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Effects of pristine or contaminated polyethylene microplastics on zebrafish development

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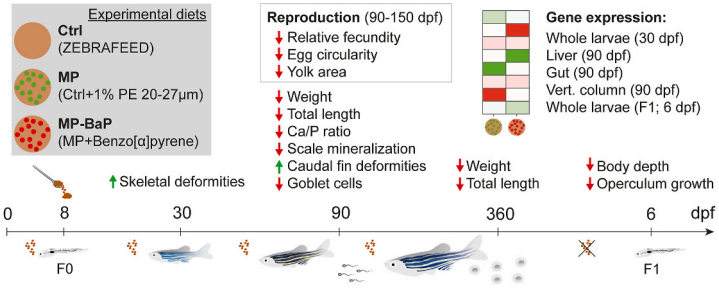
HIGHLIGHTS

- Dietary exposure to polyethylene microparticles negatively affects zebrafish growth and bone development.
- BaP contaminated microplastics impair zebrafish reproductive performance.
- Parental exposure to microplastics affect bone mineralization in offspring larvae.
- Genes involved in BaP metabolism are differentially expressed upon exposure to contaminated microplastics.
- Genes involved in oxidative stress response are differentially expressed upon exposure to contaminated microplastics

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of microplastics in the aquatic ecosystem represents a major issue for the environment and human health. The capacity of organic pollutants to adsorb onto microplastic particles raises additional concerns, as it creates a new route for toxic compounds to enter the food web. Current knowledge on the impact of pristine and/or contaminated microplastics on aquatic organisms remains insufficient, and we provide here new insights by evaluating their biological effects in zebrafish (*Danio rerio*). Zebrafish larvae were raised in ZEB316 stand-alone housing systems and chronically exposed throughout their development to polyethylene particles of 20–27 µm, pristine (MP) or spiked with benzo[α]pyrene (MP-BaP), supplemented at 1% w/w in the fish diet. While they had no effect at 30 days post-fertilization (dpf), MP and MP-BaP affected growth parameters at 90 and 360 dpf. Relative fecundity, egg morphology, and yolk area were also impaired in zebrafish fed MP-BaP. Zebrafish exposed to experimental diets exhibited an increased incidence of skeletal deformities at 30 dpf as well as an impaired development of caudal fin/scales, and a decreased bone quality at 90 dpf. An intergenerational bone

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formation impairment was also observed in the offspring of parents exposed to MP or MP-BaP through a reduction of the opercular bone in 6 dpf larvae. Beside a clear effect on bone development, histological analysis of the gut revealed a reduced number of goblet cells in zebrafish fed MP-BaP diet, a sign of intestinal inflammation. Finally, exposure of larvae to MP-BaP up-regulated the expression of genes associated with the BaP response pathway, while negatively impacting the expression of genes involved in oxidative stress. Altogether, these data suggest that long-term exposure to pristine/contaminated microplastics not only jeopardizes fish growth, reproduction performance, and skeletal health, but also causes intergenerational effects.

1. Introduction

Plastic pollution has greatly increased over the last decades and represents a major issue for both the environment and human health. Pollution with plastic debris is ubiquitous and massive with more than 5.25 trillion particles weighing 268,940 tons floating in the world's oceans (Eriksen et al., 2014). Aquatic ecosystems are particularly impacted by plastic pollution and high densities of plastic particles, in particular microplastics (<5 mm), which are observed throughout the globe thus raising concerns on the welfare of aquatic organisms (Chae and An, 2017; Wright et al., 2013a). Released into the aquatic environment through the runoff of urban/industrial effluents (e.g., nurdles, scrubbers) or the breakdown of larger debris (physical, biological, chemical degradation), microplastics are found in many rivers and oceans worldwide. The presence of microplastics has been reported not only on ocean's surface but also in deep oceans at 10,890 m (Jamieson et al., 2019), and in sediments. Plastic debris have reached even the most remote places on earth such as the Arctic snow (Bergmann et al., 2019) and Antarctic ice (Kelly et al., 2020). Microplastics are heterogeneous in composition (they are manufactured with different polymers, e.g., polyethylene, polystyrene, polyvinylchloride and polyurethane), have different densities (they float, are neutrally-buoyant or sink to the river/ocean floor), and often incorporate additives (fillers, plasticizers, colorants, and stabilizers) (Cole et al., 2011). The ingestion of microplastics by aquatic organisms may have a physical impact by disturbing feeding habits, blocking the feeding appendages and the passage of food through the intestine, causing false satiation or damaging the gastrointestinal track, resulting in decreased food intake (Wright et al., 2013a; Lusher et al., 2013). On the other hand, microplastics can chemically affect internal organs through the leaching of manufacturing additives (e.g., polybrominated diphenyl ethers, nonylphenol and triclosan), or the release of contaminants (e.g., heavy metals, endocrine-disrupting chemicals, persistent organic pollutants and halogenated organic contaminants) that are easily adsorbed onto microplastic surface before ingestion (Wang et al., 2016; Koelmans et al., 2014).

In this regard, ubiquitous contaminants such as benzo[α]pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAHs) – known to be carcinogenic and to disrupt developmental, reproductive, hepatic, endocrine and immunological processes (Collier et al., 2013; Williams et al., 2015), but also to be harmful to skeletal development and bone homeostasis (Laizé et al., 2018; Tarasco et al., 2021) – have the ability to adsorb onto the surface of different types of microplastic polymers, especially onto polyethylene (Ziccardi et al., 2016; Rochman et al., 2013; Schönlau et al., 2019). Upon ingestion of the contaminated microplastics, PAHs can leach out, diffuse throughout animal body and accumulate into a range of tissues (Batel et al., 2016, 2018; Pittura et al., 2018; O'Donovan et al., 2018), raising additional concerns about the adverse effects produced by microplastics.

Beside the damage that they may cause to the aquatic life, microplastics (and adsorbed chemicals) pose a threat to human health as they are becoming omnipresent in the food web and in our daily life (e.g., table salt, drinking water, honey, beer, etc.) (Chae and An, 2017; Avio et al., 2017; Diaz-Basantes et al., 2020; Zhang et al., 2020). Thus, many international environmental and food agencies (United Nations Environment Programme (UNEP), European Food Safety Authority (EFSA) and Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP)) have placed microplastics as one of the top priority research topics (Kass et al., 2017; GESAMP Kershaw and Rochman, 2016; UNEP, 2016). Scientific data evidencing the negative impact of microplastics on the physiology of aquatic organisms is rapidly increasing, however cellular and molecular mechanisms underlying microplastic toxicity are still poorly understood and more data are needed to further support environment management issues. Adverse effects such as growth inhibition (Lönnstedt and Eklöv, 2016; Au et al., 2015; Sjollema et al., 2016; Missawi et al., 2021), behavioral disorders (Lönnstedt and Eklöv, 2016; Rehse et al., 2016), reproductive dysfunctions (Sussarellu et al., 2016; Lee et al., 2013), feeding disorders (Wright et al., 2013b; Green et al., 2016; Abidli et al., 2021) and mortality (Lönnstedt and Eklöv, 2016; Au et al., 2015) have been described for different trophic levels (Chae and An, 2017). However, data on specific biological processes (i.e., development, reproduction, transgenerational studies) and in key species such as fish, are still missing or are controversial to fully assess the environmental and biological risks associated with microplastics and adsorbed pollutants.

Among aquatic organisms that may be exposed to microplastics, fish are central to the ocean food web and to human nutrition, and the presence of microplastics potentially contaminated has been widely reported in fish, including in species marketed for human consumption (Neves et al., 2015; Thiele et al., 2021), opening a new route for toxicity in humans. Although the occurrence and detection of microplastic in fish can be assessed in wild populations, studying the effects of microplastic at the biological level requires the use of animal models in laboratory conditions. In this regard, to better evaluate adverse effects of microplastics in fish and, by extension, in vertebrates, zebrafish has been widely used to gain insights into the biological effects of microplastic ingestion (Bhagat et al., 2020). It is a well-established ecotoxicological model organism to assess risks associated with toxicity, reproduction and osteotoxicity (Fernández et al., 2018; Laizé et al., 2014), but also to study the cellular and molecular mechanisms underlying toxicant effects.

The majority of the studies performed in zebrafish evaluated the effects of microplastic exposure (pristine or in combination with environmental pollutants) on development (Jemec Kokalj et al., 2019; Malafaia et al., 2020; Zhao et al., 2020; LeMoine et al., 2018), neurotoxicity (Mak et al., 2019; Sheng et al., 2021), behavior (Qiang and Cheng, 2019; Limonta et al., 2019), oxidative stress (Santos et al., 2021; Wan et al., 2019; Lu et al., 2018; Xu et al., 2021), intestinal damage (Gu et al., 2020; Lei et al., 2018) and microbiome changes (Wan et al., 2019; Zhang et al., 2021) (see also the review by Bhagat et al., 2020 and references therein). In this regard, while several studies have reported microplastic toxicity (normally higher in combination with contaminants), others showed limited or no impact. Variability in biological response may be due to the different type and size of microplastic tested, different exposure duration, concentration, and route. Due to laboratory limitations (i.e., static conditions, absence of recirculating housing systems to study microplastic), most of the data originated from short-term exposure trials (i.e., up to 21 days), mostly through waterborne exposure, and during specific developmental stages. Thus, the impact of microplastics on zebrafish remain to be better assessed especially upon long-term exposure throughout the full developmental process and on future generations.

In this work, the biological effects of a long-term exposure to

polyethylene (PE) microparticles were assessed. Zebrafish were fed from larvae to adulthood with experimental diets supplemented with microplastics pristine or contaminated with BaP, and zebrafish growth, reproduction, bone development, expression of selected marker genes and intergenerational effects were evaluated.

2. Materials and methods

2.1. Ethics statement

Procedures involving animals were performed following the EU and Portuguese legislation for animal experimentation and welfare (Directives 86/609/CEE and 2010/63/EU; Portaria 1005/92, 466/95 and 1131/97; Decreto-Lei 113/2013). Animal handling and experimentation were performed by qualified operators accredited by the Portuguese Direção-Geral de Alimentação e Veterinária under the authorization no. 012769/2021. All efforts were made to minimize pain, distress, and discomfort. Experiments were terminated (fish were returned to normal conditions or euthanized) whenever adverse effects were observed.

2.2. Chemicals and materials

All chemicals were bought to Sigma-Aldrich (St. Louis, MI, USA) unless otherwise stated. Plastic material was avoided as much as possible to limit any cross-contamination during experimental procedures and replaced by soda-lime glass or stainless steel material. Fluorescent polyethylene microspheres (20–27 μm; 1.005 g/cc) were purchased from Cospheric (Santa Barbara, CA, USA). Microspheres manufacturing composition: polyethylene (CAS number 9002-88-4; >70% w/w) and proprietary additive (trade secret; <30% w/w).

2.3. Spiking of benzo[α]pyrene on microplastics and chemical analysis

250 mg of polyethylene microspheres were placed in a glass flask filled with 50 mL of 10 μM benzo[α]pyrene (BaP) dissolved in dimethyl sulfoxide (DMSO) and incubated at 22 °C for 30 h under strong agitation. Microspheres were washed with DMSO and vacuum dried. To confirm the adsorption of BaP onto microplastics, 50 mg of microplastics exposed to BaP or DMSO (control vehicle) were analyzed. Briefly, microplastics were resuspended in 2.5 mL of hexane and ultra-sonicated for 1 h at 50 °C. Mixtures were centrifuged for 10 min at 2000 *g* and the hexane was transferred into new vials. Microplastics were further extracted 2 times with 2.5 mL of hexane and hexane supernatants were pooled. Hexane was evaporated under a gentle nitrogen gas flow and dry residues were resuspended in 100 μL of gas chromatography-grade dichloromethane. A recovery standard (2 μg/mL of perylene D12) was added in each sample to evaluate the efficiency of the extraction steps. Extracts were analyzed using an Agilent GC-MS (Agilent Technologies 6890 Network GC System coupled with a 5973 inert Mass Selective Detector) using an Agilent Tech DB-5MS column (length: 25 m; internal diameter: 0.250 mm; film: 0.25 μm). To achieve better sensitivity and selectivity, compounds were analyzed by selected ion monitoring mode (SIM, BaP *m/z*: 252; perylene D-12 *m/z*: 264). The ion source temperature was held at 240 °C, and the transfer line temperature was kept at 280 °C. Compounds were identified by comparing the retention times of standard samples and the mass spectra compared to the NIST library. BaP determination was performed by the elaboration of an individual calibration curve using four concentrations (0.00001–0.01 mM) from the initial standard. The quantity of BaP adsorbed on polyethylene microspheres was 2.68 ± 0.13 μg of BaP per g of microplastics (no BaP was detected in DMSO treated microplastics). Bap concentration, spiking procedure and chemical analysis were adapted from previous studies (Pittura et al., 2018; Batel et al., 2020; Cormier et al., 2019).

2.4. Experimental diets

Experimental diets were manufactured by Sparos Lda. (Olhão, Portugal) and used to feed zebrafish throughout the experimental procedures. The commercial microdiet ZEBRAFEED (Sparos) was used as control diet (Ctrl) and as the basis of the diets supplemented with pristine microplastics (MP) or with microplastics spiked with benzo[α]pyrene (MP-BaP). Four different grain sizes – i.e., <125 μm, 125–180 μm, 180–355 μm and 355–700 μm – were prepared for each diet to meet the mouth size of the different developmental stages. Briefly, microplastics (1% w/w) were incorporated and mixed in ZEBRAFEED microdiet (grain size <100 μm), hydrated with 50% of distilled water, then mixed again until complete homogenization and dried at 60 °C overnight. MP-BaP diet was similarly prepared but with microspheres spiked with benzo[α]pyrene (see above), before being incorporated into the diet. The successful incorporation of microplastics into the experimental diets was confirmed in 5 aliquots of each diet size. Diet aliquots of approximately 5 mg were solubilized in 50 mL of distilled water and vacuum filtered through a 11 μm paper filter (Grade 1; Whatman, Maidstone, UK). Fluorescence images of each paper filter Ctrl, MP and MP-BaP (at different grain size) were acquired using a MZ10F fluorescence stereomicroscope (Leica, Wetzlar, Germany) equipped with GFP filters ET470/40x (EX) and ET525/50 m (EM), and a DFC7000T color camera (Leica). The number of microplastic particles present in each aliquot was assessed using the built-in function *Find maxima* in ImageJ 1.52v (Schneider et al., 2012) and found to be homogenous among the different diets and for the different grain size (Supplementary Table 1).

2.5. Zebrafish maintenance

Sexually mature zebrafish (AB wild-type strain; ZFIN ID: ZDB-GENO-960809-7) were housed in a ZebTEC housing system (Tecniplast, Buguggiate, Italy) under optimal water quality (i.e., temperature 28 ± 0.5 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 μS, ammonia and nitrites lower than 0.1 mg/L, nitrates at 5 mg/L) and crossed following an in-house breeding program. Breeders were fed twice a day with rotifers (*Brachionus plicatilis* type L; 230 rotifers/mL) enriched with 2 mL of Phytobloom Green Formula (Necton S.A., Olhão, Portugal) and ZEBRAFEED microdiet *ad libitum*. Fertilized eggs (N = 300) were placed into a 1-L breeding tank containing system water and 0.0002% (w/v) methylene blue to prevent fungal growth. At 3 days post-fertilization (dpf), hatched larvae were transferred into 3.5-L glass tanks at a density of 100 larvae/L and maintained under static water conditions (90% of the water renewed every 2 days) at 28 °C. At 15 dpf, post-larvae were distributed into new tanks to achieve a density of 26 post-larvae/L and maintained in a ZEB316 custom-made recirculating housing system (Tarasco et al., 2020a) with a water flow of 8.4 L/h. At 30 dpf, density was adjusted to 5 juveniles/L. All fish were subjected to a 14-h light/10-h dark photoperiod throughout the duration of the experimental work.

2.6. Dietary exposure of zebrafish to microplastics

A total of 600 larvae (200 per replicate in glass tanks) were exposed to experimental diets Ctrl, MP or MP-BaP – dispensed in similar quantity using stainless steel micro-spoons – throughout development. Larvae from 5 to 8 dpf were fed 3 times a day, twice with enriched rotifers (see above) and once with the experimental diet (size <125 μm, 20% body weight). Larvae from 8 to 30 dpf were fed 3 times a day exclusively with the experimental diet (size 125–180 μm from 8 to 15 dpf and size 180–355 μm from 15 to 30 dpf, 20% body weight). Juveniles and adult fish were fed 2 times a day with experimental diet (size 355–700 μm, 3–5% body weight).

2.7. Assessment of growth parameters

Dry weight was determined in 30 dpf juveniles (pools of 6 fish) and 6 dpf larvae (offspring; pools of 30 larvae). Fish pools were placed in 1.5-mL tubes, lyophilized and weight using a precision scale (0.00001 g). Wet weight was determined in 90 and 120 dpf adults (individually) using a precision scale. Total length, standard length, head area and body depth (i.e., the vertical distance from the dorsal margin of the body to the ventral margin of the body measured at the base of the pectoral fin) were assessed in 6 dpf larvae (offspring) by image analysis of brightfield images using ImageJ.

2.8. Assessment of reproduction capacity

Sexually mature zebrafish (>3 months old) fed the different experimental diets were placed 12 h prior to the experiment in breeding tanks (Tecniplast), with males and females separated by a tank divider following a 1:1 sex-ratio. For each breeding experiment, 2 couples were randomly selected from each replicate tank (3 tanks per experimental diet), resulting in 18 independent breeding events. A total of 4 breeding experiments were performed with a resting period of 2 weeks in-between each breading event. Breeding was initiated by removing the divider separating males and females and breeding success was scored as follows: score = 1 when a couple successfully laid eggs and score = 0 when no eggs were laid, or eggs aborted. Eggs from successful breeding events were kept in glass Petri dishes containing fish water and methylene blue at a density of 50 eggs/40 mL. For each breeding event, all laid eggs were imaged at 2 and 24 hpf (hours post-fertilization) using a Leica MZ10F stereomicroscope equipped with a DFC7000T color camera. Egg number, viability, area and circularity were assessed through image analysis using a macro developed in ImageJ (Supplementary Fig. 1). Yolk area was measured at 2 hpf using ImageJ built-in tools. Relative fecundity was calculated as the total number of eggs normalized to female body weight. Hatching rate was calculated as the number of larvae hatched at 3 dpf normalized to the total number of fertilized eggs. Hatched larvae were kept in fish water up to 6 dpf to assess intergenerational effects.

Sperm quality was assessed in 6 males per condition. Fish were anesthetized with 0.6 mM tricaine methanesulfonate, rinsed with phosphate buffered saline (PBS) and sperm was collected using a 25 μL glass capillary tube (Blaubrand, Wertheim, Germany) attached to a mouthpiece while performing an abdominal massage. Sperm (1–3 μL per fish) was immediately diluted in 10 μL of 0.2 μm filter-sterilized Hank's balanced salt solution (HBSS) and maintained at 4 °C until further used but no longer than 1 h. Sperm concentration and motility were evaluated using a computer-assisted sperm analysis (CASA) system (Proiser, Valencia, Spain) coupled to a E200 microscope (Nikon, Tokyo, Japan) equipped with a x10 negative phase contrast objective. Images were captured with a ISAS camera (Proiser) and processed with CASA software. For sperm concentration, samples were diluted 1:20 in HBSS and the number of spermatozoa was determined from 3 field images. For sperm motility analysis, 0.5 μL of sperm was placed on a Makler chamber (Microptics S.L., Barcelona, Spain) and immediately activated with 5 μL of sterilized system water at 28 °C. Each sample was measured twice. Sperm motility was characterized from 0 to 60 s post-activation through the acquisition of several parameters i.e., total motility (%), progressive motility (%), curvilinear velocity (μm/s), straight line velocity (μm/s) and linearity (%). To assess sperm viability, 2 μL of sperm were diluted in 200 μL of HBSS in flow cytometry tubes and labelled with 1 μL of propidium iodide (PI; final concentration 5 ng/mL). Labelled sperm was analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with a 488nm laser for excitation and a 690/50-nm bandpass filter (FL3) for fluorescence emission. Settings for zebrafish spermatozoa acquisition and analysis were previously established (Diogo et al., 2019). A total of 10,000 events were counted for each sample.

2.9. Assessment of osteotoxicity

2.9.1. Axial skeleton development

At 30 dpf, a total of 120 juveniles (40 per replicate tank) were randomly collected for each experimental diet (i.e., Ctrl, MP and MP-BaP) and euthanized with a lethal dose of anesthetic. Juveniles were fixed for 1 h in 4% paraformaldehyde (PFA; prepared in PBS at pH 7.4) at room temperature, washed with 1X PBS, preserved in increasing ethanol series (up to 75%) and stored at −20 °C until further used. Fish were rehydrated in increasing 1X PBS series, stained for 2 h in 0.05% alizarin red S (AR-S) prepared in 1% potassium hydroxide (KOH), washed for 12 h with 1% KOH and preserved in increasing glycerol series (up to 75%) until image acquisition. The incidence, typology (such as shape variation, anomalous supernumerary elements, fusions or platyspondyly) and charge of deformities in the axial skeleton were determined through stereomicroscopy observation. The terminology used is according to Bird and Mabee (2003). Representative images were acquired using a LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) equipped with a 10x/0.30 M27 objective. Images were acquired at 1024 × 1024 pixels using 4% laser power at 555 nm, acquisition speed = 7.75 μs, mCherry channel (λex = 587 nm, λem = 610 nm) and a GaAsP detector set to gain = 650 V and digital gain = 0.3. Z slices were recorded at 4 μm per section and adjusted to cover the full depth of the region of interest.

2.9.2. Caudal fin development

At 90 dpf, a total of 24 adult fish (8 per replicate tank) were randomly collected for each experimental diet (i.e., Ctrl, MP and MP-BaP). Fish were sacrificed with a lethal dose of anesthetic and their caudal fin amputated, fixed for 1 h in 4% PFA, washed 3 times with 1X PBS, preserved in increasing ethanol series (up to 75%) and stored at −20 °C until further used. Caudal fins were rehydrated in increasing 1X PBS series, stained for 30 min in 0.1% AR-S prepared in 1% KOH, washed 3 times with 1% KOH and preserved in increasing glycerol series (up to 75%). Caudal fins were imaged using a Leica MZ10F stereomicroscope equipped with a DFC7000T color camera. Total number of rays and number of fused, bended, or developmentally impaired rays were determined in bright-field images.

2.9.3. Scale development

At 90 dpf, a total of 3 adult fish (1 per replicate tank) were randomly collected from each experimental diet (i.e., Ctrl, MP and MP-BaP), sacrificed with a lethal dose of anesthetic and 30–40 ontogenetic scales were plunked from the left flank of each specimen with stainless steel forceps. Scales were fixed for 30 min in 4% PFA at 4 °C, washed 3 times with 1X PBS, preserved in increasing ethanol series (up to 75%) and stored at −20 °C until further used. Mineral deposition was assessed in von Kossa-stained scales. Briefly, scales were rehydrated in increasing 1X PBS series, washed with 1X PBS, immersed in 5% (w/v) silver nitrate and exposed to U.V. light for 1 h. Scales were then rinsed with distilled water for 5 min and immersed for 5 min in 2.5% (w/v) sodium thiosulfate. Finally, scales were rinsed thoroughly with distilled water and preserved in increasing glycerol series (up to 75%) until image acquisition. Bright-field images of stained scales were acquired using a Leica MZ10F stereomicroscope equipped with a DFC7000T color camera. Scales area, circularity and mineral deposition were assessed through image analysis using ZFBONE ImageJ toolset (Tarasco et al., 2020b).

2.9.4. Calcium and phosphorus content

30dpf juveniles (n = 3 per diet; pools of 6 fish), 90dpf adults (n = 3 per diet), 6dpf offspring (n = 30 per replicate tanks, n = 90 per diet) were randomly sampled and sacrificed with a lethal dose of anesthetic. Fish were dried for 24 h at 60 °C, placed in quartz digestion vials and their weight was determined. Samples were digested with 6 ml of 65% nitric acid (HNO_3; Fisher Scientific, Pittsburgh, PA, USA) using a Discover SP-D 80 microwave digestion system (CEM, NC, USA).

Digested samples were diluted appropriately with ultrapure Milli-Q water (pH 7.4; Merck Millipore, Burlington, MA, USA). Mineral content was determined using a Microwave Plasma-Atomic Emission Spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA). A six-point calibration curve was carried out for each element in 5% HNO_3 (Phosphorus standard, Agilent 213.318; Calcium standard Agilent 393.366). Samples were analyzed in duplicate, and the concentration calculated using the external standard calibration method. Blank samples, containing only the decomposition reagents, were included to control contamination, and results were subtracted from the sample values.

2.9.5. Offspring operculum development

Offspring born from multiple breeding events (10 larvae per successful breeding event; see reproduction section) were sacrificed at 6 dpf with a lethal dose of anesthetic. Larvae were stained for 15 min at room temperature with 0.01% AR-S prepared in Milli-Q water and washed twice with Milli-Q water for 5 min (protocol adapted from Bensimon--Brito et al., 2016). Stained larvae were placed in a lateral plane on top of a 2% agarose gel and imaged using a Leica MZ10F fluorescence stereomicroscope equipped with a with mCherry filters ET560/40x (EX) and ET630/75 m (EM), and a DFC7000T color camera. Morphometric analysis of the operculum was performed using ZFBONE ImageJ toolset (Tarasco et al., 2020b). Total length and body depth were measured using ImageJ built-in tools.

2.10. Histology

Adult zebrafish (n = 5) were demineralized for 10 days in 10% ethylenediamine-tetraacetic acid (EDTA) and 1% PFA (pH 7.0) at 4 °C with the demineralizing solution changed every 2 days. Sample were washed in 1X PBS, incubated in 10% sucrose (in 1X PBS) for 1 h then in 30% sucrose (in 1X PBS) for 12 h at 4 °C. Specimens were then embedded for 1 h in 7.5% gelatin (gelatin from porcine skin, gel strength 300, Type A) prepared in 15% sucrose (in 1X PBS). Samples were placed at 4 °C in a histological box until gelatin solidified, then frozen in isopentane cooled in liquid nitrogen. Using a Leica microtome (CM3050S), fish were longitudinally sectioned at 10 μm under constant temperature (−24 °C). Histological sections were then maintained at −20 °C until further used. For intestinal goblet cell staining, sections were thawed for 15 min at room temperature, washed twice in 1X PBS preheated at 37 °C for 10 min. Slides were post-fixed in 4% PFA for 10 min, washed in 1X PBS, incubated in 3% acetic acid for 3 min and finally stained for 30 min with 1% alcian blue (prepared in 3% acetic acid, pH 2.5). Slides were then washed in distilled water and mounted with mowiol mounting medium. The number of goblet cells was assessed in each villus using a macro developed in ImageJ (Supplementary Fig. 2).

2.11. RNA extraction and gene expression analysis by qPCR

For each experimental diet (i.e., Ctrl, MP and MP-BaP), total RNA was prepared from the whole body of 30dpf juveniles (n = 3, pools of 10 specimen), from tissues (liver, gut and vertebral column) of 3 months adults (n = 3, pools of 5 organs) and from 6 dpf offspring larvae (n = 3, pools of 30 larvae) in triplicates. Total RNA was extracted using NZYol (NZYTech, Lisbon, Portugal), following manufacturer instructions. RNA quantity was measured using a NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA, USA) and integrity was confirmed by running 0.5 μg of total RNA on a 1.5% agarose gel. Total RNA (1 μg) was reverse-transcribed for 1 h at 37 °C using M-MLV reverse transcriptase (Invitrogen), oligo-d(T) primer and RiboLock RNase inhibitor (Thermo Scientific) according to manufacturer instructions. All quantitative real-time PCR (qPCR) reactions were performed using SensiFAST SYBR Hi-ROX kit (Meridian Bioscience, Cincinnati, OH, USA) according to manufacturer instructions, 10 μM of gene-specific primers (Supplementary Table 2) and 1:10 dilution of reverse-transcribed RNA, in a CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). PCR amplification was as follows: an initial denaturation step of 2 min at 95 °C and 40 cycles of amplification (10 s at 95 °C and 20 s at 65 °C). Efficiency of amplification was above 95% for all primer sets. Levels of expression for genes involved in xenobiotic metabolism (i.e., *ahr2*, *nr1i2*, *cyp1a* and *gstp1*), oxidative stress (i.e., *cat*, *gpx1a*, *sod1* and *sod2*), cellular stress (i.e., *hsp70* and *hsp90*) and bone formation (i.e., *sp7*, *oc2*, *spp1*, *col1a1a* and *col10a1a*) were calculated using the ΔΔCt comparative method (Pfaffl, 2001) and normalized using the average of housekeeping genes *eef1a1l1* and *rps18*, previously validated in zebrafish larvae (Tang et al., 2007).

2.12. Statistical analysis

Data were analyzed using Prism version 8.2.1 (GraphPad Software, Inc. La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, or an unpaired Student's t-test were used for data sets presenting a normal distribution. Kruskal-Wallis test followed by Dunn's multiple comparison test were used for data sets that did not follow a normal distribution. In all cases, differences were considered significant for $p < 0.05$.

3. Results

3.1. Both pristine and contaminated microplastics impair zebrafish growth

Mortality, weight, and total length were determined in zebrafish fed the experimental diets from 8 to 30, 90 or 360 dpf. Fish fed MP and MP-BaP diets exhibited a similar survival rate to that of control fish for the three time-windows considered ($p > 0.05$; data not shown). While at 30 dpf dry weight and total length were not different among diets, wet weight and total length were affected at 90 and 360 dpf (Fig. 1). At 90 dpf, fish fed with MP and MP-BaP showed a reduction in their weight (19.1 ± 5.4% and 28.7 ± 6.1%, respectively) and length (7.1 ± 2.0% and 9.3 ± 2.3%, respectively) over the control group. At 360 dpf, fish fed with MP and MP-BaP showed a reduction in their weight (38.5 ± 8.5% and 44.4 ± 8.8%, respectively) and length (15.1 ± 2.9% and 8.7 ± 3.1%, respectively) over the control group. No differences were found between MP and MP-BaP treatments ($p > 0.05$). The analysis of growth parameters in relation to fish gender showed that while weight and length were affected in males, only females fed with MP-BaP showed a reduction in their weight at 90 dpf. Growth parameters were affected at 360 dpf in both males and females fed with MP and MP-BaP diets (Supplementary Fig. 3). Altered growth parameters clearly demonstrate the developmental effect of an exposure to pristine and contaminated microplastics in zebrafish. Although not quantified, it is worth noting that microplastic particles were detected exclusively in the gut and in all the fish evaluated, independently of their developmental stage.

3.2. Contaminated microplastics affect female reproductive capacity

Fish fed the experimental diets until sexual maturity were evaluated for their reproductive performances i.e., breeding success, egg shape parameters (egg area, yolk area and circularity), relative fecundity and embryo hatching rate in females, and sperm viability and motility parameters in males. While embryo viability, area (at 2 and 24 hpf) and hatching rate were not altered by any of the experimental diets (Supplementary Fig. 4A-B-D), other parameters were affected in fish fed MP-BaP. In this regard, the success of breeding events was slightly reduced by 26.3 ± 16.1% (although not statistically different) (Fig. 2A) and the relative fecundity of the females fed MP-BaP was reduced by 53.0 ± 18.4% towards the control diet (Fig. 2B). Egg circularity at 2 hpf (Fig. 2C) and 24 hpf (Supplementary Fig. 4C) and yolk area at 2 hpf (Fig. 2D and 2E) were also affected when parents were fed MP-BaP, showing a reduction of 3.8 ± 1.4%, 2.1 ± 0.9% and 8.0 ± 2.2%,

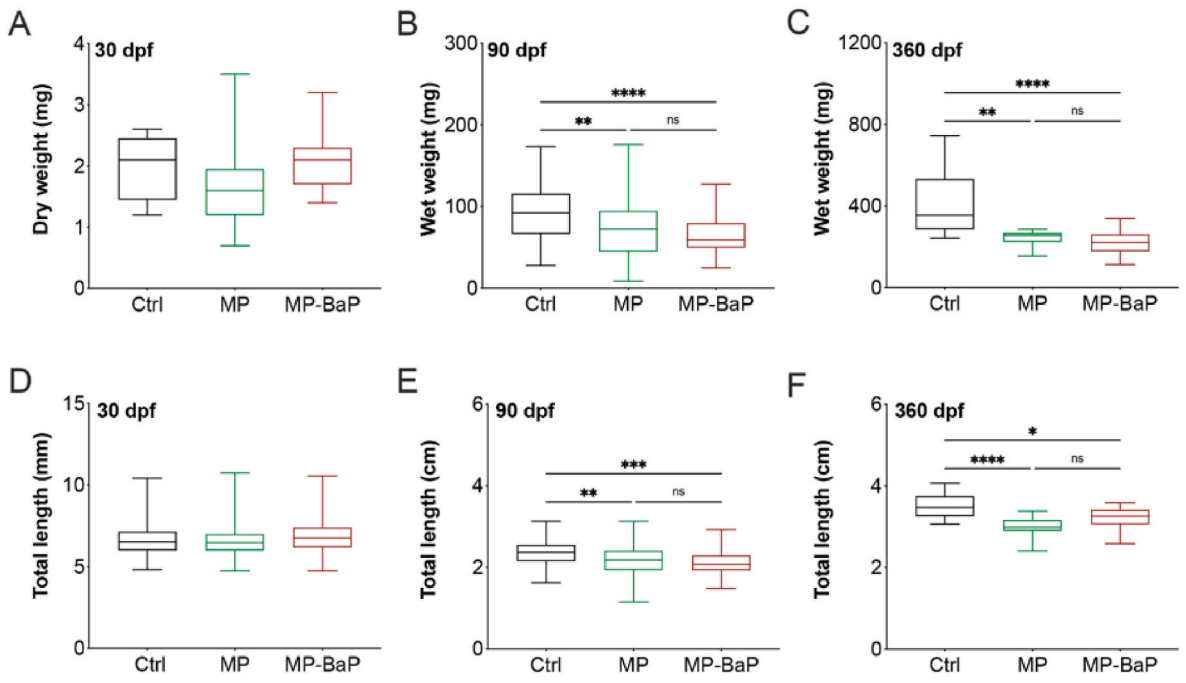

Fig. 1. Weight (**A-C**) and total length (**D-F**) at 30 (**A, D**), 90 (**B, E**) and 360 (**C, F**) days post-fertilization (dpf) of zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP) diets. *Asterisks* indicate values statistically different between diets according to one-way ANOVA followed by Tukey's multiple comparison test or Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). Values are presented as the median ± min and max value. At 30 dpf, n = 9 for dry weight and n = 54 for total length; at 60 dpf, n ≥ 66 for both wet weight and total length; at 360 dpf, n ≥ 13 for both wet weight and total length.

respectively, compared to the control group. Neither sperm viability nor motility parameters (total motility, progressive motility, curvilinear velocity, straight line velocity and linearity) were affected by the experimental diets (Supplementary Fig. 5). No differences were found between zebrafish fed MP and MP-BaP. These data show that long-term exposure of zebrafish broodstock to contaminated microplastics may impair embryo quality.

3.3. Microplastic exposure leads to osteotoxicity

3.3.1. Microplastic affects the development of zebrafish axial skeleton

AR-S stained zebrafish juveniles fed the experimental diets for 22 days (i.e., from 8 to 30 dpf) were examined to assess the effects of microplastic exposure during development of the axial skeleton. Fish fed MP and MP-BaP presented an increased incidence of deformed fish (75.0 ± 2.5% and 88.3 ± 2.3%, respectively) compared to control fish (53.0 ± 0.7% of deformed fish), and fish fed MP-BaP were significantly more deformed (13.3 ± 1.6%) than fish fed MP (Fig. 3A). The number of skeletal deformities per fish also increased in fish fed MP and MP-BaP, with a majority of fish presenting more than two deformed skeletal structures, while control fish had more cases with only one deformed structure (Fig. 3B). Several types of deformities were observed – e.g., partial/total fusion of the vertebrae or neural/hemal arches and malformations of the operculum, urostyle, parhypural and hypurals – and while most of the axial skeleton regions presented a similar distribution of the deformities among treatments, the caudal fin complex was the region more differentially affected by MP and MP-BaP diets, with an increased number of deformities of 300.0 ± 50.0% over the control diet (Fig. 3C). These data show that chronic exposure to pristine microplastic affects zebrafish skeletal development and incidence of skeletal deformities increases when microplastics are contaminated with BaP (see representative images in Fig. 3D).

3.3.2. Caudal fin structure is impaired in adult zebrafish fed with microplastics

Because the caudal fin complex was severely deformed in 30 dpf juveniles exposed to both pristine and contaminated microplastics, the structure of the caudal fin was further assessed in 90 dpf adults fed the experimental diets through the determination of the number of rays and the number of fused, bended or developmentally impaired rays. Microscopic examination of AR-S stained fish revealed an increased incidence of caudal fins showing a reduced number of rays in zebrafish fed MP-BaP (29.2 ± 5.9% and 16.7 ± 5.9% over Ctrl and MP, respectively; Fig. 4A). The number of fused rays – but not the number of bended or developmentally impaired rays (a slight effect was seen though) – also increased in the caudal fin of fish fed MP and MP-BaP (20.8 ± 5.9% over Ctrl; Fig. 4B–E). These data indicate that pristine or contaminated microplastics also trigger skeletal defects in the caudal fin of adult fish chronically exposed since larval stage.

3.3.3. Microplastic exposure affects ontogenetic scales

Since scales are central to fish mineral homeostasis, morphological parameters such as total area, circularity, and extent of demineralized area were assessed in ontogenetic scales, plunked from 90 dpf adult zebrafish fed the experimental diets. Scale area was reduced by 17.8 ± 1.9% and 12.9 ± 2.0% over Ctrl in fish fed with MP and MP-BaP, respectively, and this decrease was significantly more pronounced in fish exposed to MP (Fig. 5A). On the contrary, scale circularity was slightly increased in fish fed MP-BaP (0.7 ± 0.2% over Ctrl and by 0.7 ± 0.2% over MP; Fig. 5B). Finally, demineralized area was increased by 639.2 ± 31.2% over Ctrl and by 618.4 ± 29.9% over MP in fish fed MP-BaP (Fig. 5C-D). These data suggest that exposure to microplastics reduces scale growth, and that the presence of BaP induces a strong demineralization of the scales.

3.3.4. Exposure to contaminated microplastics decreases calcium to phosphorus ratio

To further investigate the reduction in bone mineralization observed

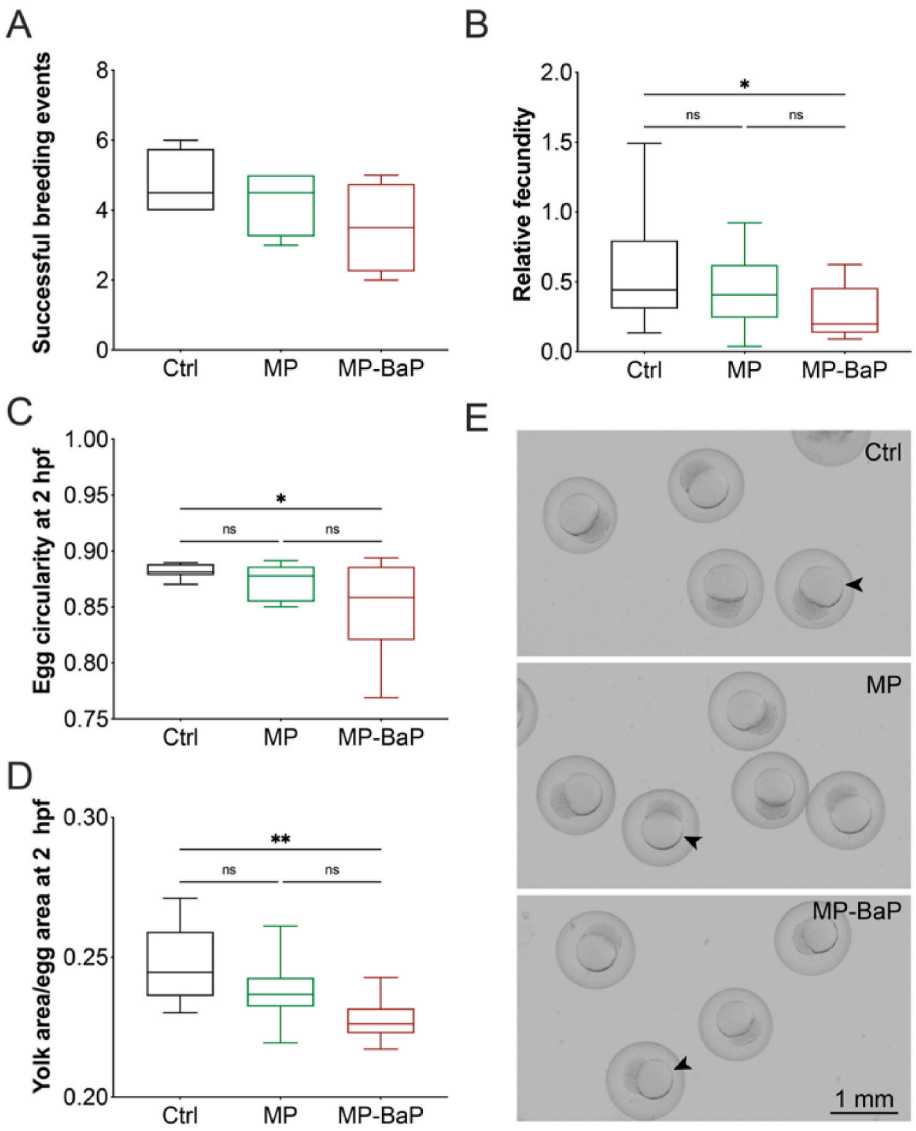

Fig. 2. Reproductive performance of sexually mature 3–4 months zebrafish fed from 8 dpf onward with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). (**A**) Average number of successful breeding events. A value of 0 or 1 was given to breeding events for which couples did not lay eggs (or eggs aborted) or for which couples successfully laid eggs, respectively (n = 4). (**B**) Relative fecundity calculated as the total number of eggs/female body weight (n ≥ 14). (**C**) Egg circularity and (**D**) yolk area assessed at 2 h post-fertilization (hpf) through the morphometric analysis of bright-field images. Yolk area was normalized using egg area (n ≥ 8; pools of 20 eggs each). (**E**) Representative images of egg at 2 hpf collected from breeding events. Arrowheads indicate egg yolk. *Asterisks* indicate values statistically different between diets according to one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$; **$p < 0.01$). Values are presented as the median ± min and max value.

in ontogenetic scales, calcium and phosphorus contents were assessed in the whole body of 30 dpf juveniles and 90 dpf adults fed the experimental diets, and calcium to phosphorus ratio – a proxy for bone quality – was determined. While calcium and phosphorus contents were not significantly altered in fish exposed to pristine or contaminated microplastics at any of the developmental stages evaluated (results not shown), Ca/P ratio was reduced by 8.3 ± 3.1% over Ctrl in fish exposed to MP-BaP until 90 dpf. There was however no difference in the Ca/P ratio calculated for fish fed MP and MP-BaP (Fig. 6). These data show that a long-term exposure (i.e., up to 90 dpf) to BaP-contaminated microplastics affects body Ca/P ratio, and suggests that bone of fish chronically exposed may suffer from a reduced mineral density and thus may be of lower quality, which could trigger skeletal deformities.

3.3.5. *Parental exposure to microplastics affects offspring bone growth*

To assess the intergenerational effect of microplastics exposure, in particular on fish development and bone growth, operculum area, head area and body standard length and depth were determined through morphometric analysis of 6 dpf offspring larvae born from parents fed the experimental diets (Fig. 7). While standard length and head area were not affected in offspring of parents exposed to pristine or contaminated microplastics, body depth (trunk height) was reduced in offspring of parents fed MP-BaP by 4.4 ± 1.7% (Fig. 7B), suggesting that larvae fitness may be affected. The corrected operculum area was also reduced by 12.1 ± 3.8% and 23.4 ± 3.9% over Ctrl in larvae born from parents fed MP and MP-BaP, respectively, suggesting that microplastics osteotoxicity is inherited by future generations (Fig. 7D-E). Reduction was more pronounced in offspring of parents fed MP-BaP, indicating that BaP osteotoxicity can also be transmitted by exposed parents to unexposed offspring, but also that microplastics and BaP have additive osteotoxic effects.

3.4. *Exposure to contaminated microplastics decreases intestinal goblet cell number*

Following recent studies in zebrafish (Limonta et al., 2019) and European seabass (Espinosa et al., 2019) showing alterations in the population of intestinal goblet cells upon exposure to microplastics, the intestine of 90 dpf adult zebrafish fed the experimental diets was examined for the number of goblet cells per villus (Fig. 8). While the number of goblet cells was not significantly different in fish fed MP versus Ctrl ($p > 0.05$), it was reduced by 32.1 ± 10.7% in fish fed MP-BaP versus Ctrl, suggesting that contaminated microplastics alter intestinal homeostasis and trigger inflammation.

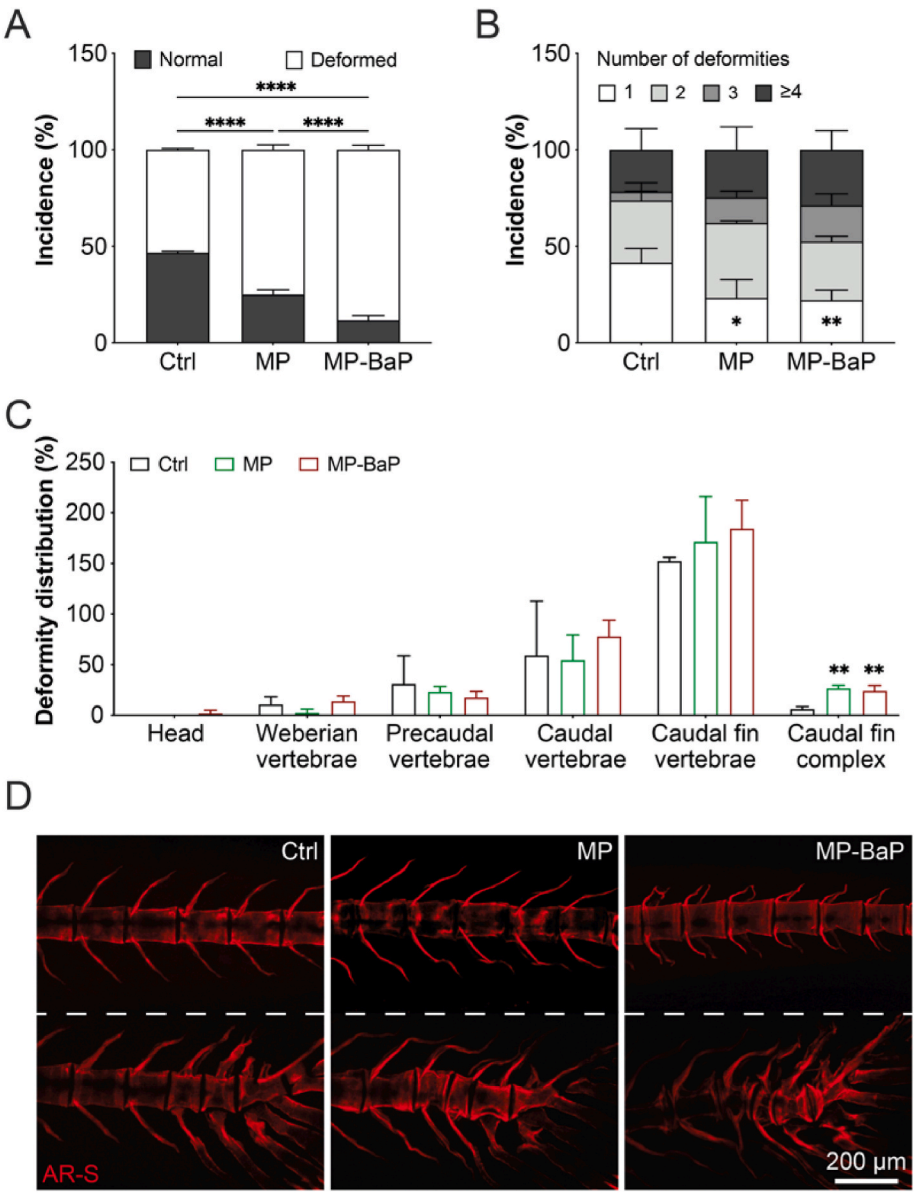

Fig. 3. Deformities in the axial skeleton of 30 dpf zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). (**A**) Percentage of normal and deformed fish. (**B**) Percentage of deformities per deformed larvae. (**C**) Distribution of the deformities along the axial skeleton. Deformed structures of each individual were summed and divided by the number of fish. (**D**) Representative fluorescence images of AR-S stained skeletal structures in fish fed experimental diets. In A and B, *asterisks* indicate values statistically different between diets according to two-way ANOVA, followed by Šídák's multiple comparisons test, (*$p < 0.05$; **$p < 0.01$, ****$p < 0.0001$). In C, *asterisks* indicate values statistically different between diets according to one-way ANOVA, followed by Tukey's multiple comparisons test, (**$p < 0.01$), n = 120.

3.5. Gene expression

To gain insights into the molecular mechanisms underlying the effects of pristine and contaminated microplastics on zebrafish, the expression of several marker genes was assessed by qPCR in 30 dpf juveniles and 6 dpf offspring larvae, and in tissues (i.e., gut, liver, and vertebral column) of 90 dpf adults (Fig. 9 and Supplementary Fig. 6). At a first glance, gene expression was highly variable among different tissues and treatments. A closer look at the expression data revealed that the pregnane X receptor gene (*nr1i2*; xenobiotic receptor) was upregulated in the vertebral column and in the liver (3.38 fold-change over control) of adult zebrafish fed MP-BaP and in offspring born from parents fed MP-BaP, suggesting the activation of xenobiotic signaling in response to BaP. Interestingly, the expression of cytochrome P450 1 A gene (*cyp1a*; phase I metabolizing enzyme) was upregulated in offspring born from parents fed MP and MP-BaP, while it was downregulated in gut, liver and vertebral column of adult zebrafish fed the experimental diets, indicating a different regulation depending on the tissue involved. Expression of genes involved in oxidative stress (*cat*, *gpx1a*, *sod1* and *sod2*) were downregulated in tissues of adult fish fed MP and MP-BaP, suggesting an impaired antioxidant response. In contrast, the expression of osteoblast differentiation marker genes (*sp7* and *oc2*) and genes involved in ECM mineralization (*spp1*, *col1a1* and *col10a1*) were upregulated in 30 dpf zebrafish fed MP-BaP, indicating a feedback response to MP osteotoxicity. Additionally, the expression of cellular stress marker genes (*hsp70* and *hsp90*) was downregulated in most of the treatments, indicating an alteration in cellular homeostasis.

4. Discussion

Microplastic pollution is one of the biggest threats to the aquatic environment, and effects on aquatic organisms have been subject to increased scrutiny over the past decade. The present work is an additional contribution to the already long list of the adverse effects of microplastics in fish and provides new insights on parameters that have been overlooked such as long-term exposure, nutritional route for microplastics exposure, contamination of the microplastics with adsorbed organic pollutants and a focus on osteotoxic effects.

Exposure to experimental diets supplemented with pristine and contaminated microplastics affected zebrafish growth at 90 and 360 dpf. Effects of microplastics on growth parameters such as body length and weight have already been assessed using aquatic model species (e.g.,

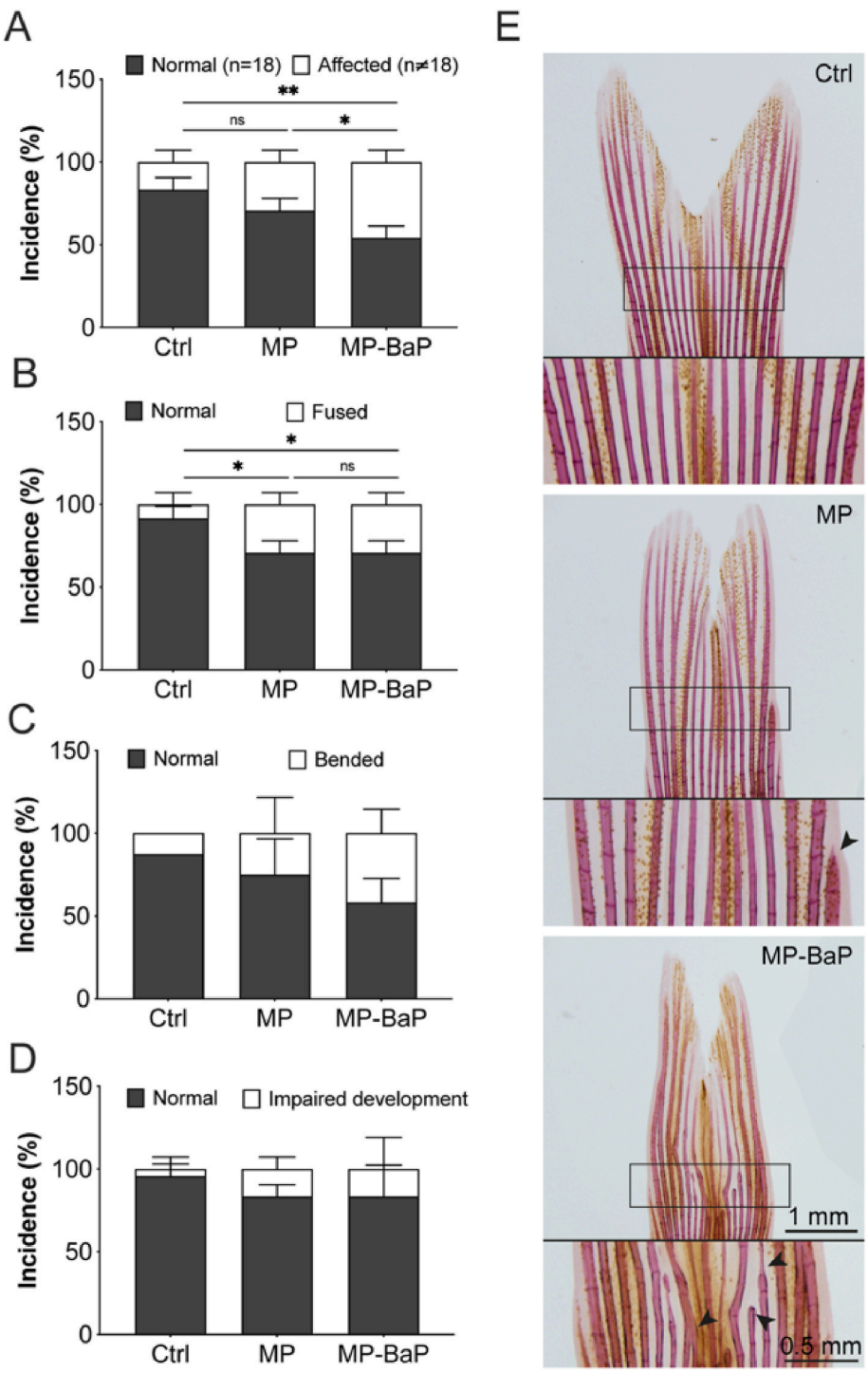

Fig. 4. Deformities in the caudal fin of 90 dpf zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). (**A**) Percentage of caudal fin with a normal number of rays (equal to 18) or not (number of rays different from 18). (**B**) Percentage of caudal fin with individualized rays or presenting fused rays. (**C**) Percentage of caudal fin with rays normally shaped or presenting bended rays. (**D**) Percentage of caudal fin with normally developed rays or presenting rays with an impaired development. (**E**) Representative bright-field images of AR-S stained caudal fin from fish fed experimental diets. *Asterisks* indicate values statistically different between diets according to two-way ANOVA, followed by Šídák's multiple comparisons test, (*$p < 0.05$; **$p < 0.01$), n = 24.

zebrafish and medaka) or farmed species (e.g., gilthead seabream and European seabass) (LeMoine et al., 2018; Santos et al., 2020, 2021; Batel et al., 2020; Khosrovyan et al., 2020; Yang et al., 2020; Pannetier et al., 2020; Jovanović et al., 2018; Capó et al., 2021; Mazurais et al., 2015), but most of these studies reported a limited impairment, if any. Among the studies that support the effect observed here, a reduction of body length or weight was observed in zebrafish larvae exposed (waterborne) to 1–5 μm microplastics (polymer not specified, pristine or spiked with copper) from 2 hpf to 14 dpf or to 5μm polystyrene microparticles (pristine or spiked with F–53 B) from 6 to 13 dpf, respectively (Santos et al., 2020, 2021; Yang et al., 2020). Similarly, body length was reduced in Japanese medaka (*Oryzias latipes*) larvae fed from 3 days after hatching for 30 days with a diet supplemented with a mix of commercial microplastics (PP, PS, HDPE and LDPE) or microplastics sampled in natural environments (Pannetier et al., 2020). The duration of the exposure seems to be a critical aspect of impaired fish growth by microplastics as most of the published data available to date was collected from relatively short-term exposures (maximum 21 days or 30 days of exposure for zebrafish or medaka, respectively). In this manner, the present study confirmed that a short exposure from 8 to 30 dpf does not affect zebrafish growth, while longer exposures (i.e., up to 90 and 360 dpf) triggered a reduction in body weight and total length. Using exposure parameters similar to those used in the present study (i.e., nutritional exposure through a diet supplemented with 1% w/w of

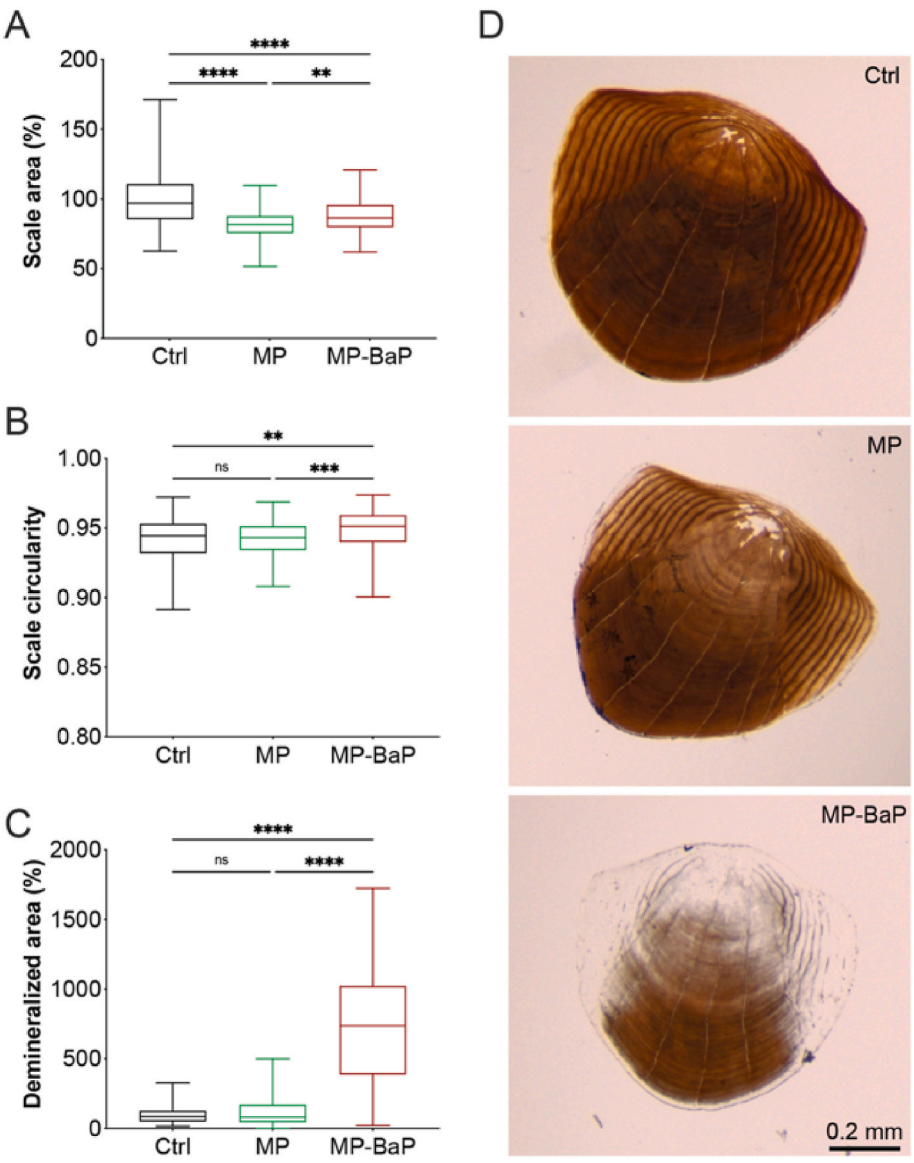

Fig. 5. Morphology and mineral content of ontogenetic scales from 90 dpf zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). (**A**) Scale area and (**B**) circularity were assessed through the morphometric analysis of ontogenetic scales in bright-field images. (**C**) Mineral content was evaluated through von Kossa staining. (**D**) Representative images of von Kossa stained scales collected from fish fed experimental diets. *Asterisks* indicate values statistically different between diets according to Kruskal-Wallis test followed by Dunn's multiple comparison test (**$p < 0.01$; ***$p < 0.001$; ***$p < 0.0001$). Values are presented as the median ± min and max value. n ≥ 112.

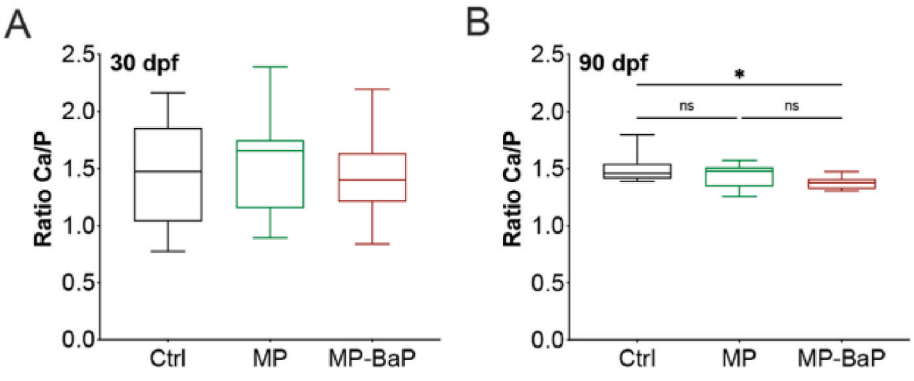

Fig. 6. Calcium to phosphorus ratio (Ca/P) in 30 dpf juveniles (**A**) and 90 dpf adults (**B**) fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). At 30 dpf, n = 9, pools of 6 fish; at 90 dpf, n = 9 dpf. *Asterisks* indicate values statistically different between diets according to one-way ANOVA followed by Tukey's multiple comparison test (*$p <$ 0.05). Values are presented as the median ± min and max value.

11–13 μm polyethylene particles), Cormier et al. (2021) reported that the growth of the zebrafish and marine medaka (*Oryzias melastigma*) was not impaired in fish fed the experimental diets for 60 days. Although this needs to be clarified, we propose that nutritional exposure to microplastics (1% w/w) only impact fish growth if exposure time is equal or longer than 3 months. We propose that the growth effect of microplastics may be related to their ingestion and an impression of false satiation, reducing feeding activity and impairing energy balance (Wright et al., 2013a; Zhang et al., 2021; Cormier et al., 2021; Jovanović, 2017; Zhu et al., 2020), although we cannot exclude the possibility that leaching of polyethylene particle manufacturing additives can also affect zebrafish growth.

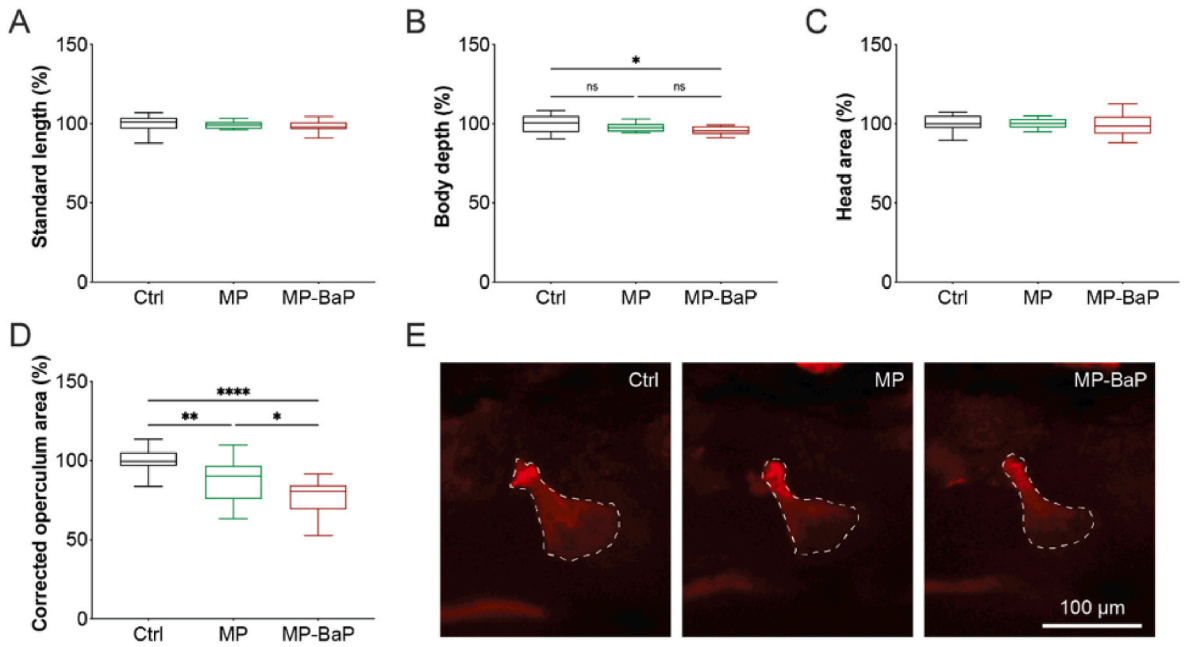

Fig. 7. Growth parameters of 6 dpf offspring larvae born from parents fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). Standard length (**A**) and body depth (**B**) were assessed through the morphometric analysis of bright-field images. Head area (**C**) and operculum growth (**D**) were assessed through the morphometric analysis of fluorescence images. (**E**) Representative images of AR-S stained operculum of 6 dpf offspring larvae. *Asterisks* indicate values statistically different between diets according to one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$). Values are presented as the median ± min and max value, n ≥ 14, 10 larvae from each breeding event.

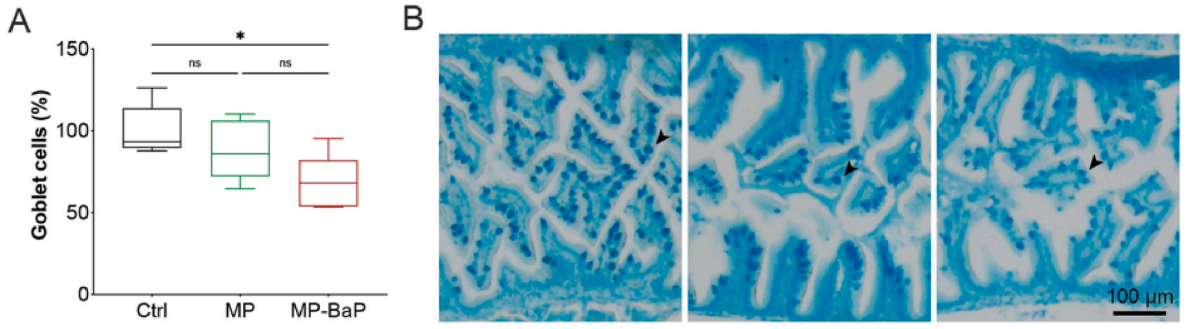

Fig. 8. Number of goblet cells in 90 dpf zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or MP-BaP (ZEBRAFEED supplemented with microplastics spiked with BaP). (**A**) Number of goblet cells/villus expressed in % over Ctrl. (**B**) Representative images of intestine cryosections stained with alcian blue to mark goblet cells (*arrowheads*). *Asterisks* indicate values statistically different between diets according to one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). Values are presented as the median ± min and max value, n = 5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Exposure to pristine microplastics did not impair female reproduction capacity, but relative fecundity and embryo quality were both affected when microplastics were contaminated with BaP. Importantly, sperm quality was not affected by pristine or contaminated microplastics. Studies on the effects of ingested microplastics are still scarce, and most of the data available in the literature have only assessed the effect of polystyrene waterborne exposure using the Japanese medaka. In this regard, waterborne exposure of sexually mature medaka to 2µm polystyrene particles for 21 days had no impact on the number of eggs and embryo survival (Assas et al., 2020), but dietary exposure of medaka during maturation (from 28 dpf juveniles to spawning adults) to bigger polystyrene particles (10 µm) for 10 weeks induced a decrease in egg number (Zhu et al., 2020). A delayed maturation of the gonads and decreased fecundity were also reported in 3 month old marine medaka exposed to 10 µm polystyrene particles for 60 days, while hatching rate and offspring body length were also affected (Wang et al., 2019).

In the present work, relative fecundity (number of spawned eggs) and embryo quality was affected in fish fed MP-BaP but not MP, suggesting that BaP may play a critical role in disrupting female reproductive performance. In this regard, waterborne exposure to BaP decreased the number of spawned eggs, the fertilization rate and the hatching success in both zebrafish and medaka (Sun et al., 2020; Corrales et al., 2014; Gao et al., 2018). In agreement with the present data, Cormier et al. (2021) reported a decrease in the number of eggs produced by zebrafish and medaka females fed diets supplemented with 1% w/w of pristine and contaminated microplastics (e.g., BaP, BP3 or PFOS). In the present work, the reproductive capacity of sexually mature 3-4-months fish exposed to pristine microplastics was not impacted (although a tendency was observed for most of the parameters evaluated). The microplastic particles used by Cormier et al. (2021) were smaller than those used in this study (11–13 *vs* 20–27 µm) and could have a stronger impact on fish reproductive capacity since a higher

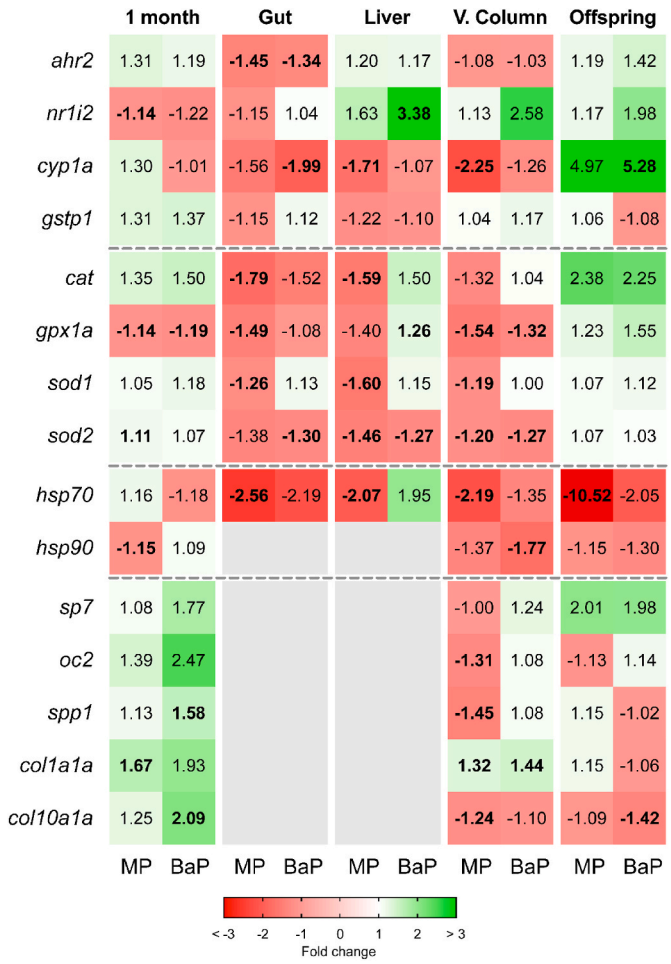

Fig. 9. Heatmap representation of gene expression levels in 30 dpf juveniles (1 month) and tissues (gut, liver, and vertebral column) of 90 dpf adult zebrafish fed from 8 dpf onwards with Ctrl (ZEBRAFEED), MP (ZEBRAFEED supplemented with pristine microplastics) or BaP (ZEBRAFEED supplemented with microplastics spiked with BaP), and in 6 dpf offspring larvae born from parents fed experimental diets. The averaged expression of *eef1a1l1* and *rps18* housekeeping genes was used to normalize gene expression levels. Values in ***bold*** are statistically different from Ctrl values according to Student's t-test ($p < 0.05$). Values are presented as fold-change over the control, n = 3.

number might have been ingested and accumulated. Our data also indicated that the yolk area was reduced in eggs laid by females fed MP-BaP. Eggs also presented a slight decrease in their circularity, probably a consequence of the reduced yolk area. When yolk reserves are disrupted, it is more likely that offspring development will be impaired, resulting in reduced hatching rate and morphological deformities which may lead to their death (Sant and Timme-Laragy, 2018).

In the present study, the only growth parameter affected in the offspring born after parental exposure was the body depth, which was reduced in larvae from parents fed MP-BaP (i.e., larvae were slimmer), while standard length and head area were not affected. Reduced body depth could be related to the reduced yolk area observed in eggs from females fed MP-BaP and consequently a shortage in the nutrients to meet the metabolic requirements needed for a healthy development. Interestingly, growth of the opercular bone was also impaired in offspring born from parents fed pristine/contaminated microplastics, indicating that microplastic and BaP osteotoxicity can be inherited by future generations. Although BaP levels were not determined in the eggs, impaired operculum growth in offspring larvae born from parents fed MP-BaP is similar to the effect recently observed in larvae exposed to BaP waterborne (Tarasco et al., 2021), indicating the probable presence of BaP in egg yolk. In this regard, it has been shown that eggs are able to accumulate lipophilic xenobiotics into their yolk by maternal or waterborne exposure (Sant and Timme-Laragy, 2018). Indirect transgenerational effects or epigenetic mechanisms that may affect gametogenesis could also be involved into intergenerational BaP osteotoxicity, as previously proposed in medaka (Mo et al., 2020, 2021; Seemann et al., 2015, 2017). Operculum growth was also reduced in offspring born from parents fed MP, suggesting that additives used in the preparation of the microplastics could also impact bone development or that microplastics may have intergenerational bone effects through mechanisms that remain to be determined.

The present study also provides clear evidence that pristine and contaminated microplastics have the potential to affect the development of zebrafish axial skeleton resulting in an increased incidence of skeletal deformities, in particular in the caudal fin region. Although the adverse effects of microplastics have been reported in many biological processes, only scarce data are available on a possible osteotoxic effect, i.e., impaired bone formation that may lead to skeletal defects and altered mineral density. Assessing osteotoxic effects is critical as bone defects may impair fish swimming performance and thus their feeding activity and their capacity to escape predation. In this regard, Zhao et al. (2020) reported that the exposure of 4 hpf zebrafish embryos for 7 days to 20 μm polystyrene microplastics spiked with butylated hydroxyanisole (BHA), a synthetic antioxidant widely used to prevent aging of plastics, increased the rate of morphological deformities (i.e., pericardial edema, yolk sac edema, hyperemia and spinal deformity), while pristine microplastics (MPs) did not show any defects. On the contrary, De Marco et al. (2022) recently showed that exposure of zebrafish embryos to 10 μm pristine polystyrene particles for up to 120 hpf increased the incidence of pericardial edema, spinal curvature and column deformation. A longer exposure (12 days) to smaller contaminated particles (0.065 μm) caused a decrease in the number of calcified vertebrae, while pristine MPs had again no effect. Zhao et al. (2020) proposed that the osteotoxic effect observed upon the exposure of fish to contaminated microplastics may be related to thyroid-stimulating hormone (TSH) which was increased upon microplastic exposure. Although TSH levels were not determined here, BaP was shown to increase TSH also in Abu mullet (Movahedinia et al., 2018) and we propose that microplastics contaminated with BaP may exert their osteotoxic effect through a mechanism that may involve TSH. In this regard, it has been shown that TSH is a negative regulator of skeletal remodeling in mice, which can inhibit osteoblast differentiation (Abe et al., 2003). Impaired osteoblast maturation could also be at the origin of the osteotoxic effects – i.e., impaired development of the axial skeleton and caudal fin, impaired scale mineralization, and reduced bone quality – observed in fish fed microplastics contaminated with BaP. In this regard, we recently showed that waterborne exposure of zebrafish to BaP impaired osteoblast maturation and affected bone remodeling through the activation of the xenobiotic and metabolism pathways and an increase in inflammatory response (Tarasco et al., 2021). Although the presence of BaP in bony tissues or in the surrounding tissues was not evaluated, we propose that, upon their ingestion, contaminated microplastics may release BaP in the stomach and/or intestinal tissues; once unbound to the microplastic particles, BaP diffuses throughout the fish body as already showed for zebrafish and bivalves (Batel et al., 2016, 2018; Pittura et al., 2018; O'Donovan et al., 2018) and may affect organism physiology, such as bone homeostasis, either by direct inhibition of bone cell maturation/activity or by indirect activation of xenobiotic/inflammatory pathways.

Although to a lower extent, pristine microplastics also increased the incidence of deformities in the axial skeleton and the caudal fin and triggered a reduction in scale mineralization. While we cannot exclude that PE manufacturing additives may have leached, those findings suggest that other mechanisms – not associated with organic pollutants – are involved into the osteotoxic effects observed. In this regard, abnormal bone homeostasis can result from many different factors such as temperature, water acidification, water flow, genetic background,

nutrition, and exposure to environmental pollutants (Laizé et al., 2018; Pimentel et al., 2014; Boglione et al., 2013a, 2013b; Tarasco et al., 2019), thus an impact upon microplastic ingestion cannot be excluded. In this study, the exposure to contaminated microplastics caused a depletion of goblet cells at 90 dpf, suggesting that intestinal homeostasis may be impaired by BaP. Although less pronounced, exposure to pristine microplastics also affected goblet cell numbers. A similar effect was observed in European seabass larvae fed a diet supplemented with 0.1 or 0.5% w/w of PE (40–150 μm) for 21 days (Espinosa et al., 2019) and in adult zebrafish fed pristine HDPE + PS microplastics (25–90 μm) for 20 days, which additionally showed alterations of the intestinal mucosa, such as epithelial detachment and mucous hypersecretion (Limonta et al., 2019). Goblet cells maintain the intestinal mucosal barrier through the secretion of mucin which protects the intestine from pathogens and chemical injury (Kim and Ho, 2010; Zhao and Pack, 2017). Depletion of goblet cells was shown to increase inflammation and cause disruption of the intestinal homeostasis, which is essential for digestion and nutrient absorption (Brugman et al., 2009; Farré et al., 2020). Although nutrient absorption was not measured, impairment of the intestinal barrier may jeopardize zebrafish growth.

Finally, the expression of several marker genes was assessed to gain insights into the molecular mechanisms underlying the effects of pristine and contaminated microplastics on zebrafish. Although gene expression levels were variable among the different tissues and treatments, genes involved in antioxidant response were generally downregulated in zebrafish exposed to the experimental diets up to 90 dpf. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the activity of antioxidant defenses. In this sense, when the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) is inhibited, ROS accumulation may cause physiological impairments (Hoseinifar et al., 2020; Picardo and Dell'Anna, 2010). In agreement with the data presented here, a down-regulation of *cat*, *sod1*, *gpx1a* was reported in brain/liver (Umamaheswari et al., 2021a) and gills (Umamaheswari et al., 2021b) of adult zebrafish waterborne exposed to polystyrene (PS) microparticles (0.10–0.12 μm) for 35 days and in liver of marine medaka exposed to PS microparticles (10 μm) for 60 days (Wang et al., 2019). Enzymatic inhibition of CAT and SOD activity was also reported in the liver of adult zebrafish exposed to PE microparticles (146.20 ± 8.86 μm) for 10 days (Rangasamy et al., 2021) and European seabass larvae fed PE (40–150 μm) for 3 weeks (Espinosa et al., 2019). We propose that long-term exposure to microplastics impairs the activity of antioxidant enzymes and leads to increase oxidative stress, which has been associated also with an impairment of bone cells function and quality (Wauquier et al., 2009; Domazetovic et al., 2017). Interestingly, expression of cellular stress marker genes (*hsp70* and *hsp90*) was down-regulated in zebrafish fed MP and MP-BaP, both in individual tissues and whole specimen. In agreement with the present data, expression of *hsp70* was down-regulated in European seabass fed PE microparticles for 21 days (Espinosa et al., 2019) and in *Daphnia magna* exposed to a mix of MPs for 48 h (Imhof et al., 2017). Yu et al. (2019) also reported a decrease in the levels of HSP70 and HSP90 in mice exposed for 10 days to the plasticizer dibutyl phthalate concomitantly to histological alterations and decreased number of intestinal goblet cells, suggesting intestinal inflammation and damage. In fish, the expression of heat shock-proteins is normally increased to restore the cell status upon exposure to stressful conditions (i.e., heat, salinity, and environmental pollutants) (Mohanty et al., 2018). Therefore, we cannot exclude that a decrease in the expression of proteins involved in cell repair may have impaired fish physiology upon exposure to MPs, leading to an increase in oxidative damage and intestinal inflammation.

Genes related to xenobiotic metabolism were also differentially regulated in zebrafish exposed to experimental diets. In particular, *nr1i2,* a nuclear receptor known to be activated by BaP and other PAHs (Laizé et al., 2018; Tarasco et al., 2021) and responsible for the activation of detoxifying mechanism, was up-regulated in liver and vertebral column of adult zebrafish fed MP-BaP, another indication of the possibility that BaP may have leached from MP and accumulate in surrounding tissues. In the offspring, we observed a general increase in expression of genes related to xenobiotic metabolism, such as nuclear receptors and a stronger up-regulation of *cyp1a,* suggesting that xenobiotic metabolism may be activated and may cause intergenerational effects. Surprisingly, *cyp1a,* which is expected to be activated upon exposure to environmental pollutants, was down-regulated in gut, liver and vertebral column of zebrafish exposed to pristine and contaminated MPs up to 90 dpf. On the contrary, several studies have reported increased activity or transcription of *cyp1a* in zebrafish exposed to pristine or contaminated MPs (Batel et al., 2018; Xu et al., 2021; Cormier et al., 2019; Rainieri et al., 2018) from 1 to 21 days, depending on the study. In agreement with our data, Xia et al. (2020) showed that *Cyprinus carpio* larvae exposed to PVC microplastics (100–200 μm) for up to 60 days had *cyp1a* expression upregulated after 30 days of exposure but downregulated at the end of the experiment. We propose that effects of short-term exposures to MPs may be counteracted by xenobiotic metabolism enzymes, while longer exposures may be more effective due to the impairment of cellular detoxifying mechanisms. To the best of our knowledge, this is the first time that an effects of pristine/contaminated MPs is reported on bone related gene expression, i.e., a general upregulation in zebrafish larvae fed MP and MP-BaP and a downregulation of *col10a1*a, *oc2* and *spp1* in adult vertebral column. However, this is not the first time that genes involved in bone metabolism are differentially regulated in zebrafish exposed to PAHs. Indeed, bone marker genes were upregulated in zebrafish larvae exposed (waterborne) to 3-methylcholanthrene (i.e., *fgf20a*, *vdrb*, *bmp2a* and *alpl*) (Laizé et al., 2018) and to BaP (i.e., *vdrb* and *alpl*) (Tarasco et al., 2021). Furthermore, waterborne exposure to BaP also increased proliferation of immature osteoblast as reported by the increased number in *sp7* positive cells. As already seen for marker genes involved in xenobiotic response pathway (see above), it seems that short-term and long-term exposure to MPs elicit opposite effects on the expression of bone related genes. This may indicate that the transcriptional machinery will initially answer the osteotoxic signal through a compensatory/feedback mechanism, which will be override by the toxic signal in the long run. It is also possible that long-term microplastic osteotoxicity initiates indirect mechanisms such as oxidative damage and activation of xenobiotic metabolism. Further studies should aim at gaining additional knowledge on the mechanisms underlying short and long-term osteotoxicity of microplastics leading to an impairment of bone homeostasis. In a more global context, it would be of utmost interest to investigate whether gender differences reported in this study at reproductive and growth level are also observed during skeletal development.

5. Conclusions

This study demonstrated that long-term dietary exposure to pristine/contaminated polyethylene microplastics has the potential not only to jeopardize fish growth and reproductive performance, but also to produce an intergenerational effect. The data presented here also provided the first evidence of adverse effects on fish axial skeleton and bone compartment upon the ingestion of microplastics, with osteotoxic outcomes aggravated by the presence of organic contaminants adsorbed on the surface of the microplastics. Gene expression analysis evidenced an involvement of the xenobiotic pathway and antioxidant response in the mechanisms underlying the harmful effects triggered by pristine and/or contaminated microplastics. Future studies should aim at providing additional evidence on the adverse effects following a long-term exposure of fish to microplastics, and at testing a wider range of plastic polymers and contaminants with the capacity to absorb onto microplastics.

Author contribution

Marco Tarasco: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft preparation, review & editing. **Paulo J. Gavaia**: Conceptualization, Investigation, Methodology, Validation, review & editing, Funding acquisition. **Anabela Bensimon-Brito**: Investigation, Methodology, review & editing. **Fabrice P. Cordelières**: Methodology, Resources, Software, review & editing. **Tamára Santos**: Methodology. **Gil Martins**: Investigation, review & editing. **Daniela Castro**: Investigation, review & editing. **Nádia Silva**: Investigation, Methodology, review & editing. **Elsa Cabrita**: Investigation, Methodology, review & editing. **Maria J. Bebianno**: Supervision, review & editing. **Didier Y. R. Stainier**: Resources, Supervision, review & editing, Funding acquisition. **M. Leonor Cancela**: Resources, Supervision, review & editing, Funding acquisition. **Vincent Laizé**: Conceptualization, Methodology, Resources, Supervision, Validation, Visualization, review & editing, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135198.

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