* after 21 days, although PAHs alone or sorbed to MPs failed to significantly induce *cyp1a* and *gstp1* transcription in zebrafish, as reported by Batel et al. (2020). Rainbow trout exposed to 100–400 μm PS MPs (5 pellets/fish/day) contaminated with sewage or harbour waters did not show significant differences in mRNA levels of *cyp1a*, *gstp1* nor *cat*, compared to fish exposed to pristine PS MPs (Ašmonaitė et al., 2018). These results indicate that assayed PAH concentrations were not high enough to lead to effective bioaccumulation that could activate biotransformation metabolism, in agreement with results reported previously. In Batel et al. (2016), 252 μg/L B(*a*)P, but not 126 μg/L, induced EROD activity significantly. Moreover, exposure to 25 % WAF of the same NNS oil used herein did neither cause up-regulation of *cyp1a* transcription nor induction of EROD activity, as expected by the low MW PAH composition of the WAF (Esteban-Sánchez et al., 2021).

The tumour suppressor protein P53 has a central role in triggering the cellular stress response (Hanahan and Weinberg, 2000), activating different pathways such as programmed cell death (apoptosis) and DNA repair (Kim et al., 2005). Therefore, the tumour suppressor gene *tp53* has often been used in fish toxicology as a biomarker for genotoxicity

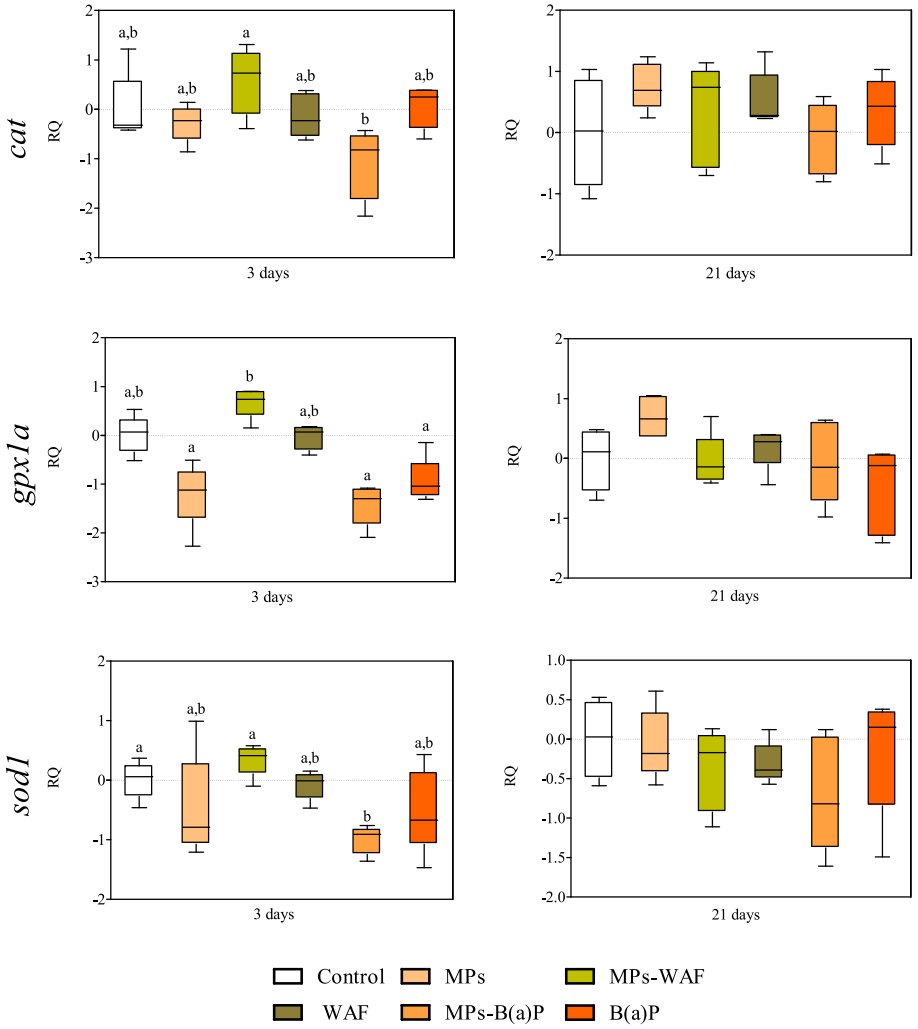

Fig. 6-. Relative quantification (RQ) of transcription levels of the oxidative stress related genes *cat*, *gpx1a* and *sod1* in adult zebrafish liver after 3 and 21 days of exposure. Data representation as in Fig. 4. Different letters indicate statistically significant differences ($p < 0.05$) within each exposure time according to the Kruskal-Wallis test followed by the post hoc Dunn's test (*cat, gpx1*) and one-way ANOVA followed by the post hoc Tukey test (*sod1*).

Table 4-
Prevalence (%) of histopathological alterations in the liver of zebrafish. Asterisks indicate statistically significant differences ($p < 0.05$) according to the Fisher's exact test compared to the control group at the same exposure day.

		n	Vacuolisation	Megalocytosis	Necrotic focus	Total
Control	3 d	10	20	n.o.	n.o.	20
	21 d	10	20	n.o.	n.o.	20
NPs	3 d	10	30	10	n.o.	30
	21 d	10	40	n.o.	10	50
MPs	3 d	10	40	n.o.	n.o.	40
	21 d	10	80*	n.o.	n.o.	80*
MPs-WAF	3 d	9	33.33	11.11	n.o.	44.44
	21 d	9	11.11	11.11	n.o.	22.22
WAF	3 d	10	70	n.o.	20	80*
	21 d	9	44.4	11.11	n.o.	55.55
MPs-B(*a*)P	3 d	10	20	n.o.	n.o.	20
	21 d	10	50	n.o.	n.o.	50
B(*a*)P	3 d	9	55.56	n.o.	n.o.	55.56
	21 d	10	30	n.o.	n.o.	30

n: number of individuals per experimental group (in some cases $n < 10$ because the liver tissue was not always present in the histological sections used for the analysis); n.o.: not observed.

(Bhaskaran et al., 1999; Ruiz et al., 2012). In this study, significant differences in its transcription levels were not observed, although higher mean values were measured in all fish groups exposed to PAHs for 21 days than in control fish or fish exposed to pristine MPs. To the best of our knowledge, there are not previous data on the effects of NP exposure on adult fish *tp53* transcription levels. Only some studies have been carried out on zebrafish embryos (Karami et al., 2017; Zhou et al., 2023) or in adults exposed to MPs or to PAHs (Martins et al., 2018; Perrichon et al., 2016; Esteban-Sánchez et al., 2021). Karami et al. (2017) also did not see differences in zebrafish embryos exposed to <18 μm low-density PE (5–500 μg/L) fragments for 10 and 20 days compared to control embryos. In zebrafish embryos exposed to the WAF of a light crude oil, significant upregulation of *tp53* transcription levels only occurred at 100 % WAF (Perrichon et al., 2016). In adult zebrafish, exposure to 5 % or 25 % WAF of the same oil used in the present study, even in the presence of a chemical dispersant, did not alter the transcription levels of *tp53* (Esteban-Sánchez et al., 2021) For individual PAHs, exposure of zebrafish or medaka to 0.5 or 1.21 μg/L of B(*a*)P, respectively, did not cause effects on *tp53* transcription levels (Zhao et al., 2013; Martins et al., 2018). The WAF and B(*a*)P concentrations used in the present study were again maybe too low to produce significant changes in *tp53* transcription levels.

Metabolism of organic pollutants can enhance the production of reactive oxygen species (ROS), leading to oxidative stress, which has also been addressed regarding potential MP and NP toxicity (Karami et al., 2017; Chen et al., 2017a; Espinosa et al., 2019; Ding et al., 2018; Bhagat et al., 2020). The antioxidant enzymes CAT and GPx are in charge of cell protection against peroxidation by decomposing reactive species and SOD is responsible for the control of superoxide transformation into hydrogen peroxide (Di Giulio et al., 1989). In this work, statistically significant downregulation of liver *cat* and upregulation of *gpx1a* and *sod1* was specifically observed in zebrafish exposed to NPs for 21 days. Down-regulation of *cat* transcription levels was also reported in exposed to 10 mg/L of 55 nm NPs (Singh et al., 2021). Chen et al. (2017a) reported reduced levels of glutathione in zebrafish embryos exposed to 1 mg/L of PS NPs (50 nm) for 5 days whilst no significant changes were recorded in CAT and GPx activities. Exposure to 100 nm PS MPs induced SOD liver activity in red tilapia at 1, 6, 10 and 14 days when fish were exposed to 1–100 μg/L of PS MPs (Ding et al., 2018). For larger plastic particles, downregulation of *cat* was reported in zebrafish larvae (5 h post fertilisation) exposed to 5, 50 and 500 μg/L of low-density PE fragments (<18 μm) for 20 days (Karami et al., 2017). Dietary exposure to PE, but not to PVC, MPs of 40–150 μm (500 mg MPs/Kg diet) inhibited liver SOD and CAT enzyme activities, as well as *sod1* transcription levels, in European sea bass (Espinosa et al., 2019). In our study, exposure to pristine PS MPs did not significantly alter transcription levels of genes coding for the antioxidant enzymes, suggesting that polymer type and particle size can modulate the response of the fish antioxidant system. Plastic size, in particular, seems to be an important characteristic being a potential factor of increased oxidative stress in zebrafish. A size dependent toxicity of PS MPs was observed in adult zebrafish exposed for 21 days to 100 nm, 5 μm and 200 μm PS MPs. Decreasing size altered the expression of genes related to phagocyte-produced ROS generation in zebrafish intestine (Gu et al., 2020).

Cat transcription levels were significantly reduced in fish exposed for 3 days to MPs-B(*a*)P compared to fish exposed to MPs-WAF. In addition, downregulation of *sod1* was observed in fish exposed to MPs-B(*a*)P compared to control fish. The absence of oxidative stress in zebrafish embryos co-exposed to MPs/NPs and persistent organic pollutants (POPs) was reported in the literature, suggesting that MPs/NPs alleviated the reported oxidative stress in embryos exposed to POPs alone (Chen et al., 2017a). Oxidative stress is an effect commonly reported in organisms exposed to xenobiotics, including PAHs (Vieira et al., 2008; Salazar-Coria et al., 2019). Exposure to WAF of Maya crude oil prepared in a proportion of 100 g/L for 24 h and then diluted at 10, 100 and 1000 mg/L caused significant inhibition of SOD activity in Nile tilapia liver only at the highest concentration while no effect was reported for CAT or GPx in zebrafish liver (Salazar-Coria et al., 2019). The exposure of common goby for 96 h to B(*a*)P (1–16 μg/L) and to anthracene (0.25–4 μg/L) caused a significant induction of CAT, SOD, GPX and GR activities in liver, while a mixture of PAHs from the WAF of fuel-oil #4 (7.5–30 % of 100 g fuel-oil/L) only provoked significant induction of CAT activity (Vieira et al., 2008). In the present study, exposure to MPs alone or to PAHs alone did not alter the transcription levels of the genes coding for the main antioxidant enzymes but, interestingly, exposure to MPs-B(*a*)P provoked downregulation of *sod1* suggesting a differential effect of the combined exposure. Similar downregulation of *sod1* transcription levels in zebrafish liver was reported after 12 days of treatment with a co-exposure of 150 μm MPs (3 mg/L) and phenanthrene (200 μg/L) (Xu et al., 2021).

4.4. Histopathological alterations on the gill and liver tissues

Liver is the main organ for metabolic activation and detoxification of PAHs and fish liver histopathology has been studied after exposure to pollutants, showing multiple alterations and liver vacuolisation being one of the most frequently reported (Chae et al., 2018; Chen et al., 2018; Hook et al., 2018; Espinosa et al., 2019; Mai et al., 2019; De Sales-Ribeiro et al., 2020). In the present study, liver vacuolisation was the only pathological condition showing statistically higher prevalence in fish exposed to MPs for 21 days compared to control fish. Lipid accumulation has previously been reported in adult zebrafish exposed to both 5 μm and 70 nm PS MPs (Lu et al., 2016; Du et al., 2023). This finding, together with the results of the metabolomic analysis suggested that exposure to MPs induced alterations of metabolic profiles in fish liver and disturbed the lipid and energy metabolism (Lu et al., 2016; Du et al., 2023). Similar significant increase in the prevalence of liver vacuolisation in fish exposed to WAF was observed in the short term (3 days). High concentration of PS NPs (5 mg/L) was also found to alter liver histology in the freshwater fish *Zacco temminckii*, causing liver vacuolisation and cell destruction after 7 days of exposure (Chae et al., 2018). Liver vacuolisation was also observed in the livers of European sea bass exposed to 40–150 μm PE and PVC MPs (100 and 500 mg/Kg) for 21 days (Espinosa et al., 2019). Similar pathology was observed in

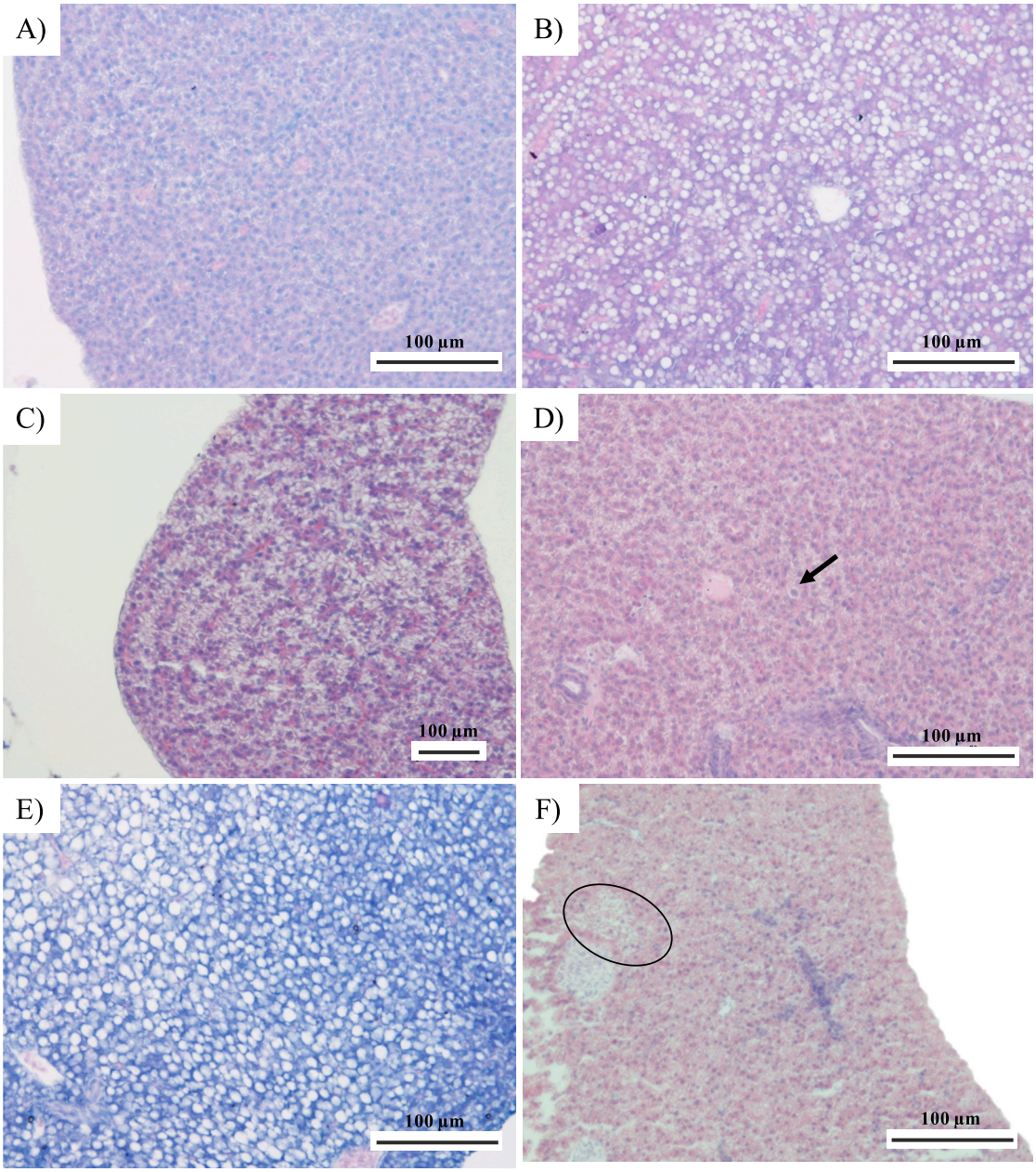

Fig. 7-. Micrographs of histological sections of zebrafish liver. A) Liver of unexposed adult zebrafish at 21 days showing normal morphology; B) Liver of a zebrafish exposed to NPs for 21 days showing vacuolisation; C) Liver of a zebrafish exposed to MPs for 21 days showing vacuolisation; D) Liver of a zebrafish exposed to MPs-WAF for 21 days showing megalocytosis (black arrow); E) Liver of a zebrafish exposed to WAF for 21 days showing liver vacuolisation; F) Liver of a zebrafish exposed to WAF for 3 days showing a necrotic focus (black circle).

the livers of zebrafish exposed for longer periods (30 and 45 days) to 1–5 µm PE fragments and fibres (2.1 10^{-2} g/cm^3, 2 % of the total food delivered) but this time without significant differences among treatments (De Sales-Ribeiro et al., 2020). When adult zebrafish were exposed for 3 weeks to a combination of 125–250 µm PE MPs (4 mg/g food) with persistent organic pollutants (PCBs. PBDEs, PFCs and methylmercury, 77.9 ng/g food), a greater liver vacuolisation was provoked than when fish were exposed to MPs or POPs alone (Rainieri et al., 2018).

In gills, a significant increase in the prevalence of pathological lesions was not recorded after 3 nor 21 days of exposure, but some pathologies were observed, such as inflammation, which is known as a mechanism of defence in fish exposed to MPs and B(*a*)P (Martins et al., 2018; Limonta et al., 2019). Accordingly, Martins et al. (2018) reported that zebrafish exposure to 0.5 µg/L of B(*a*)P for 14 days also provoked gill inflammation. A mix of PS and high-density PE MPs at environmentally relevant concentrations (100 and 1000 µg/L) caused damage on zebrafish gill integrity (adhesion and partial fusion of secondary lamellae and mucous hypersecretion) after 21 days of exposure (Limonta et al., 2019). Gills are the first barrier to be impacted on fish because of their direct contact with pollutants present in water. However, relevant effects were not observed on this study suggesting, again, that the pollutant concentrations used were not toxic enough toxic to impair gill structure.

5. Conclusions

In the conditions assessed in the present study, 4.5 µm PS MPs did not act as efficient vectors of PAHs to adult zebrafish, but results show a differential effect on zebrafish depending on the size of PS plastics. 4.5

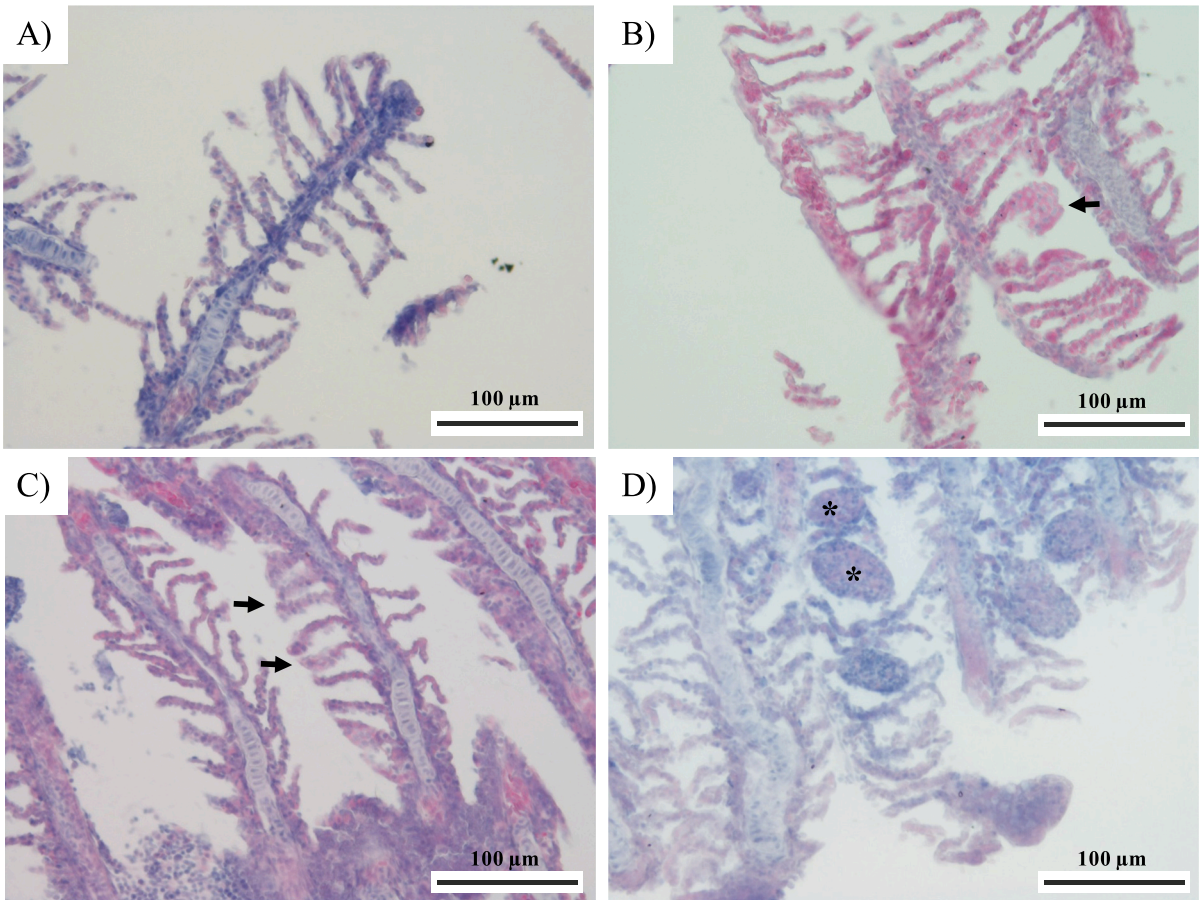

Fig. 8-. Micrographs of histological section of zebrafish gills. A) Gills of unexposed zebrafish at 21 days showing normal morphology; B) Gills of zebrafish exposed to NPs for 3 days, showing inflammation (arrow); C) Gills of a zebrafish exposed to MPs for 3 days, showing inflammation (arrows); D) Gills of a zebrafish exposed to MPs for 21 days showing aneurism (asterisks).

µm PS MPs induced biotransformation metabolism in the liver, showing further liver injury characterised by tissue vacuolisation, whereas 50 nm NPs mainly affected the antioxidant system. These findings agree with previous results reported in the literature, where alteration of lipid metabolism and oxidative stress are repeatedly reported responses to MP and NP exposure and highlight the importance of particle size to assess the environmental hazard of plastic pollution in aquatic environments. Moreover, the assessment of the ecotoxicological impact of real environmental NPs and MPs must be urgently addressed.

Ethical statement

This work has received approval from the Ethics Committee in Animal Experimentation of the University of the Basque Country and authorisation from the Local Competent Authority and a proof/certificate (M20/2017/152) of approval is available upon request.

CRediT authorship contribution statement

Ignacio Martínez-Álvarez: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Karyn Le Menach:** Validation, Resources, Methodology, Investigation. **Miren P. Cajaraville:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Hélène Budzinski:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Amaia Orbea:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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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