

## Article

# Toxicity and Functional Tissue Responses of Two Freshwater Fish after Exposure to Polystyrene Microplastics

Martha Kaloyianni <sup>1</sup>, Dimitra C. Bobori <sup>2,\*</sup>, Despoina Xanthopoulou <sup>1,2,†</sup>, Glykeria Malioufa <sup>1,†</sup>, Ioannis Sampsonidis <sup>3</sup>, Stavros Kalogiannis <sup>3</sup>, Konstantinos Feidantsis <sup>1</sup>, Georgia Kastrinaki <sup>4</sup>, Anastasia Dimitriadi <sup>5</sup>, George Koumoundouros <sup>5</sup>, Dimitra A. Lambropoulou <sup>6</sup>, George Z. Kyzas <sup>7</sup> and Dimitrios N. Bikiaris <sup>8,\*</sup>

- <sup>1</sup> Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; kaloyian@bio.auth.gr (M.K.); despant@bio.auth.gr (D.X.); mglykeri@bio.auth.gr (G.M.); kfeidant@bio.auth.gr (K.F.)
- <sup>2</sup> Laboratory of Ichthyology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
- <sup>3</sup> Department of Nutritional Sciences and Dietetics, International Hellenic University, 57001 Thessaloniki, Greece; isampson@nutr.teithe.gr (I.S.); kalogian@nutr.teithe.gr (S.K.)
- <sup>4</sup> Laboratory of Inorganic Materials, CERTH/CPERI, 57001 Thessaloniki, Greece; georgiak@cperi.certh.gr
- <sup>5</sup> Biology Department, University of Crete, 70013 Herakleion, Greece; adimitriadibio@gmail.com (A.D.); gkoumound@uoc.gr (G.K.)
- <sup>6</sup> Laboratory of Environmental Pollution Control, Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; dlambro@chem.auth.gr
- <sup>7</sup> Department of Chemistry, International Hellenic University, 65404 Kavala, Greece; kyzas@chem.ihu.gr
- <sup>8</sup> Laboratory of Polymer Chemistry and Technology, Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
- \* Correspondence: bobori@bio.auth.gr (D.C.B.); dbic@chem.auth.gr (D.N.B.)
- † Equal contribution.



**Citation:** Kaloyianni, M.; Bobori, D.C.; Xanthopoulou, D.; Malioufa, G.; Sampsonidis, I.; Kalogiannis, S.; Feidantsis, K.; Kastrinaki, G.; Dimitriadi, A.; Koumoundouros, G.; et al. Toxicity and Functional Tissue Responses of Two Freshwater Fish after Exposure to Polystyrene Microplastics. *Toxics* **2021**, *9*, 289. <https://doi.org/10.3390/toxics9110289>

**Academic Editors:**  
Markus Brinkmann and  
Farhan R. Khan

Received: 4 September 2021  
Accepted: 29 October 2021  
Published: 2 November 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Abstract:** Microplastics (MPs)' ingestion has been demonstrated in several aquatic organisms. This process may facilitate the hydrophobic waterborne pollutants or chemical additives transfer to biota. In the present study the suitability of a battery of biomarkers on oxidative stress, physiology, tissue function and metabolic profile was investigated for the early detection of adverse effects of 21-day exposure to polystyrene microplastics (PS-MPs, sized 5–12 µm) in the liver and gills of zebrafish *Danio rerio* and perch, *Perca fluviatilis*, both of which are freshwater fish species. An optical volume map representation of the zebrafish gill by Raman spectroscopy depicted 5 µm diameter PS-MP dispersed in the gill tissue. Concentrations of PS-MPs close to the EC<sub>50</sub> of each fish affected fish physiology in all tissues studied. Increased levels of biomarkers of oxidative damage in exposed fish in relation to controls were observed, as well as activation of apoptosis and autophagy processes. Malondialdehyde (MDA), protein carbonyls and DNA damage responses differed with regard to the sensitivity of each tissue of each fish. In the toxicity cascade gills seemed to be more liable to respond to PS-MPs than liver for the majority of the parameters measured. DNA damage was the most susceptible biomarker exhibiting greater response in the liver of both species. The interaction between MPs and cellular components provoked metabolic alterations in the tissues studied, affecting mainly amino acids, nitrogen and energy metabolism. Toxicity was species and tissue specific, with specific biomarkers responding differently in gills and in liver. The fish species that seemed to be more susceptible to MPs at the conditions studied, was *P. fluviatilis* compared to *D. rerio*. The current findings add to a holistic approach for the identification of small sized PS-MPs' biological effects in fish, thus aiming to provide evidence regarding PS-MPs' environmental impact on wild fish populations and food safety and adequacy.

**Keywords:** polystyrene; microplastics; *Danio rerio*; *Perca fluviatilis*; gills; liver; metabolomics; oxidative stress biomarkers

**Key Contribution:** Polystyrene microplastics (PS-MPs) effects were compared on freshwater fish tissues. PS-MPs caused elevation of oxidative stress indices and provoked metabolites reduction in both fish gills and liver. The toxicity PS-MPs caused was species and tissue specific.

## 1. Introduction

Polystyrene is a thermoplastic synthetic polymer with appropriate thermal and mechanical properties and can be used in many applications even though it is characterised as a hard and brittle material. The worldwide production of polystyrene was close to 15.61 million metric tons in 2019 and it is estimated to be stabilized at these levels for the next years, while it covers about 5–6% of the global plastic production [1]. It is a colourless transparent polymer used in household applications, electronics, packaging, isolation foams, single used items like disposable cutlery, etc. [2]. Due to the lightness of most of its products, it is very difficult to recycle and unfortunately the majority of it is disposed of in the environment, contributing significantly to the formation of microplastics (MPs). These MPs are plastic fragments with sizes less than 5 mm and can cause serious health problems for all living organisms including human beings [3–8].

Research on freshwater ecosystems has started to gain attention since rivers act as the main pathways for plastic transport to seas [9,10]. In a recent literature search, only 16.2% of the published papers concerning microplastic pollution, were focused on freshwater environments [11].

It is clear, independently from the ecosystem concerned (marine/freshwater or even terrestrial), that plastic debris are being broken down by numerous procedures, such as UV degradation, oxidation and erosion, resulting in smaller fragments, with a vast range in sizes [12,13]. Ingestion of MPs, which may facilitate the hydrophobic waterborne pollutants' or chemical additives' transfer to biota, is a process which has been demonstrated in a range of aquatic organisms belonging to different taxonomic groups, including invertebrates and vertebrates such as, amphibians [14], fish, sea turtles, seabirds and marine mammals [15–21].

The information concerning MPs biological effects on freshwater organisms is to date much limited [22–25]. Among others, studies have already demonstrated the existence of plastic chemicals in fish tissues [26,27]. This evidence has alarmed researchers to examine the transfer of MPs through trophic food chains and study the impacts of MPs on biota that constitute food supply for humans [27–29]. This increase in awareness about the MPs ecological impacts is owned to their small size that enables absorption by biota and as a result aggregation in the food chain occurs; in addition, MPs can assimilate contaminants on their surfaces [30], exerting thus additive effects to biota [31,32] and becoming more resistant to deterioration by microorganisms [33].

Fish consume plastic fragments accidentally, usually mixed with their natural prey [12,13]. Micro and nano plastic particles can be transferred to living cells through the lymphatic or circulatory system. This results to MPs' dispersion in the whole body and the induction of severe effects such as decreased feeding activity [34], impeded growth and development [35–37], endocrine disruption [38] and energy destruction [39], oxidative stress (for a review see [40], immunity and neurotransmission disorders [40,41], genotoxicity [42] and even mortality [37]. Internalization of MPs can also occur after they are adhered to fish skin or other tissues, such as gills [26]. Accordingly, MPs are concentrated through the circulation mainly in the gastrointestinal track [43], causing further histopathological alterations in the intestine, physical damage, changes in fish behavior, while translocation to liver tissue may occur, inducing a lot of unfavorable effects [44,45].

Several biomarkers have so far been suggested and examined in laboratory experimental exposure (via water or food) on fish thus offering a first set of implements that can be used to quantify the consequences that chemicals exert [46–48]. Among them oxidative stress biomarkers (as usually directly measured through free radical production, protein oxidation increase, lipid peroxidation), genotoxicity (DNA damage increase) and metabolic

biomarkers (definition of differentiations in metabolic pathways) are considered as the most commonly used in such studies [49–52].

In the present study the effects of PS-MPs sized 5–12  $\mu\text{m}$  on two tissues, liver and gills, of two freshwater fish species, zebrafish *Danio rerio* and perch, *Perca fluviatilis*, are monitored. The comparison of the effects on multiple levels on the two vital tissues in two quite distinct fish species will improve our perception of the biochemical and physiological mechanisms implicated in MPs toxicity in order to assess the effects on fish health and population dynamics as well as the suitability of a battery of biomarkers for the early detection of adverse effects of MPs on the environment. Gills were selected as an appropriate marker for evaluating toxicity effects of PS-MPs since they are highly perfused, while liver tissue acts as a detoxifying organ of contaminants entering fish body [53,54], both tissues used in estimating the adverse pollutants effects imposed to organisms [55]. The ultimate goal of our research is to suggest the most suitable fish species, tissue and set of biomarkers to be audited with regards to PS-MPs pollution. Both fish species have been used in biomonitoring studies against pollutants previously [36,46,47,56,57]. To our knowledge, it is the first time that the effects of PS-MPs on the gills and liver of two freshwater fish are investigated by using the combined metabolomic and toxicity methods.

## 2. Materials and Methods

### 2.1. Synthesis and Characterization of Polystyrene Microplastics (PS-MPs)

The preparation of PS microparticles was described in our previous work using the water oil emulsion technique [52]. As found by scanning electron microscopy, spherical PS-MPs with mean average diameter about  $8 \pm 3 \mu\text{m}$  were prepared.

### 2.2. Characterization of Polystyrene Microplastics (PS-MPs) in Fish Parts

Regarding the Fourier transform infrared microscopy, the control and exposed gill and liver were pestled in a mortar with 0.24 g spectroscopic grade KBr powder and dehydrated in room temperature in a desiccator until any moisture was removed. The mixture was then pressed to a pellet form under 6 tons pressure. The microspheres were mixed with KBr in 1% wt for the PS pellet formation. A Jasco spectrometer (Jasco FTIR-6700, Tokyo, Japan) was employed for pellet measurement. In the spectral range of  $4000\text{--}400 \text{ cm}^{-1}$  seventy scans with a resolution of  $4 \text{ cm}^{-1}$  were collected in the absorbance mode. A 19-point Savitzky–Golay algorithm calculated the second derivative absorption spectrum by the Spectra Manager 2.15.12 software (Jasco Corporation, Tokyo, Japan) by a 17-point Savitzky–Golay algorithm according to relative processing [58]. The HR (high resolution) volume mapping video was calculated by a micro-Raman in Via Instrument coupled with a solid state  $532 \text{ cm}^{-1}$  laser and  $\times 10$  Leica lens. The zebrafish gill was dehydrated in a desiccator chamber for 48 h prior to measurement; the analyzed area was  $255 \times 192 \times 10 \mu\text{m}$  and the step resolution was  $1 \mu\text{m}$ . Next, 517,400 spectra were collected and analyzed by the Wire 5.3 software for the digital construction of the HR volume mapping video (Supplementary Material). The color map was created in respect to the intensity of the spectrum at the characteristic PS peak at  $995 \text{ cm}^{-1}$ .

### 2.3. Fish Husbandry

Adult specimens of zebrafish (*D. rerio*, ZF WT 2 F10, Wageningen Agricultural University, Wageningen, The Netherlands) were provided by the Department of Biology of the University of Crete. Fish had (mean  $\pm$  SD) total length and body weight equal to  $33.5 \pm 2.7 \text{ mm}$  and  $0.25 \pm 0.07 \text{ g}$  respectively, aged 6 months, while no sex separation was conducted. All specimens were acclimatized in aerated fish tanks (3 individuals per liter) with water pump circulation through filters, under 14:10 light:dark cycle, at  $28 \pm 0.2 \text{ }^\circ\text{C}$  temperature,  $8.57 \pm 0.23 \text{ pH}$ ,  $8.94 \pm 0.14 \text{ mg L}^{-1}$  dissolved oxygen,  $560 \pm 37.9 \mu\text{S cm}^{-1}$  conductivity and  $0.2 \pm 0.06 \text{ psu}$  salinity.

Specimens of both sexes of wild fish species *P. fluviatilis* (mean  $\pm$  SD total length  $13.5 \pm 3.08 \text{ cm}$  and body weight  $27.2 \pm 19.67 \text{ g}$ ) were provided alive from Lake Volvi

(Northern Greece) by a commercial fisherman, while no sex separation was conducted. Immediately they were placed in aquariums of 150 L water volume (six individuals per liter), equipped with circulated pumps for filtering and cleaning water. Fish were maintained at well aerated water of  $19.2 \pm 0.94$  °C, pH  $7.4 \pm 0.92$ , dissolved oxygen concentration  $10.6 \pm 0.33$  mg L<sup>-1</sup>, conductivity  $609 \pm 41.1$  µS cm<sup>-1</sup> and salinity  $0.5 \pm 0.29$  psu. The photoperiod was adjusted to a 14:10 light:dark cycle.

During the 7-day acclimatization period zebrafish were fed once per day with commercial flakes (Cichlid Omni Flakes, Ocean Nutrition Europe, Essen, Belgium) while fish excrement in aquariums was removed manually, every day with a net. Perch were acclimatized for 25 days, their feeding was based on commercial dry shrimps *Gammarus pulex* (Tropical company, Dover, DE, USA) and applied once per day.

#### 2.4. Ethical Statement

All the experimental procedures involving handling and exposure of fish were performed in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation for animal experimentation and welfare. All protocols were approved by the Animal Care Committee of the Biology Department of the University of Crete (Permit Number: 285586(2020)).

#### 2.5. Food Preparation

Food was prepared following the procedure described previously [46]. In brief, an amount of PS-MPs (5–12 µm in size) in water suspension was incorporated into commercial powder fish food (different for each species as given above), to obtain the target concentrations (see below). Subsequently the mixture was well homogenized and dried in oven for about 2 h at 50 °C. Food for control groups was prepared following the same procedure as in previous study [52], omitting the addition of MPs. The heating of food could degrade food nutrient composition and possibly affect fish sensitivity to contaminants. However, since the control food was treated the same, this may not affect our results. In all experiments each fish was fed once per day with food corresponding to 3% of its wet weight [59], that is equivalent to 0.0075 g and 0.85 g for zebrafish and perch respectively. Fish were inspected during feeding, ensuring thus that all food was consumed.

#### 2.6. EC<sub>50</sub> Estimation (1st Experimental Design)

EC<sub>50</sub> value has been largely employed as a potent and reliable index for the estimation of the sublethal concentration of various toxicants in several studies of our group, in aquatic animals and snails [46,47].

For the estimation of EC<sub>50</sub> in zebrafish, 24 individuals were divided into three groups (eight individuals per group, each group in different tank). Each group was fed, once per day, with food containing nominal concentrations of 1, 50 and 100 mg PS-MPs per g dry weight for 21 days. This period is within the range of 4 h to 2 months used by several researchers as an exposure time in laboratory experiments to assess the toxicological effects of MPs in fish (see review by Phuong et al. [60]) and was selected to depict the associated toxicity mechanisms in organisms corresponded to highly pronounced contaminant stress. An additional group of eight zebrafish, fed with commercial food without PS-MPs addition under the same conditions, served as control. After the end of exposure time, blood samples were collected from the tail and centrifuged at  $3000 \times g$  for 10 min at 4 °C. Specifically, tail was dissected, and blood removed from the dorsal aortic canula was collected in heparinized Eppendorf tubes. Subsequently the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) levels were measured [61] in the blood samples in order to assess mitochondrial redox capacity. The mean values of MTT obtained from eight zebrafish were calculated for each feeding concentration and the EC<sub>50</sub> value was estimated using the SPSS software. The EC<sub>50</sub> value for zebrafish was calculated at 10 mg of PS-MPs g<sup>-1</sup> of dry food and this concentration was used for preparing zebrafish food for the consecutive in vivo exposure experiments.

For the estimation of EC<sub>50</sub> in perch, a similar experimental procedure as that described before was followed. In specific, nine individuals were divided into three groups (three individuals per group, per tank). In addition, three specimens were used as the control group. Each group was fed, once per day, with food containing nominal concentrations of 1, 50 and 100 mg PS-MPs g<sup>-1</sup> dry weight for 21 days. After the end of exposure time, the destabilization of the hemocyte's lysosomal membranes was assayed in the blood samples by the Neutral Red Retention Time (NRRT) assay [62] as modified by Dailianis et al. [63]. The EC<sub>50</sub> value for perch was calculated at 134 mg of PS-MPs g<sup>-1</sup> of dry food, which was used in the subsequent experiment.

MTT and NRRT uptake assays are both cellular biomarkers. MTT represents mitochondrial redox capacity and NRRT provides information on the lysosomal activity of the cells. Since zebrafish blood volume was much less in relation to that of perch, MTT assay was preferred over NRRT due to the less blood required for conducting the assay.

Since data about the environmental concentrations of PS-MPs in freshwater aquatic ecosystems is limited [42], our experiments were designed mainly to assess the response of both fish species under sublethal PS-MPs concentrations, simulating a highly pessimistic pollution scenario. Our approach was to provoke accelerated effects on fish exposed to higher concentrations than those reported in the field. The use of higher concentrations than those found in the field, in order to depict the associated MPs toxicity mechanisms is very common in such laboratory studies, thus, the ingestion and toxic effects observed in organisms correspond to highly pronounced contaminant stress (see review by Phuong et al. [60]). In accordance with the concentrations of MPs used in the present study, the use of 40 mg g<sup>-1</sup> has been reported for zebrafish experiments [64], while Solomando et al. [65] exposed *Sparus aurata* to 100 mg MPs g<sup>-1</sup>.

### 2.7. Fish Feeding Exposure to PS-MPs (2nd Experimental Design)

Experiments for assessing the response of both fish to PS-MPs were run in parallel. Zebrafish control ( $n = 30$ –10 individuals per aquarium) and exposed individuals ( $n = 30$ , 10 individuals per aquarium) were kept in aquariums of 30 L with circulated water, external oxygenation and the same conditions as in the acclimatization stage. Fish were fed once per day with food containing 10 mg PS-MPs g<sup>-1</sup> of dry food for 21 days while control animals were fed with food without added MPs. Accordingly, perch specimens were divided into two groups, the control ( $n = 6$ , 2 individuals per aquarium) and the experimental group ( $n = 6$ , 2 individuals per aquarium) and kept in aquariums under the same conditions as those previously described. Fish were fed once per day with pellets containing 134 mg PS-MPs g<sup>-1</sup> of dry food for 21 days, except the control group which was fed with commercial food for percid, without the addition of PS-MPs. During the treatment period the water in aquariums was kept at a constant volume by adding the appropriate quantity of water. No fish mortality was observed, either in the control or the exposed groups for both species.

### 2.8. Tissue Sampling

After the treatment period, control and exposure fish of both species were anaesthetized (zebrafish in cold water and perch in ethanol clove oil diluted in water), immediately placed on ice and blood samples were taken from the caudal area and placed in tubes with heparin. Gills and liver tissues were consequently extracted from both fish, placed in tubes and stored at  $-30\text{ }^{\circ}\text{C}$  (for approximately 1 month) until further analyses and were used for the estimation of lipid peroxidation, protein carbonylation, DNA damage, ubiquitin conjugates, autophagic and apoptotic processes and metabolomics analysis.

### 2.9. Molecular and Biochemical Analyses

All analyses described below were assessed in the liver and gills of the whole population ( $n = 30$  individuals of *Danio rerio*) divided in 3 pools of 10 fish and each pool was analyzed separately ( $n = 3$  pools). For *Perca fluviatilis*  $n = 6$  individuals per experimental

condition (control and exposure) were used and the tissues of 2 fish were pooled and analyzed together forming 3 different pools.

The estimation of lipid peroxidation in gills and liver tissues followed the method described by Niehaus and Samuelsson [66]. Frozen tissues were immediately homogenized in 50 mmol L<sup>-1</sup> phosphate buffer (pH 7.4). The homogenate was then centrifuged (2000 × *g*, 4 °C, 15 min), and immediately 250 µL of 20% TCA and 500 µL of 0.67% thiobarbituric acid were added in 250 µL of supernatant. The mixture was vortexed, boiled for 60 min, and cooled at room temperature. Thereafter, 2 mL of butanol was added and the mixture was again centrifuged (3000 × *g*, 15 min). The results are expressed as nmol malondialdehyde (MDA) per mg protein (protein concentration was determined by using the BioRad protein assay), since one of the terminal products of lipid peroxidation is MDA. The concentration of MDA was detected at 535 nm ( $\epsilon = 156 \text{ mM}^{-1} \text{ cm}^{-1}$ ) [67].

The content of protein carbonylation (PCC) was determined according to Buss et al. [68] and Alamdari et al. [69]. However, the procedure followed herein is modified since protein samples are first absorbed to an ELISA 96 well plate through overnight incubation at 4 °C and then react with 2,4-dinitrophenylhydrazine (DNPH). Quantification of PCC was based on a standard curve produced by measuring at 450 nm 5 µg bovine serum albumin (BSA) instead of 60 µg proposed by Buss et al. [68]. Forms of reduced and oxidized BSA were employed for the creation of a standard curve [69]). The PCC content was quantified according to the standard curve of BSA ( $y = 1.4033x + 0.002$ ),  $R^2 = 0.9916$ , bovine serum albumin concentrations used were 0–0.25 µM. The results were expressed as nmol carbonyl groups mg<sup>-1</sup> of protein.

The levels of ubiquitinated proteins and caspases conjugates in gills and liver of both fish species were quantified using well established methodology. Frozen tissues were immediately homogenized in 3 mL g<sup>-1</sup> of cold lysis buffer (20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM benzamidine, pH 7, 200 µM leupeptin, 10 µM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM dithiothreitol, 300 µM phenyl methylsulfonyl fluoride (PMSF), 50 µg mL<sup>-1</sup> pepstatin and 1% *v/v* Triton X-100), and extracted on ice for 30 min. Samples were centrifuged (10,000 × *g*, 10 min, 4 °C) and the supernatant was boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris-HCl, 13% *v/v* glycerol, 133 mM DTT, 10% *w/v* SDS, 0.2% *w/v* bromophenol blue). Protein concentration was determined by using the BioRad protein assay. Thereafter, samples were immersed in a nitrocellulose membrane (0.45 µm, Schleicher & Schuell, Stockbridge, GA, USA), set in a dot blot (BioRad, Hercules, CA, USA) vacuum apparatus. As antibodies were used a polyclonal anti-ubiquitin rabbit antibody (Cat. No. 3936, Cell Signaling, Beverly, MA, USA) and a monoclonal anti-cleaved caspase rabbit antibody (Cat. No. 8698 Cell Signaling, Beverly, MA, USA). Thereafter, nitrocellulose membranes were washed with TBST (3 × 5 min). Then, an 1 h incubation with a horseradish peroxidase linked secondary antibody (7074, 7076, Cell Signaling, Beverly, MA, USA) followed and membranes were washed with TBST (3 × 5 min). The dots were detected using enhanced chemiluminescence (Chemicon) on Fuji Medical X-ray film and quantified by densitometry scanning laser (GelPro Analyzer Software, GraphPad, San Diego, CA, USA).

The method modified by Dailianis et al. [70] was applied for the estimation of DNA damage in both fish tissues examined. After gill and liver cells were treated with collagenase, DNA lysis and electrophoresis under neutral conditions, and DNA staining with acridine orange [71], the presence of comets was examined and counted under fluorescent microscope (Olympus CKX41) following the criteria of Ritter and Knebel [72]. Detailed description of the procedure of DNA damage is referred by Dimitriadi et al. [52]. In brief, six slides per pool (zebrafish) and six slides per individual (perch) were measured, in order to represent technical replicates. Randomly selected 100 cells were scored from each slide (Tritek Cometscore™ 1.5, TriTek Corporation, Wilmington, DE, USA). Moreover, PS-MPs free cells were exposed to H<sub>2</sub>O<sub>2</sub> (1 µM) in order to verify the comet assay method electrophoresis conditions as well as the genotoxicity of H<sub>2</sub>O<sub>2</sub> (positive control) as previously published [73]. The results are expressed as % DNA in tail (percentage of DNA in comet

tail). % DNA in tail and Olive moment in positive control data ( $1 \mu\text{M H}_2\text{O}_2$ ) were  $28.3 \pm 5.2$  and  $40 \pm 6.3$ , respectively. The results are expressed as percentage of DNA in tail (% DNA in tail).

Autophagic and apoptotic indicators were quantified by SDS/PAGE immunoblot techniques. Frozen gill and liver tissue samples from control and PS-MPs treated individuals of both fish species were homogenized in  $3 \text{ mL g}^{-1}$  of cold lysis buffer (20 mM  $\beta$ -glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM benzamidine, pH 7, 200  $\mu\text{M}$  leupeptin, 10  $\mu\text{M}$  trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM dithiothreitol, 300  $\mu\text{M}$  phenyl methylsulfonyl fluoride (PMSF), 50  $\mu\text{g mL}^{-1}$  pepstatin, 1% *v/v* Triton X-100), and extracted on ice for 30 min. Samples were centrifuged ( $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ) and the supernatant was boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris-HCl, 13% *v/v* glycerol, 133 mM DTT, 10% *w/v* SDS, 0.2% *w/v* bromophenol blue). Protein concentration was determined by using the BioRad protein assay. Thereafter, equivalent amounts of proteins (50  $\mu\text{g}$ ) were separated on 10% and 0.275% or 15% and 0.33% (*w/v*) acrylamide and bisacrylamide slab gels respectively, followed by electrophoretic transfer onto nitrocellulose membranes (0.45  $\mu\text{m}$ , Schleicher & Schuell, Stockbridge, GA, USA). Nitrocellulose membranes were dyed with Ponceau staining for ensuring good quality results of protein transfer and loading and subsequently they were left overnight for incubation with the appropriate antibodies (Monoclonal rabbit anti-LC3B (3868, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-p62/SQSTM1 (5114, Cell Signaling, Beverly, MA, USA), anti-Bcl2 (7973, Abcam, Cambridge, UK) and anti-Bax (B-9) (2772, Cell Signaling, Beverly, MA, USA). Thereafter, nitrocellulose membranes were washed with TBST ( $3 \times 5$  min). Then, an 1 h incubation with a horseradish peroxidase linked secondary antibody (7074, 7076, Cell Signaling, Beverly, MA, USA) followed and membranes were washed with TBST ( $3 \times 5$  min). The blots were detected using enhanced chemiluminescence (Chemicon) on Fuji Medical X-ray film and quantified by densitometry scanning laser (GelPro Analyzer Software, GraphPad, San Diego, CA, USA).

## 2.10. Metabolomics

### 2.10.1. Sample Preparation

Polar metabolite extraction was performed by adding 5 mg of tissue to 150  $\mu\text{L}$  of an ice cold methanol:water (1:1) mixture. The tissue was then ground using a chilled mortar and pestle. The ground extract was transferred into an Eppendorf tube and subjected to ultrasonic treatment using a sonication rod for a total of 3 min, divided in six fractions of 30 s intervals separated by 2-min intervals, in ice water bath in order to avoid a significant rise of the temperature. Following sonication the extract was centrifuged at 5000 rpm for 10 min at room temperature and was then stored at  $-20^\circ\text{C}$  until analysis. Before analysis, each extract was allowed a brief thaw time and was then filtered through a 20  $\mu\text{m}$  syringe filter. L-Alanine-3,3,3- $\text{d}_3$  was added as an injection standard to each sample to a concentration of 10 ppm before analysis.

### 2.10.2. LC-MS/MS Analysis

Each sample was analysed on a Thermo Scientific™ TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer coupled to an Accela™ 1250 UHPLC pump and an Accela™ autosampler employing a Waters™ ACQUITY UPLC BEH Amide Column (1.7  $\mu\text{m}$ ,  $2.1 \times 150$  mm). The applied analysis method was based on previously developed methods [74,75]. Briefly, the flow rate was set to  $300 \mu\text{L min}^{-1}$ . Solvent A was 95:5% Acetonitrile:H<sub>2</sub>O, 10 mM CH<sub>3</sub>COONH<sub>4</sub> and solvent B was 30:70% Acetonitrile:H<sub>2</sub>O, 10 mM CH<sub>3</sub>COONH<sub>4</sub>. A gradient elution program was applied as follows: 100% A (hold for 4 min), then to 60:40% A:B (over 21 min), then to 15:85% A:B (over 4 min and then hold for 3 min) then to 100% A (hold for 15 min). The injection volume was 5  $\mu\text{L}$ .

Standards of analytical grade were purchased from Sigma-Aldrich, Alfa Aesar and Acros Organics were used for all compounds in order to verify transitions and conditions. Retention times were verified in the analytical run by injecting a global quality control stan-

dard that included all of the aforementioned compounds at a concentration of  $5 \mu\text{g mL}^{-1}$ . Blanks were injected after each sample in order to check for any carryover effects.

### 2.10.3. Data Analysis and Interpretation

Sample data was analysed using Thermo Scientific™ Qual Browser, Thermo Xcalibur version 3.063. Sample comparison was performed using response ratios of the analyte peak area to the area of the injection standard. Metabolites were correlated to metabolic pathways using publicly available databases Metaboanalyst 5.0, Small Molecules Pathway Database (SMTDB) and Human Metabolome Database (HMDB).

### 2.11. Statistical Analyses

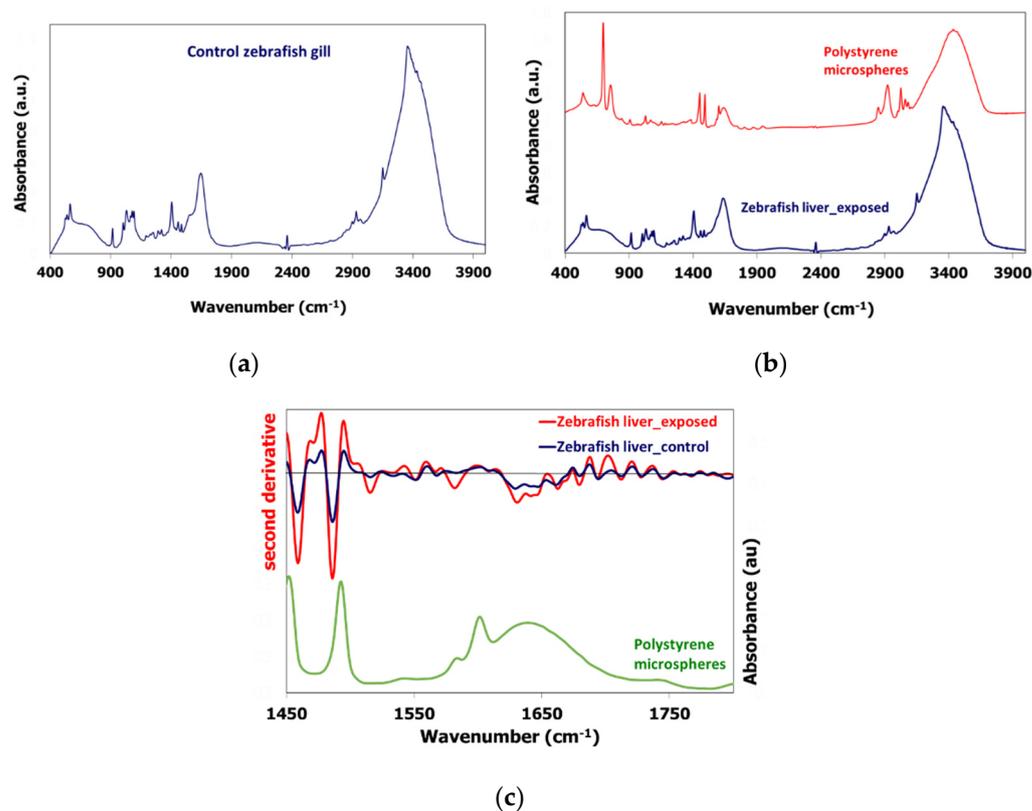
Molecular and biological analyses results were expressed as mean ( $\pm$  standard deviation, SD) of mean. The non-parametric Mann–Whitney U test ( $p < 0.05$ ) was used to assess significant differences ( $p < 0.05$ ) between control and treated specimens. Moreover, a two-level nested Anova model was applied to investigate the effect of species and tissue examined (each species, each tissue and species-tissue combination) on the parameters studied. Spearman's rank correlation analysis was also applied for extracting intercorrelations of the parameters measured in both tissues and fish species. The above analyses were performed using the SPSS software (ver. 27, Inc. Chicago, IL, USA).

## 3. Results

### 3.1. PS-MPs Characterization

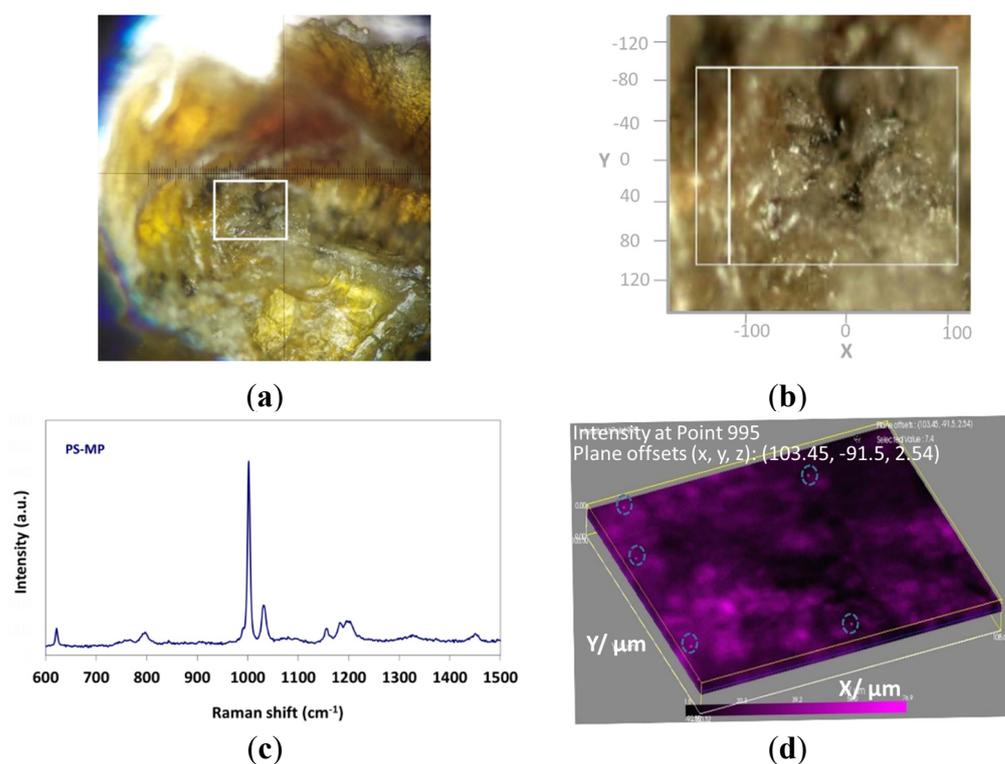
PS-MPs are spherical with average diameter size about  $8 \mu\text{m}$  and as was found in our previous work are completely amorphous [52]. During zebrafish feeding we are expecting these to be entered by food to their bodies and accumulated to several organs. This was evaluated by FTIR and micro-Raman spectroscopies. The control and exposed liver and gill samples from zebrafish and perch samples were characterized by FTIR spectroscopy. Both fish liver and gill samples exhibit similar control and exposed spectra (Figure 1, shown only spectra of zebrafish), depicting peaks corresponding to proteins; between  $900$  and  $1300 \text{ cm}^{-1}$  are phosphates mainly associated with RNA and DNA related nucleic acids, while in the  $1300$  and  $1800 \text{ cm}^{-1}$  region are protein (Amide I, II) bonds and in the  $2700$ – $3900$  wavenumbers are peaks related to N–H stretching vibration of proteins [76]. The control zebrafish gill FTIR spectrum is shown in Figure 1a, while the zebrafish liver exposed samples with the PS-MPs is shown in Figure 1b. Both spectra exhibit similar peaks, while the potential PS characteristic peaks are not evident at the exposed spectra.

The PS characteristic peaks are  $3025 \text{ cm}^{-1}$  for aromatic C–H stretching vibration, C–H stretching at  $2921 \text{ cm}^{-1}$ , three peaks at  $1600$ ,  $1492$  and  $1451 \text{ cm}^{-1}$  respectively indicates aromatic C–H bond stretching vibration and  $1260$ ,  $1017$ ,  $796$ ,  $749$  and  $695 \text{ cm}^{-1}$  corresponds to aromatic C–H deformation vibration. The PS-MPs characteristic peaks coincide with the control peaks, thus only high PS presence in the exposed samples would allow their spectra exhibition, as it has been reported in other FTIR studies of particles in biological media [77]. A second derivative analysis of the exposed (orange line) and control (blue line) zebrafish liver sample, which can determine minor changes in the spectra peaks, showed an increase of the peaks at  $1451$  and  $1492 \text{ cm}^{-1}$ , as shown in Figure 1c, which can be attributed to absorbance enhancement of the exposed sample due to PS presence. PS concentration in the liver and gill samples of both zebrafish and perch species is not high enough to be evident to the primary absorbance spectra, further spectrum analysis and comparison of the exposed and control sample though can allow determining a possible limited concentration of PS.



**Figure 1.** (a) FTIR spectrum of the control zebrafish gill, (b) FTIR spectrum of the exposed zebrafish liver sample and the PS microspheres, (c) Second derivative analysis of the exposed and control zebrafish liver samples and the absorbance spectrum of the PS microspheres.

Even though Raman spectroscopy has been used for the characterization of MPs in zebrafish organs [78], in the current study an advanced 3D mapping characterization was performed by Raman spectroscopy detecting the polystyrene (PS) microparticles directly in the zebrafish gill without further dissolution or destruction of the organ, providing information on location, concentration and size date of the measured microplastic by an ex situ technique with micrometer resolution. An area of  $255 \times 192 \times 10 \mu\text{m}$  of the zebrafish gill (Figure 2a) was selected for HR volume study by micro-Raman spectroscopy. The gill organic material (Figure 2b) exhibited no Raman intensity signal, while the regions where the PS-MPs were detected exhibited the characteristic PS Raman peak at  $995 \text{ cm}^{-1}$ , as shown in Figure 2c. A still image of the HR volume map exhibiting the x-y plane at the depth of  $2.54 \mu\text{m}$  is observed in Figure 2d; the distinct spherical pink dots are attributed to PS-MPs presence. The size of the PS-MPs was calculated from the sequential images of the z axis video movie, having a mean diameter of  $\sim 5 \mu\text{m}$ . On the current analyzed area of  $490,000 \mu\text{m}^3$ , 19 PS-MPs were detected.



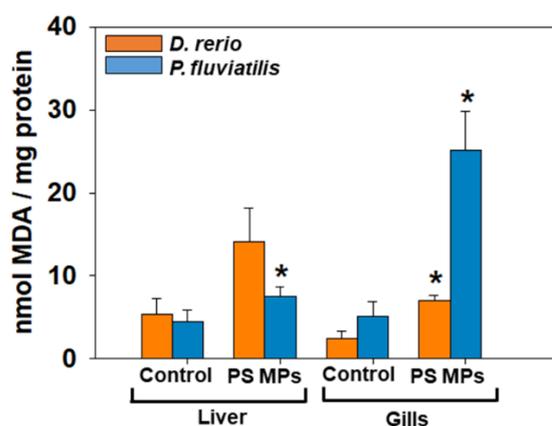
**Figure 2.** (a) Zebrafish gill as observed from the microscope lens, indicating the measured area with the white rectangle, (b) The measured area of Figure 2a in the x, y, z axes, (c) Raman spectrum of PS-MS, (d) Still image of the HR volume video map indicating the PS-MS in depth 2.54  $\mu\text{m}$  of the gill area (the HR volume video map can be found in the Supplementary Material).

### 3.2. Molecular and Biochemical Responses

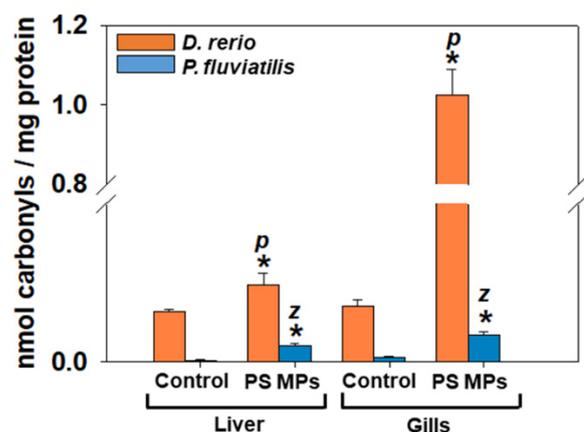
#### 3.2.1. Oxidative Stress Biomarkers

**Lipid peroxidation.** PS-MPs caused significant increase in lipid peroxidation (which is measured by MDA increase) only in the liver of *P. fluviatilis* ( $p = 0.014$ , Figure 3). However, the increase observed in the gills was significant for both species (zebrafish:  $p = 0.046$ ; perch:  $p = 0.004$ ). Comparing the two fish response, the highest sensitivity, considering the size of response was observed in gills of *P. fluviatilis* (5 times increase for perch compared to 2.8 times for zebrafish). When comparing the tissue response in each fish, gills are more susceptible to MPs than liver in both in *D. rerio* and *P. fluviatilis* (Figure 3).

**Protein oxidation and proteolysis (carbonyl groups and ubiquitin).** Our results showed that exposure to PS-MPs revealed a significant (liver, zebrafish:  $p = 0.032$ ; perch:  $p = 0.001$ ; gills, zebrafish:  $p = 0.004$ ; perch:  $p < 0.001$ ) increase in carbonyl groups in liver and gills of both fish in relation to the respective controls (Figure 4). The highest sensitivity against MPs concerning the size of response, between the two fish was observed in the liver of *P. fluviatilis* compared to *D. rerio* (10 times and 1.5 times increase respectively compared to control) and in gills of *D. rerio* in comparison to *P. fluviatilis* (14.3 times and 5.5 times increase respectively compared to control). When comparing the tissue response in each fish, gills are more susceptible to MPs than liver in *D. rerio* and liver more sensitive than gills in *P. fluviatilis* against PS-MPs. Tissue carbonyls responses seem to be opposite than those of MDA.



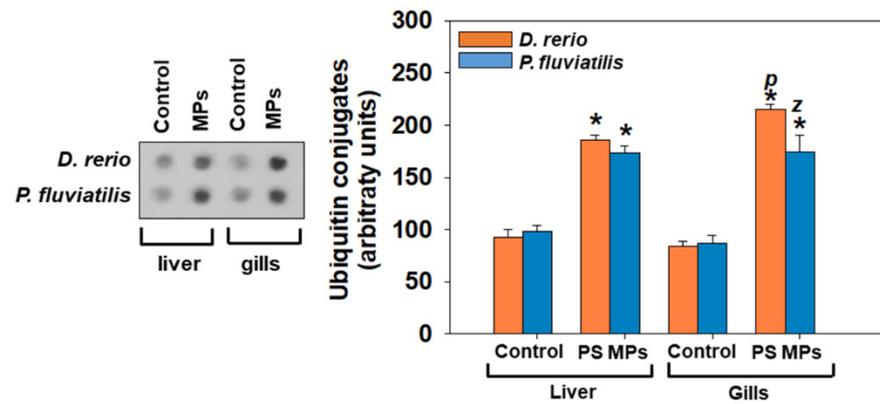
**Figure 3.** Lipid peroxidation was expressed as MDA concentrations (mean  $\pm$  SD nmol mg<sup>-1</sup> protein), in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals) and *Perca fluviatilis* ( $n = 6$ ). Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively).



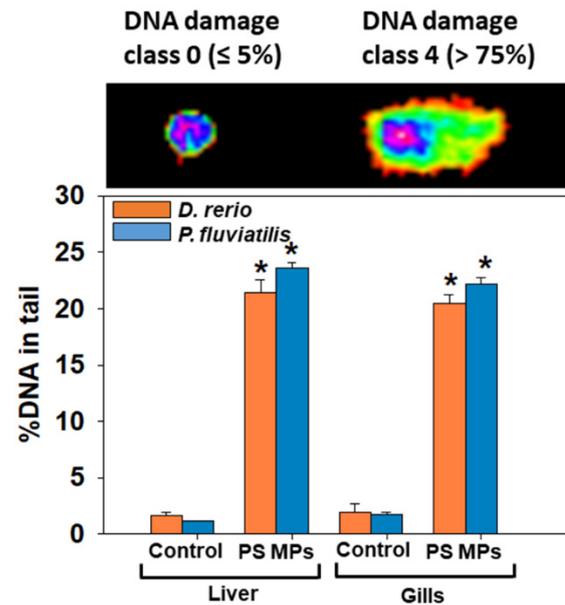
**Figure 4.** Protein carbonylation (mean  $\pm$  SD nmol carbonyls mg<sup>-1</sup> protein) in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals) and *Perca fluviatilis* ( $n = 6$ ). The results were expressed as nmol carbonyl groups mg<sup>-1</sup> of protein. Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively), while  $z$  and  $p$  denote significant differences ( $p < 0.05$ ) between *D. rerio* and *P. fluviatilis* respectively.

Ubiquitin conjugates in liver and gills of *D. rerio* and *P. fluviatilis* after their exposure to PS-MPs are depicted in Figure 5. In all cases ubiquitin levels were significantly ( $p < 0.05$ ) higher in exposed animals in relation to controls.

Genotoxic responses (DNA damage). Our results showed that DNA in tail (%) after the exposure to PS-MPs was evident in both tissues of animals studied, revealing a significant (for liver, zebrafish:  $p = 0.009$ ; perch:  $p = 0.004$ , for gills, zebrafish:  $p = 0.004$ ; perch:  $p = 0.004$ ) increase compared to controls (Figure 6). The response of liver and gills of both fish species regarding the increase in DNA in tail of the comets after MPs, was in the range 12 to 20 times higher in relation to their respective controls.



**Figure 5.** Ubiquitin conjugates (mean  $\pm$  SD) in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals) and *Perca fluviatilis* ( $n = 6$ ). Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively), while z and p denote significant differences ( $p < 0.05$ ) between *D. rerio* and *P. fluviatilis* respectively.

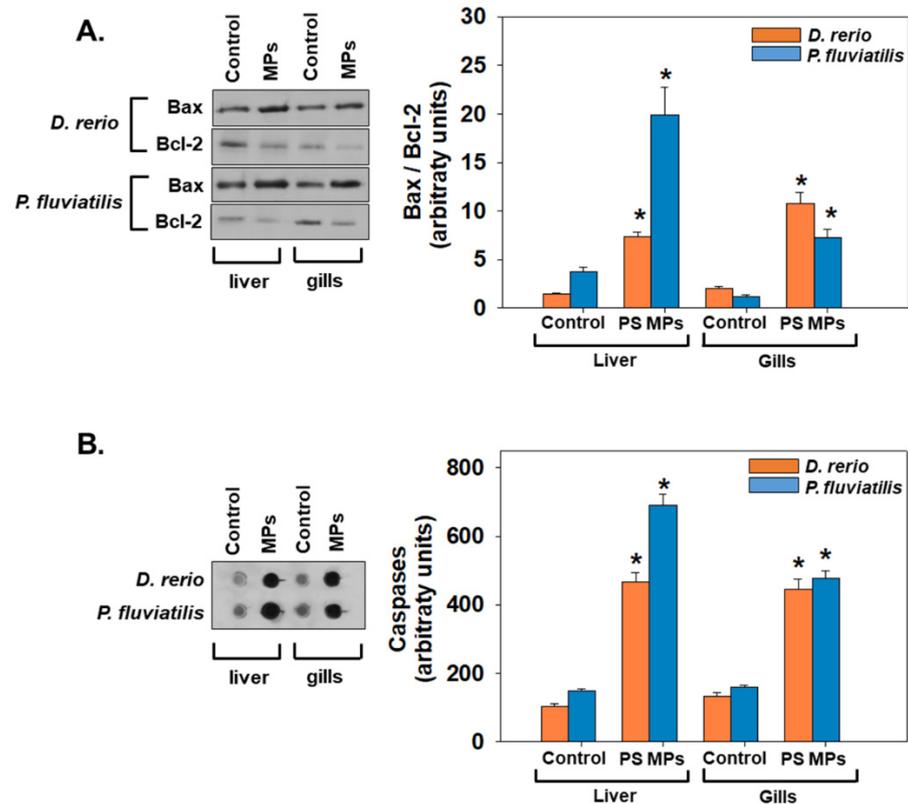


**Figure 6.** Percentage (%) of DNA damage (mean  $\pm$  SD) in tail in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals) and *Perca fluviatilis* ( $n = 6$ ). Six slides per pool (zebrafish) and six slides per individual (perch) were measured, in order to represent technical replicates. Randomly selected 100 cells were scored from each slide (TritekCometscoreTM 1.5, TriTek Corporation, Wilmington, DE, USA). Representative pictures of DNA damage are shown. Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively). % DNA in tail and Olive moment in positive control data ( $1 \mu\text{M H}_2\text{O}_2$ ) were  $28.3 \pm 5.2$  and  $40 \pm 6.3$ , respectively.

### 3.2.2. Apoptosis and Autophagy

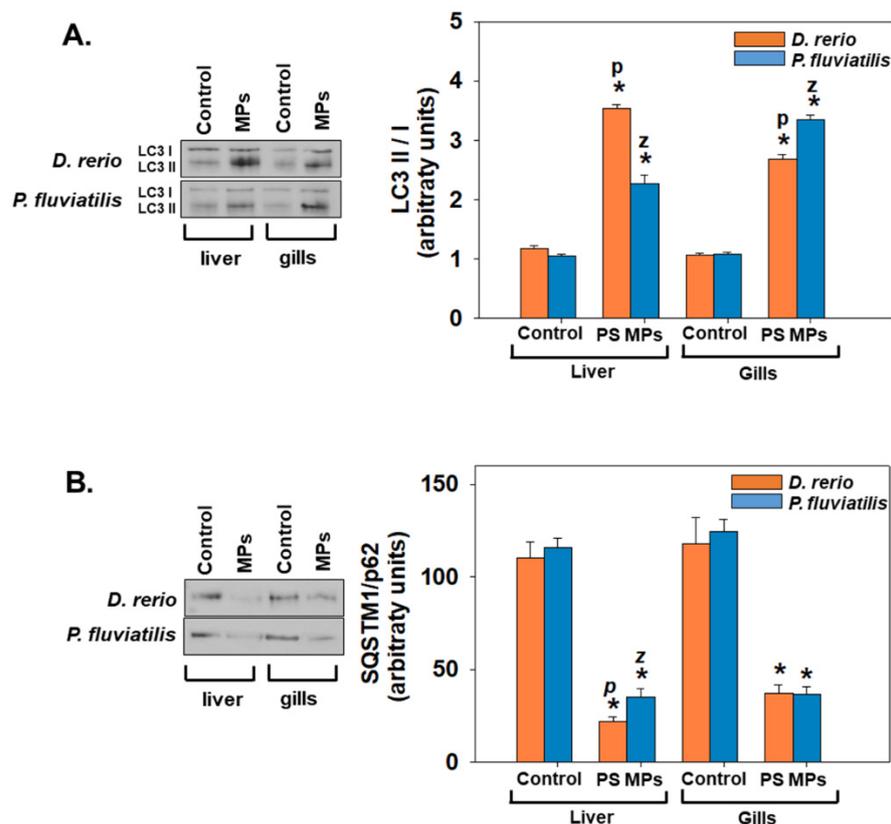
Exposure to PS-MPs triggers apoptosis in the liver and gills of both fish species is confirmed by the increased Bax/Bcl-2 ratio and caspases levels (Figure 7). Our results showed that the ratio Bax/Bcl-2 in both tissues on both fish was increased 5 to 6 times, in comparison to the respective controls (for all cases  $p < 0.05$ ) (Figure 7A). When comparing the two fish, exposure to PS-MPs resulted to a similar increase (5.2 times in the liver of both *D. rerio* and *P. fluviatilis*). The susceptibility of both fish against PS-MPs seems to be the

same. Tissues' responses also seem to follow the same profile against PS-MPs exposure for both fish. Caspases levels were also significantly increased in both tissues of both species compared to the control group ( $p < 0.05$ ) (Figure 7B). Regarding tissue responses, liver seems to be more susceptible than gills in both fish studied (4.49 and 4.63 times increase in liver vs. 3.36 and 3.02 times increase in gills in zebrafish and perch, respectively).



**Figure 7.** Bax/Bcl-2 ratio (A) and caspases levels (B) (mean  $\pm$  SD) in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals each pool) and *Perca fluviatilis* ( $n = 6$ ). Tissue extracts from all groups were immunoblotted for Bax, Bcl-2 and caspases. Blots and dots were quantified using scanning densitometry. Representative blots and dots are shown. Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively).

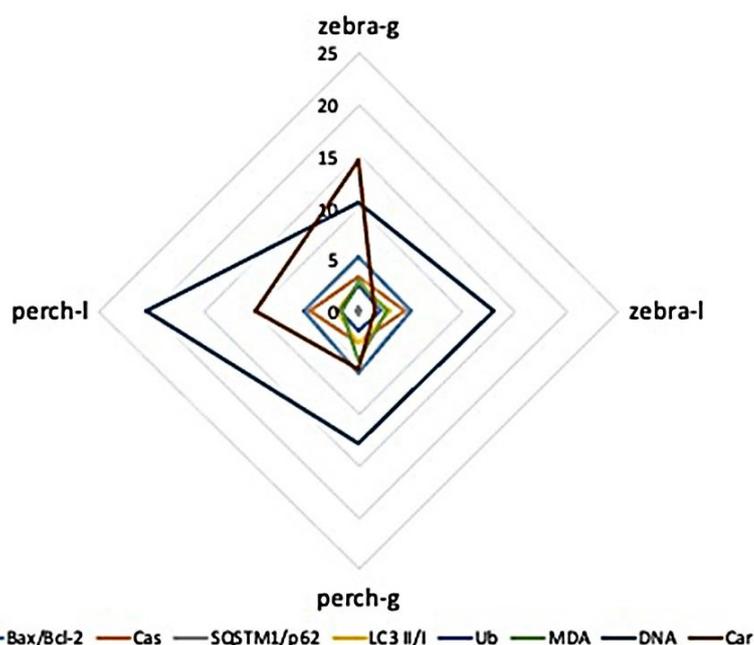
Concerning, our autophagy results, exposure to PS-MPs resulted in significant alterations (Mann–Whitney U test,  $p < 0.05$ ) of the autophagic indicators investigated herein, confirming PS-MPs' provoked initiation of autophagy (Figure 8). Concerning LC3 II/I ratio, PS-MPs exposure resulted in the range of 2 to 3 times increase in liver and gills of both examined fish species (for all cases  $p < 0.05$ ) (Figure 8A). The comparison of the responses of two fish revealed similar feedback to PS-MPs. Concerning the tissue responses, the two fish responded differently, with zebrafish liver and perch gills to be more susceptible to PS-MPs than their respective controls (Figure 8A). Regarding SQSTM1/p62 levels, exposure to PS-MPs resulted to a significant ( $p < 0.05$ ) decrease in comparison to control in both fish species, indicating the same profile of both fish against PS-MPs exposure (Figure 8B). Tissue responses also of both fish were similar (Figure 8B).



**Figure 8.** LC3II/I ratio (A) and SQSTM1/p62 levels (B) (mean  $\pm$  SD) in liver and gills of *Danio rerio* ( $n = 3$  pools of 10 individuals) and *Perca fluviatilis* ( $n = 6$ ). Tissue extracts from all groups were immunoblotted for LC3II/I and SQSTM1/p62. Blots and dots were quantified using scanning densitometry. Representative blots and dots are shown. Mann–Whitney U test was employed to test for significance at  $p < 0.05$  between all experimental groups. \* denotes significant differences ( $p < 0.05$ ) compared to the control group ( $n = 3$  pools of 10 individuals and  $n = 6$  for *D. rerio* and *P. fluviatilis* respectively), while  $z$  and  $p$  denote significant differences ( $p < 0.05$ ) between *D. rerio* and *P. fluviatilis* respectively.

### 3.3. Inter-Species and Inter-Tissue Comparisons of Molecular and Biochemical Parameters

Regarding the sensitivity of gills in comparison to liver of each fish against PS-MPs, oxidative stress biomarkers responses, do not seem to follow similar profiles. When all molecular and biochemical parameters are examined together for assessing the response among the fish species and tissues examined, gills of both species have the highest response against 5–12  $\mu\text{m}$  of PS-MPs for the majority of the parameters studied (Figure 9). Additionally, perch liver was the most liable tissue to respond to DNA damage, and zebrafish gills the most responsive to carbonyl groups (Figure 9). The results of nested Anova are presented in Table 1.



**Figure 9.** Graphical representation of molecular and biochemical biomarkers measured in the gills (g) and liver (l) of *Danio rerio* (zebrafish) and *Perca fluviatilis* (perch) (for graphical purposes caspases and ubiquitin values were divided by 10).

**Table 1.** Nested Anova of the effects of species, tissue and species-tissue combination on the molecular and biochemical indicators studied.

Parameter	Species	Tissue	Species -Tissue
MDA	ns	ns	F(1,24) = 7.14, $p = 0.013$
Car	ns	ns	F(1,24) = 36.8, $p = 0.001$
Ub	ns	ns	F(1,24) = 7.74, $p = 0.010$
DNA	ns	ns	F(1,24) = 7.67, $p = 0.011$
Bax/Bcl-2	ns	ns	ns
Cas	ns	ns	F(1,24) = 57.86, $p = 0.000$
LC3 II/I	ns	ns	F(1,24) = 144.50, $p = 0.000$

ns =  $p > 0.05$ .

### 3.4. Correlation between Biochemical Indicators

Table 2 illustrates correlation analyses between the parameters studied in the liver and gills of *D. rerio* and *P. fluviatilis* after exposure to PS-MPs. Higher number of significant ( $p < 0.05$ ) intercorrelations among the parameters studied were extracted in liver samples of perch and gill samples of zebrafish. It is also evident that parameter intercorrelation is independent of tissue and species, since no pattern is obviously followed (Table 2).



### 3.5. Metabolomics

In total the levels of 33 small polar metabolites were estimated which participate in pathways related mainly to amino acids, nitrogen and energy metabolism. The levels of all measured metabolites in zebrafish gills were decreased in comparison to the control, which is probably associated with reduced metabolic rate in gills as shown in Table 3. The levels of L-phenylalanine, L-carnitine and L-proline exhibited greatest decrease by 93, 91 and 91%, respectively, whereas salicylic acid, L-lactic acid and choline were least affected exhibiting a decrease of 30, 44 and 49%, respectively. Similarly, in exposed perch gills the levels of all metabolites decreased except salicylic acid and L-phenylalanine that were increased by 210 and 64%, respectively whereas the metabolites whose levels exhibited the greatest decrease were acetyl-L-carnitine (ALCAR), L-alanine, L-glutamic and pyruvic acid, 96, 86, 86 and 75%, respectively.

**Table 3.** Alterations of metabolites levels in gills and liver of exposed fish in comparison to the control, expressed as %.

	Gills		Liver	
	Zebrafish	Perch	Zebrafish	Perch
L-Asparagine	−78		99	61
L-Glutamine			−36	−15
L-Glutamic acid	−83	−86	−8	−21
L-Valine	−79	−63	194	21
L-Lysine	−72		−23	−14
L-Alanine	−83	−86	55	−30
L-Proline	−91	−63	96	8
L-Tyrosine	−84	3	67	45
L-Phenylalanine	−93	64	113	67
L-Arginine	−51		135	221
Ornithine			−15	−5
Citrulline	−67		88	−58
Creatine			59	−34
Creatinine			62	−57
Pyruvic acid		−75		
L-Lactic acid	−44	−66		
Succinic acid	−56	−54	20	120
2-Oxoglutaric acid			−31	−61
L-Carnitine	−91	−61	96	50
ALCAR	−80	−96	−55	−56
Butyric acid			28	28
Hypoxanthine			223	−59
Adenine			−56	−54
Adenosine			−65	120
Deoxyadenosine			124	−44
Uridine			155	−45
Salicylic acid	−30	210	−51	7
Betaine	−70	−45	−11	75
Choline	−49	−33	94	8
Putrescine		−43		
Niacinamide	−71	−59	7	17
Riboflavin				1
Trehalose			−74	−81

In zebrafish livers the greatest perturbations were observed among nucleic acids metabolites; adenine and adenosine exhibited significant increases, 185 and 127% respectively, while the greatest decreases were exhibited in hypoxanthine (69%), uridine (61%) and deoxyadenosine levels (55%). Hypoxanthine is also a known marker of exercise exhaustion [79]. Among amino acids L-valine, L-arginine, L-phenylalanine, L-asparagine and

L-proline exhibited a decrease of almost 50% or greater while L-glutamine is the only amino acid that exhibited increase greater than 50%. On the other hand L-arginine, succinic acid and adenosine levels in perch liver were increased by 221, 120 and 120%, respectively, while 2-oxoglutaric acid, hypoxanthine, citrulline, L-creatinine, ALCAR and adenine decreased to levels less than half compared to the control.

The results of nested analysis applied to nine metabolites measured in gills and liver tissue of both species are presented in Table 4. Only L-glutamic acid exhibited significant differences when species or tissue factor are considered while no significant difference was observed when species-tissue combination is examined. However, the effect of species-tissue was significant to all other metabolites examined (Table 4).

**Table 4.** Nested Anova of the effects of species, tissue and species-tissue combination on the metabolites studied.

Metabolites	Species	Tissue	Species -Tissue
Betaine	ns	ns	F(1,8) = 124.09, $p = 0.000$
Choline	ns	ns	F(1,8) = 270.74, $p = 0.000$
L-alanine	ns	ns	F(1,8) = 23.83, $p = 0.001$
L-carnitine	ns	ns	F(1,8) = 154.40, $p = 0.000$
L-glutamic acid	F(1,1) = 6384.34, $p = 0.008$	F(1,1) = 531,344, $p = 0.001$	ns
L-phenylalanine	ns	ns	F(1,8) = 139.07, $p = 0.000$
L-proline	ns	ns	F(1,8) = 14.61, $p = 0.005$
Salicylic acid	ns	ns	F(1,8) = 380.88, $p = 0.000$
Succinic acid	ns	ns	F(1,8) = 4.75, $p = 0.061$

ns =  $p > 0.05$ .

#### 4. Discussion

In the present study, PS-MPs' effects were studied by measuring and comparing the responses of biochemical and molecular parameters as well as metabolite levels in the gills and liver of two freshwater fish species, zebrafish, *D. rerio* and perch, *P. fluviatilis*. Fish were fed with food supplemented with PS-MPs with particle size 5–12  $\mu\text{m}$ , at concentrations close to those estimated for each species by the  $\text{EC}_{50}$  value by in vivo experiments. The concentrations of PS-MPs that each fish was treated were close to the physiological tolerance of each fish to PS-MPs effect. Our outcomes revealed that exposure of both fish species to PS-MPs at sublethal concentrations caused toxic effects on fish tissues after 21 days of exposure. Accordingly, the metabolic pathway analysis revealed that PS-MPs concentrations caused a significant effect to the metabolism of glycerolipid, the unsaturated fatty acids biosynthesis as well as the gluconeogenesis ability of studied fishes. The difference in the response against PS-MPs of the two species could be attributed to the difference in weight (100 times greater), length (four times greater) and lifespan (four times greater) of the examined fish.

##### 4.1. Oxidative Stress

The exposure of fish to a number of pollutants including nanoparticles, biomaterials as well as MPs causes oxidative stress, due to ROS overproduction [44,80]. Subsequently ROS promote protein and lipid peroxidation as well as genotoxic damages [46,47,81,82]. MDA is the principal biomarker of lipid peroxidation that is significantly increased in both fish tissues; MDA has already been proved to be a reliable biomarker for aquatic animals and terrestrial snails [46,47,52,82].

Another consequence due to MPs fish exposure is the alteration of cellular proteins [83,84]. Proteins that are carbonylated at a high degree are considered to be dysfunctional, are gathered as a mass of proteins, linked with a covalent bond and cannot be proteolyzed, causing several detrimental effects on the cell functions [85]. Protein carbonylation has been proposed as a sensitive indicator of protein oxidation in zebrafish, Prussian carp and

terrestrial snail [46,47,86]. Wen et al. [87] report significantly increased carbonyl levels in liver of the fish *Symphysodon aequifasciatus* after exposure to three concentrations of MPs (0, 50 and 500  $\mu\text{g L}^{-1}$ ) together with two levels of Cd (0 and 50  $\mu\text{g L}^{-1}$ ) for 30 days. Thus, it may be assumed that these diffusible products of molecular peroxidation originate from the reaction catalyzed by myeloperoxidase that produces potent oxidants, causing cell oxidative injury which successively, may produce deleterious effects on fish organism.

Oxidative stress apart from causing peroxidation of lipids and protein oxidation also produces DNA damage in fish tissues [46]. In particular, ROS can lead to several DNA modifications. These include: bases degradation, breaks of single- or double-stranded DNA, sugar-bound, purine or pyrimidine modifications, mutations which can include translocations or deletions, and finally cross-linking with proteins [88]. Our results exhibited a significant increase in DNA damage in both fish tissues, with perch liver exhibiting the highest response. Similar to our results, Pannetier et al. [89] reported oxidative DNA damage to a liver cell line of *Oryzias latipes* larvae fed for 30 days with three doses of MPs (0.01, 0.1 and 1% *w/w* in fish food). Moreover, bivalves which were exposed to polyethylene (PE) and polystyrene (PS) microplastics, sized 1000–100  $\mu\text{m}$ , at concentrations of 0.5, 5 and 50  $\mu\text{g L}^{-1}$  [90] or to PS-MPs at 1  $\text{mg L}^{-1}$  (20  $\mu\text{m}$ ) concentration for 14 days, [90,91] showed irreversible loss of DNA integrity.

Regarding the sensitivity of gills in comparison to liver of each fish against PS-MPs, MDA, protein carbonyls and DNA damage responses, do not seem to follow similar profiles. Wang et al. [38] reported higher MDA levels in the liver rather than in the gills of the marine medaka (*Oryzias melastigma*) after exposure to 2, 20 and 200  $\mu\text{g L}^{-1}$  concentrations of 10  $\mu\text{m}$  PS-MPs for 60 days. Furthermore, our results showed that tissue carbonyls responses seem to be opposite than those of MDA. Yang et al. [92] revealed lipid peroxidation and oxidative stress in zebrafish larvae when exposed to either 6:2 chlorinated polyfluorinated ether sulfonate (F-53B), 50  $\text{ng mL}^{-1}$  polystyrene microplastics (PS-MPs) or their combination for 7 days. Similar inflammatory responses were reported after long term (more than 90 days) treatment with diet enriched with 10% PS-MPs in the intestine of *Sparus aurata* [38,65,88]. In addition, regarding MPs effect on *Sparus aurata* liver, although no change in MDA levels was observed, increased protein damage due to low-density polyethylene MPs (size between 100 and 500  $\mu\text{m}$ ) exposure for 90 days has been reported, that was attributed to an increase in myeloperoxidase (MPO) activity, indicating an inflammatory response [93]. These results as well as other studies [39,94,95], suggest that MPs ingestion provoke the antioxidant defense; however, this was not enough for the prevention of oxidative damage.

Protein degradation, which can be a consequence of ribosomal dysfunction and/or disrupted structure [96], is the vital intracellular task that accounts both for housekeeping as well as for the management of various functions of the cell, including that of dealing with different types of stress [97]. Proteasomes and lysosomes constitute the most important proteolytic systems. Any change in these proteolytic systems affects many metabolic pathways of the cell. Proteolysis of different proteins of the cell is achieved by the ubiquitin–proteasome system (UPS) [98]. Furthermore, the ubiquitin–proteasome pathway (UPP), which is also activated by oxidative stress [99], holds a major part in multitude functions of the cell as DNA repair, signal transduction, as well as dealing with different types of stress, e.g., oxidation, exposure to heavy metals [97]. Our results showed significantly higher ubiquitination in the liver and the gills of both fish examined, with differences in the size of response of each tissue as well as between the two fish species. In precise, gills had a higher response compared to liver tissue, while when the two fish species compared, zebrafish seems to respond with higher sensitivity than *P. fluviatilis*. Thus, our results encourage the suggestion of ubiquitin as a biomarker against PS-MPs, as has already been proposed for other pollutants in fish [46,47].

#### 4.2. Molecular Events, Apoptosis and Autophagy

Various stimuli, including ROS generation and the subsequent oxidative stress [100,101], can trigger multiple signaling pathways, which are responsible for a cell's fate. Apoptosis,

which is triggered either by inside boosts, or by extracellular impulses, is probably the most prominent cell death mechanism. Caspase 3 is involved in the innate immune system and apoptosis, in order to protect the fish when it is under stress-induced toxicity [102]. Caspase activity has been found to be a valuable biomarker for the detection of stress-induced apoptosis in fish [46,47,52,103]. In addition, the ratio of Bax/Bcl2 reflects the activation of pro-caspase and occurrence of apoptosis [104].

Under certain circumstances, while autophagy presents a different mechanism, it can also lead to cell death. Autophagy's key role and function is the survival of the cell. Autophagy also is an adaptive response under stressful conditions [105]. Given that the life of a cell is at stake, there is molecular crosstalk between apoptosis and autophagy pathways. The nature of these interconnections is diverse and ranges from protein–protein interactions and post-translational modifications via the deterioration of molecular components by distinct proteins and organelles [106]. Excess cellular levels of ROS which lead to damage to proteins, nucleic acids, lipids, membranes and organelles, trigger cell death processes such as apoptosis [100]. Moreover, in recent years, a growing amount of evidence argues for ROS being among the main intracellular signal transducers sustaining autophagy [101]. Thus, ROS participates in the interplay between autophagy and apoptosis by its ability to mediate the redox signaling pathways. However, the molecular machinery linking autophagy to apoptosis is still being elucidated.

Microtubule-associated proteins light chain 3 (LC3) are autophagy pathway's principal proteins where they serve in selecting the autophagy substrate and autophagosome biogenesis. LC3 is the most extensively used indicator of autophagosomes [107]. In addition, Sequestosome-1 (SQSTM1), which is the ubiquitin-binding protein p62, is a protein of the autophagosome cargo which marks other proteins for discriminatory autophagy. In the process of autophagy SQSTM1 is degraded. Both the ratio LC3II/I, as well as SQSTM1 are widely used as indicators of autophagy [52,108–112]. The significant elevation of apoptosis and autophagy markers recorded in our study denotes a parallel increase of apoptosis together with autophagy generally in the tissues of both fish studied; however, no clear pattern is evident for the intertissue and interspecies differences. Accordingly, when several marine organisms are exposed to MPs, increased levels of apoptosis are observed. In specific, key genes' related to Casp3 and Tp53 transcriptional changes were increased after exposure of sheepshead minnow to polyethylene MPs microspheres with diameters 150–180  $\mu\text{m}$  [113]. Moreover, exposure of adult zebrafish to two concentrations of high-density (100 and 1000  $\mu\text{g L}^{-1}$ ) polyethylene and polystyrene microplastics for twenty days [114] and *Mytilus edulis* exposure to high density polyethylene nonuniformly shaped grains ranging >0–80  $\mu\text{m}$  in size [115] has resulted in apoptosis activation in their tissues. Similarly, concerning autophagy, a significant presence of autophagy vacuoles was observed in the enterocytes of planarians *Dugesia japonica* fed with polyethylene microsphere mixtures with a diameter ranging from 1 to 10  $\mu\text{m}$  or 10–27  $\mu\text{m}$  sized plastic particles [116]. Except for marine organisms and mammals, exposure to microparticles/plastics and specifically PS-MPs has led to apoptotic or autophagic cell death in human cell lines such as gastric cancer cells (AGS) after exposure to 500 nm and 60 nm polystyrene nanoplastics at concentrations 1, 5, 10, 50, and 100  $\text{mg L}^{-1}$  [117] and macrophages [118].

#### 4.3. Metabolomics

To our knowledge this is the first study on MPs' effects of on the metabolome of gills in fish and only the second on fish liver. In accordance with all previous studies MPs affected cellular function and metabolism in all tissues tested. Lu et al. [119] using NMR in order to study the effects of PS-MPs on the metabolome of zebrafish liver, reported that the metabolic profile was altered significantly predominantly disturbing the lipid and energy metabolism. They also showed that MPs size and concentration may be correlated with different alterations in the metabolome. Recently, Dimitriadi et al. [52] studied the effects of PS-MPs on metabolites of the heart tissue of zebrafish demonstrating similarly that metabolites related to amino acid and energy metabolism exhibited significant de-

crease. Teng et al. [120] reported metabolic alterations and inflammatory responses in the whole oyster *Crassostrea gigas* after exposure to irregular MPs composed of polyethylene and polyethylene terephthalate at concentrations of 10 and 1000  $\mu\text{g L}^{-1}$  for 21 days. Qiao et al. [121] demonstrated that when zebrafish were exposed to PS-MPs (5- $\mu\text{m}$  beads; 50  $\mu\text{g L}^{-1}$  and 500  $\mu\text{g L}^{-1}$ ) for 21 days, their gut exhibited metabolome and microbiome responses, oxidative stress and inflammation. Other studies that examined the effects of polyethylene MPs on larval zebrafish after exposure to 1 to 4  $\mu\text{m}$  at concentrations of 0, 10, 100, and 1000  $\mu\text{g L}^{-1}$  for 7 days [122,123] and in developing zebrafish when exposed to 0.02 to 200  $\text{mg L}^{-1}$  concentrations of MPs, sized 65 nm and 20  $\mu\text{m}$  for 7 days [124], also demonstrated alterations in energy, glycolipid and lipid metabolism as well as in the microbiome of the fish.

#### 4.4. Interspecies and Intertissue Comparisons

Based on the intercorrelation results of the molecular and biochemical parameters studied in both fish tissues it may be deduced that MPs toxicity mechanism is species and tissue specific. Moreover, the results of nested Anova indicated significant relations of almost all (with the exception of Bax/Bcl-2 ratio) molecular and biochemical parameters studied as well as of the metabolites (with the exception of L-glutamic acid) when species-tissue is considered as the model design. However, since the two fish species differ in size, the ability of the MPs to be translocated may be different, so thus differences in the fish responses are expected. According to our results, the tissues response between the two different organisms did not follow a similar profile as also shown for several fish species [125,126]. This could be indicative of relatively closely related toxicity mechanisms in the livers despite the differences in life span and size and the fact that toxicity was earlier shown to be species and tissue specific [46,47]. Interestingly liver is known as the major detoxifying organ in all organisms and in the present study it suffered greater DNA damage in both species than gills.

Our group has recently demonstrated PS-MPs effects also on the heart tissue and the whole fish, in specific showing frequency reduction of ventricular heart contraction, decrease of swimming velocity and internalization of the MPs in the heart of *D. rerio* [52]. Thus, it is becoming evident that PS-MPs pollution at sublethal concentrations impacts most essential organs in a mechanism that involves oxidative stress, inflammation and metabolic alterations.

#### 4.5. Internalization-Toxicity Induction by PS-MPs

The literature concerning absorption mechanism and MPs accumulation in marine and freshwater fish is limited. It has been reported for nanoparticles that several significant parameters define their absorption rates: size, aggregation, distribution, and cell sedimentation. Endocytosis, phagocytosis or pinocytosis can facilitate absorption [55,127]. In specific it has been reported that PS and polycarbonate nanoplastic particles are internalized through phagocytosis by neutrophils in the kidneys of the fathead minnow (*Pimephales promelas*) [128,129]. Kashiwada [130] detected 39 nm PS particles in liver, intestine and gonads of the medaka *Oryzias latipes*, which most possibly entered in gills and/or gut epithelium and were transported through the bloodstream. In particular for zebrafish, detection of 5- $\mu\text{m}$  and 20- $\mu\text{m}$  sized MPs was observed in the liver, gut and gills. While in some cases, MPs/NPs were accumulated in the gut of larvae or adult individuals, in other cases they are found in gill and liver [131].

In the present study, the exposed groups of treated with PS-MPs fish showed statistically significant variations from the control group, in all the examined parameters. Therefore, in line with the responses of the parameters measured, our results indicate a toxic impact PS-MPs exert on the liver and gill cells of both fish, with specific biomarkers responded greater either in gills or in liver, while DNA damage was experienced greater in liver tissue of both species than gills. Towards in understanding our findings, in relation to the increase in all the oxidative stress biomarkers, the change in apoptotic and autophagic

markers, as well as the change in metabolite profile, we rely on the fact that in general the animal exposure to certain exogenous effectors, as MPs, provoke ROS production, inflammation and immune system changes [44,80,111].

In fact, according to the latter data, MPs especially of 5–12  $\mu\text{m}$  sized, as those used in the present study, may be internalized from the food to the gut and then transferred via blood to gills and liver of fish. Uptake of MPs in liver and gills has been shown by the present study's results. MPs' high surface area could cause ROS production in the tissues leading to oxidative stress [113]. This increased oxidative stress provokes the increase of ROS production that subsequently leads to peroxidation of lipids and protein carbonyls together with increase in DNA damage [132]. Moreover, cellular components and MPs interaction can influence cell signaling, thus causing activation of proteolysis, apoptosis and autophagy processes. In parallel to the activation of all the latter events in fish tissues metabolic alterations as a result of oxidative stress also occurs. These results indicate that MPs' accumulation and distribution in both fish gills and liver dramatically influence tissues toxicity. Thus, our results reveal that PS-MPs by generating oxidative stress, alter the functionality and metabolism of liver and gills of freshwater fish, and finally affecting the fish fitness for survival.

## 5. Conclusions

The outcomes of the present study indicate that cellular components and PS-MPs interaction produce a toxic impact by generating oxidative stress on the liver and gills of both fish species studied, as shown by lipid peroxidation, protein oxidation and DNA damage measurements. In parallel, cell signaling is influenced, thus provoking molecular inductions as apoptosis, ubiquitylation, autophagy and metabolic alterations affecting mainly amino acids, nitrogen and energy metabolism. The levels of most of the metabolites in both fish tissues were reduced in comparison to the control, which is probably associated with reduced metabolic rate after PS-MPs treatment.

In general, toxicity response was species and tissue specific with each biomarker showing different responses in gills and liver. Among biochemical indices DNA damage exhibited greater response in the liver of both species compared to gills. The alterations of metabolites in gills were more profound to those observed in liver. MDA, protein carbonylation, DNA damage, ubiquitin levels, caspases, Bax/Bcl-2 ratio, LC3 II/I and SQSTM1, as well as metabolites profile continue to provide essential information on cellular functionality in biomonitoring studies against PS-MPs in freshwater fish.

In addition, our results showed that *P. fluviatilis* seems to be more liable to respond against PS-MPs compared to *D. rerio*, at the experiment's conditions.

MPs constitute an increasing environmental hazard and have been shown to affect most organs in aquatic organisms at the cellular, metabolic and functional level. The current findings provide data that promote our understanding of the interplay of the effects between tissues in fish species that may eventually lead to the selection of appropriate biomarkers for MPs pollution, food safety and fishing stocks sustainability.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/toxics9110289/s1>.

**Author Contributions:** Investigation, supervision, writing, review and editing, M.K.; Conceptualization, supervision, writing review and editing, D.C.B.; Investigation, formal analysis, D.X.; Investigation, formal analysis, G.M.; Investigation, original draft preparation I.S.; Conceptualization, validation, writing, review and editing, S.K.; Investigation, writing, K.F.; Investigation, validation, writing, G.K. (Georgia Kastirinaki); Investigation, writing—review and editing, visualization, A.D.; Investigation, validation, writing, G.K. (George Koumoundouros); Resources, D.A.L.; Validation, original draft preparation, G.Z.K.; Supervision, writing review and editing, D.N.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** We acknowledge that (a) part of this work was supported by the project “FoodOmicsGR Comprehensive Characterization of Foods” (MIS 502907) which is implemented under the Action

“Reinforcement of the Research and Innovation Infrastructure”, funded by the Operational Programme Competitiveness, Entrepreneurship and Innovation (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development Fund); (b) Part of this research was co-financed by Greece and the European Union (European Social Fund—ESF) through the Operational Programme “Human Resources Development, Education and Lifelong Learning” in the context of the project “Reinforcement of Postdoctoral Researchers—2nd Cycle” (MIS-5033021), implemented by the State Scholarships Foundation (IKY); (c) Part of this work has also been done after financial support received from the Greek Ministry of Development and Investments (General Secretariat for Research and Technology) through the research project “Intergovernmental International Scientific and Technological Innovation-Cooperation. Joint declaration of Science and Technology Cooperation between China and Greece” with the topic “Development of monitoring and removal strategies of emerging micro-pollutants in wastewaters” (Grant no: T7ΔKI-00220) and it is gratefully acknowledged.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Hellenic Republic, Region of Crete, General Directorate of Regional Agricultural Economy & Veterinary Services, Directory of Veterinary Services (protocol code 285586, 7 December 2020).

**Informed Consent Statement:** All the experimental procedures involving handling and exposure of fish were performed in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation for animal experimentation and well-fare. All protocols were approved by the Animal Care Committee of the Biology Department of the University of Crete (Permit number: 285586, 7 December 2020).

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Geyer, R.; Jambeck, J.R.; Law, K.L. Production, use, and fate of all plastics ever made. *Sci. Adv.* **2017**, *3*, e1700782. [[CrossRef](#)] [[PubMed](#)]
2. Setälä, O.; Fleming-Lehtinen, V.; Lehtiniemi, M. Ingestion and transfer of microplastics in the planktonic food web. *Environ. Pollut.* **2014**, *185*, 77–83. [[CrossRef](#)]
3. Cole, M.; Lindeque, P.; Fileman, E.; Halsband, C.; Goodhead, R.; Moger, J.; Galloway, T.S. Microplastic Ingestion by Zooplankton. *Environ. Sci. Technol.* **2013**, *47*, 6646–6655. [[CrossRef](#)]
4. Mattsson, K.; Ekvall, M.T.; Hansson, L.-A.; Linse, S.; Malmendal, A.; Cedervall, T. Altered Behavior, Physiology, and Metabolism in Fish Exposed to Polystyrene Nanoparticles. *Environ. Sci. Technol.* **2015**, *49*, 553–561. [[CrossRef](#)]
5. Della Torre, C.; Bergami, E.; Salvati, A.; Faleri, C.; Cirino, P.; Dawson, K.A.; Corsi, I. Accumulation and Embryotoxicity of Polystyrene Nanoparticles at Early Stage of Development of Sea Urchin Embryos *Paracentrotus lividus*. *Environ. Sci. Technol.* **2014**, *48*, 12302–12311. [[CrossRef](#)]
6. Hartmann, N.B.; Hüffer, T.; Thompson, R.C.; Hassellöv, M.; Verschoor, A.; Daugaard, A.E.; Rist, S.; Karlsson, T.; Brennholt, N.; Cole, M.; et al. Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris. *Environ. Sci. Technol.* **2019**, *53*, 1039–1047. [[CrossRef](#)]
7. Hale, R.C.; Seeley, M.E.; La Guardia, M.J.; Mai, L.; Zeng, E.Y. A Global Perspective on Microplastics. *J. Geophys. Res. Oceans* **2020**, *125*, e2018JC014719. [[CrossRef](#)]
8. Ricciardi, M.; Pironti, C.; Motta, O.; Miele, Y.; Proto, A.; Montano, L. Microplastics in the Aquatic Environment: Occurrence, Persistence, Analysis, and Human Exposure. *Water* **2021**, *13*, 973. [[CrossRef](#)]
9. Siegfried, M.; Koelmans, A.A.; Besseling, E.; Kroeze, C. Export of microplastics from land to sea. A modelling approach. *Water Res.* **2017**, *127*, 249–257. [[CrossRef](#)]
10. Napper, I.E.; Baroth, A.; Barrett, A.C.; Bholra, S.; Chowdhury, G.W.; Davies, B.F.R.; Duncan, E.M.; Kumar, S.; Nelms, S.E.; Hasan Niloy, M.N.; et al. The abundance and characteristics of microplastics in surface water in the transboundary Ganges River. *Environ. Pollut.* **2021**, *274*, 116348. [[CrossRef](#)] [[PubMed](#)]
11. Meng, Y.; Kelly, F.J.; Wright, S.L. Advances and challenges of microplastic pollution in freshwater ecosystems: A UK perspective. *Environ. Pollut.* **2020**, *256*, 113445. [[CrossRef](#)]
12. Zhou, R.; Lu, G.; Yan, Z.; Jiang, R.; Bao, X.; Lu, P. A review of the influences of microplastics on toxicity and transgenerational effects of pharmaceutical and personal care products in aquatic environment. *Sci. Total Environ.* **2020**, *732*, 139222. [[CrossRef](#)] [[PubMed](#)]
13. Enyoh, C.E.; Shafea, L.; Verla, A.W.; Verla, E.N.; Qingyue, W.; Chowdhury, T.; Paredes, M. Microplastics Exposure Routes and Toxicity Studies to Ecosystems: An Overview. *Environ. Anal. Health Toxicol.* **2020**, *35*, e2020004. [[CrossRef](#)]
14. Araújo, A.P.d.C.; Rocha, T.L.; e Silva, D.d.M.; Malafaia, G. Micro(nano)plastics as an emerging risk factor to the health of amphibian: A scientometric and systematic review. *Chemosphere* **2021**, *283*, 131090. [[CrossRef](#)]

15. Canniff, P.M.; Hoang, T.C. Microplastic ingestion by *Daphnia magna* and its enhancement on algal growth. *Sci. Total Environ.* **2018**, *633*, 500–507. [[CrossRef](#)]
16. Caron, A.G.M.; Thomas, C.R.; Berry, K.L.E.; Motti, C.A.; Ariel, E.; Brodie, J.E. Ingestion of microplastic debris by green sea turtles (*Chelonia mydas*) in the Great Barrier Reef: Validation of a sequential extraction protocol. *Mar. Pollut. Bull.* **2018**, *127*, 743–751. [[CrossRef](#)]
17. Zhu, L.; Wang, H.; Chen, B.; Sun, X.; Qu, K.; Xia, B. Microplastic ingestion in deep-sea fish from the South China Sea. *Sci. Total Environ.* **2019**, *677*, 493–501. [[CrossRef](#)]
18. Redondo-Hasselerharm, P.E.; Falahudin, D.; Peeters, E.T.H.M.; Koelmans, A.A. Microplastic Effect Thresholds for Freshwater Benthic Macroinvertebrates. *Environ. Sci. Technol.* **2018**, *52*, 2278–2286. [[CrossRef](#)] [[PubMed](#)]
19. Reynolds, C.; Ryan, P.G. Micro-plastic ingestion by waterbirds from contaminated wetlands in South Africa. *Mar. Pollut. Bull.* **2018**, *126*, 330–333. [[CrossRef](#)] [[PubMed](#)]
20. Nelms, S.E.; Barnett, J.; Brownlow, A.; Davison, N.J.; Deaville, R.; Galloway, T.S.; Lindeque, P.K.; Santillo, D.; Godley, B.J. Microplastics in marine mammals stranded around the British coast: Ubiquitous but transitory? *Sci. Rep.* **2019**, *9*, 1075. [[CrossRef](#)]
21. Rotjan, R.D.; Sharp, K.H.; Gauthier, A.E.; Yelton, R.; Lopez, E.M.B.; Carilli, J.; Kagan, J.C.; Urban-Rich, J. Patterns, dynamics and consequences of microplastic ingestion by the temperate coral, *Astrangia poculata*. *Proc. R. Soc. B Biol. Sci.* **2019**, *286*, 20190726. [[CrossRef](#)]
22. Scherer, C.; Weber, A.; Lambert, S.; Wagner, M. Interactions of Microplastics with Freshwater Biota. In *Freshwater Microplastics: Emerging Environmental Contaminants?* Wagner, M., Lambert, S., Eds.; Springer International Publishing: Cham, Switzerland, 2018; pp. 153–180.
23. Triebkorn, R.; Braunbeck, T.; Grummt, T.; Hanslik, L.; Huppertsberg, S.; Jekel, M.; Knepper, T.P.; Kraus, S.; Müller, Y.K.; Pittroff, M.; et al. Relevance of nano- and microplastics for freshwater ecosystems: A critical review. *TrAC Trends Anal. Chem.* **2019**, *110*, 375–392. [[CrossRef](#)]
24. Xiao, Y.; Jiang, X.; Liao, Y.; Zhao, W.; Zhao, P.; Li, M. Adverse physiological and molecular level effects of polystyrene microplastics on freshwater microalgae. *Chemosphere* **2020**, *255*, 126914. [[CrossRef](#)]
25. Banaee, M.; Gholamhosseini, A.; Sureda, A.; Soltanian, S.; Fereidouni, M.S.; Ibrahim, A.T.A. Effects of microplastic exposure on the blood biochemical parameters in the pond turtle (*Emys orbicularis*). *Environ. Sci. Pollut. Res.* **2021**, *28*, 9221–9234. [[CrossRef](#)]
26. Abbasi, S.; Soltani, N.; Keshavarzi, B.; Moore, F.; Turner, A.; Hassanaghahi, M. Microplastics in different tissues of fish and prawn from the Musa Estuary, Persian Gulf. *Chemosphere* **2018**, *205*, 80–87. [[CrossRef](#)]
27. Daniel, D.B.; Ashraf, P.M.; Thomas, S.N. Microplastics in the edible and inedible tissues of pelagic fishes sold for human consumption in Kerala, India. *Environ. Pollut.* **2020**, *266*, 115365. [[CrossRef](#)]
28. Carbery, M.; O'Connor, W.; Palanisami, T. Trophic transfer of microplastics and mixed contaminants in the marine food web and implications for human health. *Environ. Int.* **2018**, *115*, 400–409. [[CrossRef](#)] [[PubMed](#)]
29. Wang, W.; Gao, H.; Jin, S.; Li, R.; Na, G. The ecotoxicological effects of microplastics on aquatic food web, from primary producer to human: A review. *Ecotoxicol. Environ. Saf.* **2019**, *173*, 110–117. [[CrossRef](#)] [[PubMed](#)]
30. Rillig, M.C. Microplastic in Terrestrial Ecosystems and the Soil? *Environ. Sci. Technol.* **2012**, *46*, 6453–6454. [[CrossRef](#)] [[PubMed](#)]
31. Lohmann, R. Microplastics are not important for the cycling and bioaccumulation of organic pollutants in the oceans—but should microplastics be considered POPs themselves? *Integr. Environ. Assess. Manag.* **2017**, *13*, 460–465. [[CrossRef](#)]
32. Zhu, X.; Qiang, L.; Shi, H.; Cheng, J. Bioaccumulation of microplastics and its in vivo interactions with trace metals in edible oysters. *Mar. Pollut. Bull.* **2020**, *154*, 111079. [[CrossRef](#)] [[PubMed](#)]
33. Yoshida, S.; Hiraga, K.; Takehana, T.; Taniguchi, I.; Yamaji, H.; Maeda, Y.; Toyohara, K.; Miyamoto, K.; Kimura, Y.; Oda, K. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science* **2016**, *351*, 1196. [[CrossRef](#)]
34. Campos, D.; Rodrigues, A.C.M.; Rocha, R.J.M.; Martins, R.; Candeias-Mendes, A.; Castanho, S.; Soares, F.; Pousão-Ferreira, P.; Soares, A.M.V.M.; Gravato, C.; et al. Are Microplastics Impairing Marine Fish Larviculture?—Preliminary Results with *Argyrosomus regius*. *Water* **2021**, *13*, 104. [[CrossRef](#)]
35. Cormier, B.; Le Bihanic, F.; Cabar, M.; Crebassa, J.-C.; Blanc, M.; Larsson, M.; Dubocq, F.; Yeung, L.; Clérandeau, C.; Keiter, S.H.; et al. Chronic feeding exposure to virgin and spiked microplastics disrupts essential biological functions in teleost fish. *J. Hazard. Mater.* **2021**, *415*, 125626. [[CrossRef](#)] [[PubMed](#)]
36. Hansson, T.; Lindesjö, E.; Förlin, L.; Balk, L.; Bignert, A.; Larsson, Å. Long-term monitoring of the health status of female perch (*Perca fluviatilis*) in the Baltic Sea shows decreased gonad weight and increased hepatic EROD activity. *Aquat. Toxicol.* **2006**, *79*, 341–355. [[CrossRef](#)]
37. Naidoo, T.; Glassom, D. Decreased growth and survival in small juvenile fish, after chronic exposure to environmentally relevant concentrations of microplastic. *Mar. Pollut. Bull.* **2019**, *145*, 254–259. [[CrossRef](#)]
38. Wang, J.; Li, Y.; Lu, L.; Zheng, M.; Zhang, X.; Tian, H.; Wang, W.; Ru, S. Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*). *Environ. Pollut.* **2019**, *254*, 113024. [[CrossRef](#)]
39. Barboza, L.G.A.; Vieira, L.R.; Branco, V.; Figueiredo, N.; Carvalho, F.; Carvalho, C.; Guilhermino, L. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). *Aquat. Toxicol.* **2018**, *195*, 49–57. [[CrossRef](#)]

40. Kim, J.-H.; Yu, Y.-B.; Choi, J.-H. Toxic effects on bioaccumulation, hematological parameters, oxidative stress, immune responses and neurotoxicity in fish exposed to microplastics: A review. *J. Hazard. Mater.* **2021**, *413*, 125423. [[CrossRef](#)]
41. Barboza, L.G.A.; Lopes, C.; Oliveira, P.; Bessa, F.; Otero, V.; Henriques, B.; Raimundo, J.; Caetano, M.; Vale, C.; Guilhermino, L. Microplastics in wild fish from North East Atlantic Ocean and its potential for causing neurotoxic effects, lipid oxidative damage, and human health risks associated with ingestion exposure. *Sci. Total Environ.* **2020**, *717*, 134625. [[CrossRef](#)] [[PubMed](#)]
42. Guimarães, A.T.B.; Estrela, F.N.; Pereira, P.S.; de Andrade Vieira, J.E.; de Lima Rodrigues, A.S.; Silva, F.G.; Malafaia, G. Toxicity of polystyrene nanoplastics in *Ctenopharyngodon idella* juveniles: A genotoxic, mutagenic and cytotoxic perspective. *Sci. Total Environ.* **2021**, *752*, 141937. [[CrossRef](#)]
43. Critchell, K.; Hoogenboom, M.O. Effects of microplastic exposure on the body condition and behaviour of planktivorous reef fish (*Acanthochromis polyacanthus*). *PLoS ONE* **2018**, *13*, e0193308. [[CrossRef](#)] [[PubMed](#)]
44. Wang, W.; Ge, J.; Yu, X. Bioavailability and toxicity of microplastics to fish species: A review. *Ecotoxicol. Environ. Saf.* **2020**, *189*, 109913. [[CrossRef](#)]
45. Wright, S.L.; Kelly, F.J. Plastic and Human Health: A Micro Issue? *Environ. Sci. Technol.* **2017**, *51*, 6634–6647. [[CrossRef](#)]
46. Bobori, D.; Dimitriadi, A.; Karasiali, S.; Tsoumaki-Tsouroufli, P.; Mastora, M.; Kastrinaki, G.; Feidantsis, K.; Printzi, A.; Koumoundouros, G.; Kaloyianni, M. Common mechanisms activated in the tissues of aquatic and terrestrial animal models after TiO<sub>2</sub> nanoparticles exposure. *Environ. Int.* **2020**, *138*, 105611. [[CrossRef](#)] [[PubMed](#)]
47. Kaloyianni, M.; Dimitriadi, A.; Ovezik, M.; Stamkopoulou, D.; Feidantsis, K.; Kastrinaki, G.; Gallios, G.; Tsiaoussis, I.; Koumoundouros, G.; Bobori, D. Magnetite nanoparticles effects on adverse responses of aquatic and terrestrial animal models. *J. Hazard. Mater.* **2020**, *383*, 121204. [[CrossRef](#)] [[PubMed](#)]
48. Svendsen, C.; Spurgeon, D.J.; Hankard, P.K.; Weeks, J.M. A review of lysosomal membrane stability measured by neutral red retention: Is it a workable earthworm biomarker? *Ecotoxicol. Environ. Saf.* **2004**, *57*, 20–29. [[CrossRef](#)] [[PubMed](#)]
49. Prokić, M.D.; Radovanović, T.B.; Gavrić, J.P.; Faggio, C. Ecotoxicological effects of microplastics: Examination of biomarkers, current state and future perspectives. *TrAC Trends Anal. Chem.* **2019**, *111*, 37–46. [[CrossRef](#)]
50. Zhao, Y.; Qiao, R.; Zhang, S.; Wang, G. Metabolomic profiling reveals the intestinal toxicity of different length of microplastic fibers on zebrafish (*Danio rerio*). *J. Hazard. Mater.* **2021**, *403*, 123663. [[CrossRef](#)]
51. Solomando, A.; Capó, X.; Alomar, C.; Compa, M.; Valencia, J.M.; Sureda, A.; Deudero, S. Assessment of the effect of long-term exposure to microplastics and depuration period in *Sparus aurata* Linnaeus, 1758: Liver and blood biomarkers. *Sci. Total Environ.* **2021**, *786*, 147479. [[CrossRef](#)]
52. Dimitriadi, A.; Papaefthimiou, C.; Genizegkini, E.; Sampsonidis, I.; Kalogiannis, S.; Feidantsis, K.; Bobori, D.C.; Kastrinaki, G.; Koumoundouros, G.; Lambropoulou, D.A.; et al. Adverse effects polystyrene microplastics exert on zebrafish heart—Molecular to individual level. *J. Hazard. Mater.* **2021**, *416*, 125969. [[CrossRef](#)]
53. Rodrigues, E.D.L.; Fanta, E. Liver histopathology of the fish *Brachydanio rerio* Hamilton-Buchman after acute exposure to sublethal levels of the organophosphate Dimethoate 500. *Rev. Bras. Zool.* **1998**, *15*, 441–450. [[CrossRef](#)]
54. van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol.* **2003**, *13*, 57–149. [[CrossRef](#)]
55. Handy, R.D.; Al-Bairuty, G.; Al-Jubory, A.; Ramsden, C.S.; Boyle, D.; Shaw, B.J.; Henry, T.B. Effects of manufactured nanomaterials on fishes: A target organ and body systems physiology approach. *J. Fish Biol.* **2011**, *79*, 821–853. [[CrossRef](#)]
56. Huuskonen, S.; Lindström-Seppä, P. Hepatic cytochrome P4501A and other biotransformation activities in perch (*Perca fluviatilis*): The effects of unbleached pulp mill effluents. *Aquat. Toxicol.* **1995**, *31*, 27–41. [[CrossRef](#)]
57. Noaksson, E.; Linderth, M.; Gustavsson, B.; Zebühr, Y.; Balk, L. Reproductive status in female perch (*Perca fluviatilis*) outside a sewage treatment plant processing leachate from a refuse dump. *Sci. Total Environ.* **2005**, *340*, 97–112. [[CrossRef](#)]
58. Laidou, S.; Alanis-Lobato, G.; Pribyl, J.; Raskó, T.; Tichy, B.; Mikulasek, K.; Tsagiopoulou, M.; Oppelt, J.; Kastrinaki, G.; Lefaki, M.; et al. Nuclear inclusions of pathogenic ataxin-1 induce oxidative stress and perturb the protein synthesis machinery. *Redox Biol.* **2020**, *32*, 101458. [[CrossRef](#)]
59. Iheanacho, S.C.; Odo, G.E. Dietary exposure to polyvinyl chloride microparticles induced oxidative stress and hepatic damage in *Clarias gariepinus* (Burchell, 1822). *Environ. Sci. Pollut. Res.* **2020**, *27*, 21159–21173. [[CrossRef](#)] [[PubMed](#)]
60. Phuong, N.N.; Zalouk-Vergnoux, A.; Poirier, L.; Kamari, A.; Châtel, A.; Mouneyrac, C.; Lagarde, F. Is there any consistency between the microplastics found in the field and those used in laboratory experiments? *Environ. Pollut.* **2016**, *211*, 111–123. [[CrossRef](#)]
61. Nerantzaki, M.; Adam, K.-V.; Koliakou, I.; Skoufa, E.; Avgeropoulos, A.; Papageorgiou, G.Z.; Bikiaris, D. Novel Castor Oil-Derived Block Copolymers as Promising Candidates for Biological Applications: Biorelevant and Biocompatible. *Macromol. Chem. Phys.* **2017**, *218*, 1700305. [[CrossRef](#)]
62. Lowe, D.M.; Pipe, R.K. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: An in vitro study. *Aquat. Toxicol.* **1994**, *30*, 357–365. [[CrossRef](#)]
63. Dailianis, S.; Domouhtsidou, G.P.; Raftopoulou, E.; Kaloyianni, M.; Dimitriadis, V.K. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. *Mar. Environ. Res.* **2003**, *56*, 443–470. [[CrossRef](#)]
64. Rainieri, S.; Conlledo, N.; Larsen, B.K.; Granby, K.; Barranco, A. Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (*Danio rerio*). *Environ. Res.* **2018**, *162*, 135–143. [[CrossRef](#)] [[PubMed](#)]

65. Solomando, A.; Capó, X.; Alomar, C.; Álvarez, E.; Compá, M.; Valencia, J.M.; Pinya, S.; Deudero, S.; Sureda, A. Long-term exposure to microplastics induces oxidative stress and a pro-inflammatory response in the gut of *Sparus aurata* Linnaeus, 1758. *Environ. Pollut.* **2020**, *266*, 115295. [[CrossRef](#)] [[PubMed](#)]
66. Niehaus, W.G., Jr.; Samuelsson, B. Formation of Malonaldehyde from Phospholipid Arachidonate during Microsomal Lipid Peroxidation. *Eur. J. Biochem.* **1968**, *6*, 126–130. [[CrossRef](#)] [[PubMed](#)]
67. Wills, E.D. Lipid peroxide formation in microsomes. General considerations. *Biochem. J.* **1969**, *113*, 315–324. [[CrossRef](#)] [[PubMed](#)]
68. Buss, H.; Chan, T.P.; Sluis, K.B.; Domigan, N.M.; Winterbourn, C.C. Protein Carbonyl Measurement by a Sensitive ELISA Method. *Free Radic. Biol. Med.* **1997**, *23*, 361–366. [[CrossRef](#)]
69. Alamdari, D.H.; Kostidou, E.; Paletas, K.; Sarigianni, M.; Konstas, A.G.P.; Karapiperidou, A.; Koliakos, G. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Radic. Biol. Med.* **2005**, *39*, 1362–1367. [[CrossRef](#)]
70. Dailianis, S.; Piperakis, S.M.; Kaloyianni, M. Cadmium effects on ROS production and DNA damage via adrenergic receptors stimulation: Role of Na<sup>+</sup>/H<sup>+</sup> exchanger and PKC. *Free Radic. Res.* **2005**, *39*, 1059–1070. [[CrossRef](#)]
71. Liao, W.; McNutt, M.A.; Zhu, W.-G. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Methods* **2009**, *48*, 46–53. [[CrossRef](#)]
72. Ritter, D.; Knebel, J. Genotoxicity testing in vitro—Development of a higher throughput analysis method based on the comet assay. *Toxicol. In Vitro* **2009**, *23*, 1570–1575. [[CrossRef](#)]
73. Binelli, A.; Cogni, D.; Parolini, M.; Riva, C.; Provini, A. In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes. *Aquat. Toxicol.* **2009**, *91*, 238–244. [[CrossRef](#)]
74. Virgiliou, C.; Sampsonidis, I.; Gika, H.G.; Raikos, N.; Theodoridis, G.A. Development and validation of a HILIC-MS/MS multitargeted method for metabolomics applications. *Electrophoresis* **2015**, *36*, 2215–2225. [[CrossRef](#)] [[PubMed](#)]
75. Zisi, C.; Sampsonidis, I.; Fasoula, S.; Papachristos, K.; Witting, M.; Gika, H.G.; Nikitas, P.; Pappa-Louisi, A. QSRR Modeling for Metabolite Standards Analyzed by Two Different Chromatographic Columns Using Multiple Linear Regression. *Metabolites* **2017**, *7*, 7. [[CrossRef](#)] [[PubMed](#)]
76. Parker, F.S. *Applications of Infrared Spectroscopy in Biochemistry, Biology, and Medicine*, 1st ed.; Springer: Boston, MA, USA, 1971.
77. Kastrinaki, G.; Samsouris, C.; Kosmidis, E.K.; Papaioannou, E.; Konstandopoulos, A.G.; Theophilidis, G. Assessing the axonal translocation of CeO<sub>2</sub> and SiO<sub>2</sub> nanoparticles in the sciatic nerve fibers of the frog: An ex vivo electrophysiological study. *Int. J. Nanomed.* **2015**, *10*, 7089–7096. [[CrossRef](#)]
78. Sheng, C.; Zhang, S.; Zhang, Y. The influence of different polymer types of microplastics on adsorption, accumulation, and toxicity of triclosan in zebrafish. *J. Hazard. Mater.* **2021**, *402*, 123733. [[CrossRef](#)] [[PubMed](#)]
79. Yin, C.; Ma, Z.; Li, F.; Duan, C.; Yuan, Y.; Zhu, C.; Wang, L.; Zhu, X.; Wang, S.; Gao, P.; et al. Hypoxanthine Induces Muscular ATP Depletion and Fatigue via UCP2. *Front. Physiol.* **2021**, *12*, 647743. [[CrossRef](#)]
80. Xia, X.; Sun, M.; Zhou, M.; Chang, Z.; Li, L. Polyvinyl chloride microplastics induce growth inhibition and oxidative stress in *Cyprinus carpio* var. larvae. *Sci. Total Environ.* **2020**, *716*, 136479. [[CrossRef](#)]
81. Indo, H.P.; Davidson, M.; Yen, H.-C.; Suenaga, S.; Tomita, K.; Nishii, T.; Higuchi, M.; Koga, Y.; Ozawa, T.; Majima, H.J. Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage. *Mitochondrion* **2007**, *7*, 106–118. [[CrossRef](#)]
82. Taze, C.; Panetas, I.; Kalogiannis, S.; Feidantsis, K.; Gallios, G.P.; Kastrinaki, G.; Konstandopoulos, A.G.; Václavíková, M.; Ivanicova, L.; Kaloyianni, M. Toxicity assessment and comparison between two types of iron oxide nanoparticles in *Mytilus galloprovincialis*. *Aquat. Toxicol.* **2016**, *172*, 9–20. [[CrossRef](#)] [[PubMed](#)]
83. Levine, R.L.; Garland, D.; Oliver, C.N.; Amici, A.; Climent, I.; Lenz, A.-G.; Ahn, B.-W.; Shaltiel, S.; Stadtman, E.R. [49] Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **1990**, *186*, 464–478. [[CrossRef](#)]
84. Ghezzi, P.; Bonetto, V. Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* **2003**, *3*, 1145–1153. [[CrossRef](#)] [[PubMed](#)]
85. Hecker, M.; Wagner, A.H. Role of protein carbonylation in diabetes. *J. Inher. Metab. Dis.* **2018**, *41*, 29–38. [[CrossRef](#)]
86. Kaloyianni, M.; Feidantsis, K.; Nteli, I.; Stergiou, P.; Tsoulia, T.; Dimitriadi, A.; Antonopoulou, E.; Bobori, D. Biochemical and molecular responses of cyprinids in two Mediterranean lacustrine ecosystems: Opportunities for ecological assessment and biomonitoring. *Aquat. Toxicol.* **2019**, *211*, 105–115. [[CrossRef](#)] [[PubMed](#)]
87. Wen, B.; Jin, S.-R.; Chen, Z.-Z.; Gao, J.-Z.; Liu, Y.-N.; Liu, J.-H.; Feng, X.-S. Single and combined effects of microplastics and cadmium on the cadmium accumulation, antioxidant defence and innate immunity of the discus fish (*Symphysodon aequifasciatus*). *Environ. Pollut.* **2018**, *243*, 462–471. [[CrossRef](#)]
88. Birben, E.; Sahiner, U.M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ. J.* **2012**, *5*, 9–19. [[CrossRef](#)] [[PubMed](#)]
89. Pannetier, P.; Morin, B.; Le Bihanic, F.; Dubreil, L.; Clérandeau, C.; Chouvellon, F.; Van Arkel, K.; Danion, M.; Cachot, J. Environmental samples of microplastics induce significant toxic effects in fish larvae. *Environ. Int.* **2020**, *134*, 105047. [[CrossRef](#)]
90. Avio, C.G.; Gorbi, S.; Milan, M.; Benedetti, M.; Fattorini, D.; d’Errico, G.; Pauletto, M.; Bargelloni, L.; Regoli, F. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ. Pollut.* **2015**, *198*, 211–222. [[CrossRef](#)]
91. Ribeiro, F.; Garcia, A.R.; Pereira, B.P.; Fonseca, M.; Mestre, N.C.; Fonseca, T.G.; Ilharco, L.M.; Bebianno, M.J. Microplastics effects in *Scrobicularia plana*. *Mar. Pollut. Bull.* **2017**, *122*, 379–391. [[CrossRef](#)]

92. Yang, H.; Lai, H.; Huang, J.; Sun, L.; Mennigen, J.A.; Wang, Q.; Liu, Y.; Jin, Y.; Tu, W. Polystyrene microplastics decrease F-53B bioaccumulation but induce inflammatory stress in larval zebrafish. *Chemosphere* **2020**, *255*, 127040. [[CrossRef](#)]
93. Capó, X.; Company, J.J.; Alomar, C.; Compa, M.; Sureda, A.; Grau, A.; Hansjosten, B.; López-Vázquez, J.; Quintana, J.B.; Rodil, R.; et al. Long-term exposure to virgin and seawater exposed microplastic enriched-diet causes liver oxidative stress and inflammation in gilthead seabream *Sparus aurata*, Linnaeus 1758. *Sci. Total Environ.* **2021**, *767*, 144976. [[CrossRef](#)]
94. Alomar, C.; Sureda, A.; Capó, X.; Guijarro, B.; Tejada, S.; Deudero, S. Microplastic ingestion by *Mullus surmuletus* Linnaeus, 1758 fish and its potential for causing oxidative stress. *Environ. Res.* **2017**, *159*, 135–142. [[CrossRef](#)]
95. Ding, J.; Zhang, S.; Razanajatovo, R.M.; Zou, H.; Zhu, W. Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). *Environ. Pollut.* **2018**, *238*, 1–9. [[CrossRef](#)] [[PubMed](#)]
96. Magni, S.; Della Torre, C.; Garrone, G.; D'Amato, A.; Parenti, C.C.; Binelli, A. First evidence of protein modulation by polystyrene microplastics in a freshwater biological model. *Environ. Pollut.* **2019**, *250*, 407–415. [[CrossRef](#)] [[PubMed](#)]
97. Shang, F.; Taylor, A. Oxidative stress and recovery from oxidative stress are associated with altered ubiquitin conjugating and proteolytic activities in bovine lens epithelial cells. *Biochem. J.* **1995**, *307 Pt 1*, 297–303. [[CrossRef](#)] [[PubMed](#)]
98. Chondrogianni, N.; Petropoulos, I.; Grimm, S.; Georgila, K.; Catalgol, B.; Friguet, B.; Grune, T.; Gonos, E.S. Protein damage, repair and proteolysis. *Mol. Asp. Med.* **2014**, *35*, 1–71. [[CrossRef](#)]
99. Jing, K.; Shin, S.; Jeong, S.; Kim, S.; Song, K.S.; Park, J.H.; Heo, J.Y.; Seo, K.S.; Park, S.K.; Kweon, G.R.; et al. Docosahexaenoic acid induces the degradation of HPV E6/E7 oncoproteins by activating the ubiquitin–proteasome system. *Cell Death Dis.* **2014**, *5*, e1524. [[CrossRef](#)]
100. Redza-Dutordoir, M.; Averill-Bates, D.A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim. Biophys. Acta (BBA)-Mol. Cell Res.* **2016**, *1863*, 2977–2992. [[CrossRef](#)] [[PubMed](#)]
101. Filomeni, G.; De Zio, D.; Cecconi, F. Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death Differ.* **2015**, *22*, 377–388. [[CrossRef](#)]
102. Cheng, C.-H.; Yang, F.-F.; Liao, S.-A.; Miao, Y.-T.; Ye, C.-X.; Wang, A.-L.; Tan, J.-W.; Chen, X.-Y. High temperature induces apoptosis and oxidative stress in pufferfish (*Takifugu obscurus*) blood cells. *J. Therm. Biol.* **2015**, *53*, 172–179. [[CrossRef](#)]
103. Jiang, W.-D.; Feng, L.; Qu, B.; Wu, P.; Kuang, S.-Y.; Jiang, J.; Tang, L.; Tang, W.-N.; Zhang, Y.-A.; Zhou, X.-Q.; et al. Changes in integrity of the gill during histidine deficiency or excess due to depression of cellular anti-oxidative ability, induction of apoptosis, inflammation and impair of cell-cell tight junctions related to Nrf2, TOR and NF-κB signaling in fish. *Fish Shellfish Immunol.* **2016**, *56*, 111–122. [[CrossRef](#)] [[PubMed](#)]
104. Takle, H.; Andersen, Ø. Caspases and apoptosis in fish. *J. Fish Biol.* **2007**, *71*, 326–349. [[CrossRef](#)]
105. Wang, K. Autophagy and apoptosis in liver injury. *Cell Cycle* **2015**, *14*, 1631–1642. [[CrossRef](#)]
106. Fairlie, W.D.; Tran, S.; Lee, E.F. Chapter Four—Crosstalk between apoptosis and autophagy signaling pathways. In *International Review of Cell and Molecular Biology*; Spetz, J.K.E., Galluzzi, L., Eds.; Academic Press: Cambridge, MA, USA, 2020; Volume 352, pp. 115–158.
107. Klionsky, D.J.; Abdelmohsen, K.; Abe, A.; Abedin, M.J.; Abeliovich, H.; Acevedo Arozena, A.; Adachi, H.; Adams, C.M.; Adams, P.D.; Adeli, K.; et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **2016**, *12*, 1–222. [[CrossRef](#)]
108. Kabeya, Y.; Mizushima, N.; Yamamoto, A.; Oshitani-Okamoto, S.; Ohsumi, Y.; Yoshimori, T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J. Cell Sci.* **2004**, *117*, 2805–2812. [[CrossRef](#)] [[PubMed](#)]
109. Bjørkøy, G.; Lamark, T.; Johansen, T. p62/SQSTM1: A Missing Link between Protein Aggregates and the Autophagy Machinery. *Autophagy* **2006**, *2*, 138–139. [[CrossRef](#)]
110. Komatsu, M.; Waguri, S.; Koike, M.; Sou, Y.-S.; Ueno, T.; Hara, T.; Mizushima, N.; Iwata, J.-I.; Ezaki, J.; Murata, S.; et al. Homeostatic Levels of p62 Control Cytoplasmic Inclusion Body Formation in Autophagy-Deficient Mice. *Cell* **2007**, *131*, 1149–1163. [[CrossRef](#)] [[PubMed](#)]
111. Pankiv, S.; Clausen, T.H.; Lamark, T.; Brech, A.; Bruun, J.-A.; Outzen, H.; Øvervatn, A.; Bjørkøy, G.; Johansen, T. p62/SQSTM1 Binds Directly to Atg8/LC3 to Facilitate Degradation of Ubiquitinated Protein Aggregates by Autophagy. *J. Biol. Chem.* **2007**, *282*, 24131–24145. [[CrossRef](#)]
112. Dodson, M.; Darley-Usmar, V.; Zhang, J. Cellular metabolic and autophagic pathways: Traffic control by redox signaling. *Free Radic. Biol. Med.* **2013**, *63*, 207–221. [[CrossRef](#)] [[PubMed](#)]
113. Choi, J.S.; Jung, Y.-J.; Hong, N.-H.; Hong, S.H.; Park, J.-W. Toxicological effects of irregularly shaped and spherical microplastics in a marine teleost, the sheepshead minnow (*Cyprinodon variegatus*). *Mar. Pollut. Bull.* **2018**, *129*, 231–240. [[CrossRef](#)]
114. Limonta, G.; Mancía, A.; Benkhalqui, A.; Bertolucci, C.; Abelli, L.; Fossi, M.C.; Panti, C. Microplastics induce transcriptional changes, immune response and behavioral alterations in adult zebrafish. *Sci. Rep.* **2019**, *9*, 15775. [[CrossRef](#)] [[PubMed](#)]
115. von Moos, N.; Burkhardt-Holm, P.; Köhler, A. Uptake and Effects of Microplastics on Cells and Tissue of the Blue Mussel *Mytilus edulis* L. after an Experimental Exposure. *Environ. Sci. Technol.* **2012**, *46*, 11327–11335. [[CrossRef](#)]
116. Gambino, G.; Falleni, A.; Nigro, M.; Salvetti, A.; Cecchetti, A.; Ippolito, C.; Guidi, P.; Rossi, L. Dynamics of interaction and effects of microplastics on planarian tissue regeneration and cellular homeostasis. *Aquat. Toxicol.* **2020**, *218*, 105354. [[CrossRef](#)] [[PubMed](#)]
117. Yan, X.; Zhang, Y.; Lu, Y.; He, L.; Qu, J.; Zhou, C.; Hong, P.; Sun, S.; Zhao, H.; Liang, Y.; et al. The Complex Toxicity of Tetracycline with Polystyrene Spheres on Gastric Cancer Cells. *Int. J. Environ. Res. Public Health* **2020**, *17*, 2808. [[CrossRef](#)] [[PubMed](#)]

118. Lawlor, C.; O'Connor, G.; O'Leary, S.; Gallagher, P.J.; Cryan, S.-A.; Keane, J.; O'Sullivan, M.P. Treatment of Mycobacterium tuberculosis-Infected Macrophages with Poly(Lactic-Co-Glycolic Acid) Microparticles Drives NFκB and Autophagy Dependent Bacillary Killing. *PLoS ONE* **2016**, *11*, e0149167. [[CrossRef](#)] [[PubMed](#)]
119. Lu, Y.; Zhang, Y.; Deng, Y.; Jiang, W.; Zhao, Y.; Geng, J.; Ding, L.; Ren, H. Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver. *Environ. Sci. Technol.* **2016**, *50*, 4054–4060. [[CrossRef](#)]
120. Teng, J.; Zhao, J.; Zhu, X.; Shan, E.; Zhang, C.; Zhang, W.; Wang, Q. Toxic effects of exposure to microplastics with environmentally relevant shapes and concentrations: Accumulation, energy metabolism and tissue damage in oyster *Crassostrea gigas*. *Environ. Pollut.* **2021**, *269*, 116169. [[CrossRef](#)]
121. Qiao, R.; Sheng, C.; Lu, Y.; Zhang, Y.; Ren, H.; Lemos, B. Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Sci. Total Environ.* **2019**, *662*, 246–253. [[CrossRef](#)]
122. Wan, Z.; Wang, C.; Zhou, J.; Shen, M.; Wang, X.; Fu, Z.; Jin, Y. Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. *Chemosphere* **2019**, *217*, 646–658. [[CrossRef](#)]
123. Zhao, Y.; Qin, Z.; Huang, Z.; Bao, Z.; Luo, T.; Jin, Y. Effects of polyethylene microplastics on the microbiome and metabolism in larval zebrafish. *Environ. Pollut.* **2021**, *282*, 117039. [[CrossRef](#)]
124. Zhao, H.-J.; Xu, J.-K.; Yan, Z.-H.; Ren, H.-Q.; Zhang, Y. Microplastics enhance the developmental toxicity of synthetic phenolic antioxidants by disturbing the thyroid function and metabolism in developing zebrafish. *Environ. Int.* **2020**, *140*, 105750. [[CrossRef](#)]
125. Currie, S.; Moyes, C.D.; Tufts, B.L. The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: In vivo and in vitro comparisons. *J. Fish Biol.* **2000**, *56*, 398–408. [[CrossRef](#)]
126. Dietz, T.J.; Somero, G.N. The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*). *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3389. [[CrossRef](#)]
127. Vignardi, C.P.; Hasue, F.M.; Sartório, P.V.; Cardoso, C.M.; Machado, A.S.D.; Passos, M.J.A.C.R.; Santos, T.C.A.; Nucci, J.M.; Hower, T.L.R.; Watanabe, I.-S.; et al. Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish *Trachinotus carolinus* (Linnaeus, 1766). *Aquat. Toxicol.* **2015**, *158*, 218–229. [[CrossRef](#)]
128. Greven, A.-C.; Merk, T.; Karagöz, F.; Mohr, K.; Klapper, M.; Jovanović, B.; Palić, D. Polycarbonate and polystyrene nanoplastic particles act as stressors to the innate immune system of fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2016**, *35*, 3093–3100. [[CrossRef](#)] [[PubMed](#)]
129. Greven, A.-C. Polycarbonate and Polystyrene Nanoparticles Act as Stressors to the Innate Immune System of Fathead Minnows (*Pimephales promelas*, Rafinesque 1820). Ph.D. Thesis, LMU München-Tierärztliche Fakultät, Munich, Germany, 2016.
130. Kashiwada, S. Distribution of Nanoparticles in the See-through Medaka (*Oryzias latipes*). *Environ. Health Perspect.* **2006**, *114*, 1697–1702. [[CrossRef](#)]
131. Yong, C.Q.Y.; Valiyaveetill, S.; Tang, B.L. Toxicity of Microplastics and Nanoplastics in Mammalian Systems. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1509. [[CrossRef](#)]
132. Barrera, G. Oxidative Stress and Lipid Peroxidation Products in Cancer Progression and Therapy. *ISRN Oncol.* **2012**, *2012*, 137289. [[CrossRef](#)] [[PubMed](#)]