Does hydrogen-rich water mitigate MP toxicity in rainbow trout (*Oncorhyncus mykiss*)? Monitoring with hematology, DNA damage, and apoptosis via ROS/GSH/MDA pathway

by

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Abstract

Although the number of studies documenting the presence of Microplastics (MP) in fish is increasing, research studies focused on its detoxification are very limited. In this study, rainbow trout (*Oncorhyncus mykiss*) were randomly divided into two groups after being fed with MPs (15% polypropylene [PP] +15% polyethylene [PE]) for 2 months. MP excretion without any application (PP+PE) in group I fish, and excretion of MPs with hydrogen-rich water (HRW) application (PP+PE+HRW) in group 2 were investigated under semi-static conditions for 21 days. This effect was also compared by using positive and negative control groups (Control [no treatment, free PP, PE or/and HRW] and only HRW group). In this direction, the following were determined: PP+PE chronic toxicity in aquatic organisms, the toxicity mechanism and the effect of HRW as a possible treatment method in blood tissue; with hematological indices ([RBC count [RBC], leukocyte count [WBC], hemoglobin value [Hb], hematocrit ratio [Hct], platelet count [PLT], hemoglobin count per erythrocyte [MCHC], mean hemoglobin amount per erythrocyte [MCH] and mean erythrocyte volume [MCV]) in other tissues (liver, gill and brain tissue) oxidative stress response (catalase [CAT]), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), glutathione (GSH), reactive oxygen products (ROS), malondialdehyde ([MDA] levels), DNA damage (8-OHdG: 8-Hydroxy-2-Deoxyguanosine), and the apoptosis (caspase 3) levels were investigated. In addition, acetylcholinesterase enzyme (AChE) activity, which is important in neurotoxicity pathways in the brain, was determined. The presence of plastics (PP/PE) in target tissues (muscle, liver, gill and gastrointestinal tract) was also obtained.

The results showed that PP+PE caused toxicity in all three tissues. MPs showed an inhibiting effect on antioxidant enzyme activities and an inductive effect on MDA, ROS, 8-OHdG, and caspase 3 levels. HRW showed a mitigating effect on MP-mediated toxicity in *O. mykiss* brain, blood, gill, and liver by controlling the ROS/ GSH/MDA pathway. HRW can be suggested as a cost-effective and eco-friendly curative for the protection of fish from the oxidative damages produced by the ingestion of microplastics.

Key words: Oxy-hydrogen gas, hydrogen-rich water, molecular hydrogen, MPs/NPs, blood indices, rainbow trout, antioxidant status, fish

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1. Introduction

Microplastics (MPs) cause ecologic problems in both aquatic and terrestrial environments via being carried by wind, surface waters, atmosphere, and rivers (Rai et al. 2021). A recent study revealed that 80% of MP pollution in the seas comes from terrestrial environments (Sana et al. 2020). MPs have been found in a wide variety of polymers, sizes, shapes, and concentrations in salt and fresh waters, the atmosphere, agroecosystems, a wide variety of foods, drinking water and different biotas (Campanale et al. 2020).

Studies reported that aquatic flora and fauna were affected in different ways when they came into contact with MPs (Alak et al. 2022). MPs can be bound and physically digested by almost all aquatic taxa and they can affect biota via surface leachate or pathogens (Alak et al. 2022, Su et al. 2022).

In addition to the species, feeding behavior and age, the spatial niche in which the fish species or population lives can affect the uptake of MPs (Atamanalp et al. 2021, Iheanacho et al. 2023, Sun et al. 2023). Similarly, the uptake of MPs is correlated with the temporally and spatially variable MP concentration (Prinz and Korez 2020, Alak et al. 2022). After ingestion the MP can reach the fish bloodstream and lead to intestinal obstruction, reduced food consumption and physical injury ending in death (Atamanalp et al. 2021a, Alak et al. 2022, Kokturk et al. 2023). Many studies in the last few years have shown that MPs in aquatic organisms have caused oxidative stress, genotoxicity, reproductive dysfunction, behavioral changes, low survival rate, low population growth rate, and physical and chemical toxicity including genetic transmission (Hanachi et al. 2019; Barboza et al. 2020, Zakari et al. 2020). Histopathological studies in fish indicate the negative effects of MPs in disorders of lipid and energy metabolisms (liver inflammation, disruptions in the immune system, and changes in blood parameters / metabolic profiles) (Triebskorn et al. 2019, Atamanalp et al. 2021, Hamed et al. 2021, Sayed et al. 2022, Kokturk et al. 2023).

Aquatic organisms can be contaminated with MPs/nanoplastic (NPs) via the ingestion of MP-polluted water or feeding on MP-contaminated organisms (Kolandhasamy et al. 2019, Baalkhuyur et al. 2020, Li et al. 2020). Fish species were considered a good model organism for the detection of MPs in the ecotoxicity of plastics due to physiological, biochemical, and genetic changes (Rai et al. 2020). Toxicity biomarkers used in the study of MP effects on organisms include increased biotransformation enzyme activities and oxidative stress parameters, decreased activity of acetylcholinesterase, and changes in the gut microbiome and feeding behavior (Alak et al. 2019, Triebskorn et al. 2019, Uçar et al. 2020, Atamanalp et al. 2021, Alak et al. 2022).

Hematology parameters are an important detection tool for monitoring fish status in feeding, water quality, health changes and response to treatment (Fazio et al. 2019).

The hydroxyl radical (·OH) is among the most well-known oxidising free radicals. In the presence of hydrogen peroxide (H_2O_2), it is formed by the Fenton reaction between cellular reductants and iron. It can oxidise and damage PUFA (polyunsaturated fatty acids), proteins, and DNA. Reactive oxygen species and hydroxyl radicals can damage cellular defense, break the DNA chain, and put down some of the biochemical mechanisms (enzymes, proteins, membrane ion transport system, lipids). This damage may manifest itself in severe damage to the tissues (Precourt et al. 2009) and the in vitro biochemical properties of the myofibrillar protein (Harel and Kanner 1985, Park et al. 2007a, Park et al. 2007b).

Many oxidative DNA damage products such as the guanine derivative 8-OHgua and the deoxynucleoside 8-OHdG have been considered the most important indicators of DNA damage (Alak et al. 2017). Malondialdehyde (MDA) is considered an important indicator in the evaluation of oxidative stress (Alak et al. 2021, Atamanalp et al. 2021). Caspase (cysteine-aspartic acid proteases) releases endonucleases by destroying the endonuclease cross-linked proteins of DNA. Accordingly, it is produced as inactive granules that are activated by proteolytic degradation. Measurement of antioxidant enzyme activity (SOD, GPx, CAT, AR, PON, and MPO) was also used as a toxic indicator in some ecotoxicological studies (Ucar et al. 2020, Atamanalp et al. 2021b). An increase in ROS production activates NF-κB signaling pathways (Yuan et al. 2018, Lu et al. 2018). Different fish tissues such as liver, muscle, brain, gonad, kidney are used to examine the pollutants' effects in the aquatic ecosystems (Atamanalp et al. 2021). Although gill and kidney tissues are important in osmoregulation in fish, the preferred organ tissue in estimating the level of water pollution is that of the liver (Atamanalp et al. 2021c).

Depending on the pollution type, many treatment methods were developed to restore the organism's health. In most cases, although it is desired to use methods that will maximise performance and provide recovery in a short time, recent therapeutic applications using natural agents have attracted attention. As a result, to achieve the improvement purpose in the best way, strategies that will not cause

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adverse conditions to the environment and ecosystem have become increasingly important. Molecular hydrogen (H_2) is a safe gas with the smallest size and lowest density in the universe (Alwazeer et al. 2021). H_2 can be absorbed from the digestive and respiratory systems and quickly enters the circulation system of living beings. H_2 can pass biomembranes and reach the cytosol, mitochondria, and nucleus and pass the blood-brain barrier (Dixon et al. 2013).

H_2 has recently been recognised as a new therapeutic agent (Ostojic 2016). Hydrogen-rich water (HRW) is one of the different H_2 administration methods including hydrogen-rich saline, inhalation, hydrogen-eye drop, and hydrogen bath. HRW showed many beneficial health properties in human and animal models (Aoki et al. 2012, Mizuno et al. 2017, Barancik et al. 2020, Fukai 2020). The present study aimed to evaluate the potential curative role of hydrogen application as a new green agent in the detoxification mechanism towards MP toxicity. With the current study, a data pool will be created to compare the operational feasibility of the processes for the protection of the aquatic ecosystem, especially aquatic life, with other approaches.

2. Materials and methods

2.1. Animal maintenance and treatment procedure

Rainbow trout (*Oncorhynchus mykiss*, n = 240) with an average weight of 75 ± 2.5 g was used as fish material in the study. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All conditions (stocking density, water parameters, etc.) were designed according to the welfare-related assessments, suitable for *O. mykiss* (Uçar et al. 2023). After the acclimation period, these fish were fed with feeds prepared with polyethylene (PE) and polypropylene (PP) plastics obtained from the commercial company for 6 weeks and then subjected to recovery treatment with HRW for 21 days. In the study, 4 groups were formed: one control and three treatments. The fish were randomly distributed to 12 tanks, with 20 fish in each tank. During depuration (21 days), fish were fed with plastic-free feeds twice a day (morning and afternoon). At the end of the trial period, the fish were euthanised by cervical section and samples were collected from the control and treatment groups (Topal et al. 2017).

Preparation of hydrogen-rich water (HRW): The water of the aquarium was bubbled with an oxy-hydrogen gas machine (HB-33 Epoch, Taiwan) using water electrolysis to produce a mixture of hydrogen (70%) and oxygen (30%). The hydrogen concentrations in aquariums and its stability were continuously controlled during the assay period, and its value was determined in preliminary experiments using the ORP electrode (Sensorex, USA).

2.2. Polypropylene (PP) and Polyethylene (PE) microplastic (MPs) preparation and characterisation

Aquatic organisms were exposed to MPs in different size ranges in nature, and especially fiber and fragment microplastic shapes were defined as dominant. In this study, with a realistic approach, MPs in two different polymer structures of different lengths were procured from a local company (Ucar Plastic Chemistry), and fragments were prepared by blending PP and PE plastics in grinding machines. To remove possible contamination from the cut microplastics, they were repeatedly washed with deionised water and soaked in 70% ethanol for 24 hours (Zhao et al. 2021).

PP and PE polymer types were confirmed using an FTIR spectrophotometer. PP and PE samples were placed on the ATR crystal. Spectra were collected in 32 scans with a resolution of 4 cm^{-1} and a wavenumber range of 4000–650 cm^{-1}. Polymer types of PP and PE MPs were taken from the Agilent Micro Lab FTIR software library. In addition, 0.07 g of PP and PE microplastics were mixed with 10 mL of pure water and filtered through a vacuum filter assembly using filters with 1.2 µm apertures and photographed using an Olympus BX43 Stereomicroscope (maximum magnification 40×) equipped with an Olympus DP21 Microscope Digital camera. The size distribution and average sizes of the photographed microplastic particles were calculated by considering the size of 100 particles of both polymer types. During the recovery period, one control group (plastics + normal water) and three treatment groups (PP+PE, PP+PE+HRW, and HRW) were established. One of the treatment groups was treated with the self-cleaning procedure and the other with the HRW treatment. At the end of 21 days of the recovery period, hematological indices and oxidative stress response, DNA damage and apoptosis marker levels in the tissue samples (brain, gill, liver), and neurotoxicity level in the brain tissue were measured in randomly selected fish from each group. In addition, the presence of plastics (PP and PE) was evaluated in muscle, liver, and gill tissues as well as gastro-intestinal samples.

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2.2.1. Quantification of MPs

The determination of MP amount was determined according to the methods of Jovanović et al. (2018), Sayed et al. (2021), and Li et al. (2022), with some modifications. At the end of the trial, 5 fish were euthanised, having been chosen at random from tanks for the four groups: Control, PP + PE (15% Polyproylene + 15% polyethylene) and PP + PE + HRW (15% Polyproylene + 15% polyethylene + HRW), and HRW. To avoid contamination, samples were dissected first from the muscles, then from the liver, and finally from the gills and intestines. All tissue samples were placed into 250 ml glass beakers and treated with 30 ml of 4 M KOH in a shaking water bath at 100 rpm for one hour at 60°C. The samples were then filtered using 1.2 µm mesh filters (Whatman glass microfiber filters) in a vacuum filter assembly. Filter papers were placed in glass Petri dishes and kept at room temperature for 24 hours. MP particles were counted and photographed using an Olympus BX43 Stereomicroscope (maximum magnification 40×) equipped with an Olympus DP21 Microscope Digital camera. Online software (ImageJ) was used to determine the size of each particle individually in the photographs (Jovanović et al. 2018).

2.3. Control of contamination

All Petri dishes and glass beakers were washed at least 3 times with filtered distilled water before use. To protect the samples from possible contamination, the beaker and other materials in which the samples were placed were covered with aluminum foil throughout every stage of the study. Tissue removal and sample preparation were handled in a laminar cabinet, with special care taken to avoid contamination by air pollutants. The surfaces on which the study was carried out were repeatedly and regularly cleaned with a 70% alcohol and distilled water solution. Each researcher wore a cotton apron and latex gloves during the study. Two controls were performed during the trials as follows: control water (distilled water used in solution preparation and material cleaning was kept open during operation) and control air (Petri dish was kept with the lid open during operation) (Nur et al. 2022).

2.4. Determination of hematologic indexes

The hematological parameters; RBC (erythrocyte count), WBC (leukocyte count), Hb (hemoglobin value), Hct (hematocrit ratio), PLT (platelet count), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) values were determined in the blood samples (Alak et al. 2022b). In order to obtain fast and reliable results, the related parameters were taken on the Prokan (PE-6800 model) fully automatic blood count device calibrated according to fish species.

2.5. Determination of oxidative stress response levels

Homogenate preparation: Tissue samples (gill, brain, and liver) taken from fish were homogenised by adding 3 times their weight in buffer solution (KH_2PO_4) (Alak et al. 2022b). After homogenisation, the tissues were centrifuged at 19460 g for 45 min at 4°C. Supernatants were then used for enzyme activity (SOD, CAT, GPx, GR, AChE, and caspase 3) and biochemical (DNA damage, MDA, GSH, ROS) analyses (Alak et al. 2020a, b, c; Atamanalp et al. 2021b; Uçar et al. 2021; Alak et al. 2022b).

Measurement of SOD (superoxide dismutase) activity: The activity was determined spectrophotometrically at 560 nm (Sun et al. 1988). Measurement of CAT (catalase) activity: the method of Aebi (1974) was used. The activity was determined by measuring at 240 nm and was calculated by taking into account the rate of decrease in the amount of absorbance (Atamanalp et al. 2022a). Measurement of GPx (glutathione peroxidase) activity: the activity was calculated by absorbance change at 340 nm (decrease in reading values over 3 min) according to Beutler (1984). GR (Glutathione reductase) activity was measured according to Beutler's method. One enzyme unit is defined as the oxidation of 1 mmol NADPH per min under 25°C and pH 8.0 (Atamanalp et al. 2022a). ROS level in fish tissues was determined by modifying the method of Gupta et al. (2007). The 2',7'-dichlorofluorescein diacetate (DCFDA) kit (ABCAM, DCFDA Cellular ROS Detection Assay Kit, ab113851) was used to obtain the ROS levels in tissues. Tissues were homogenised using Tris-HCl buffer (50 mM, pH = 7.4) at a 1:10 w/v rate inside an ice bath. 100 µl of fish tissue homogenate samples were mixed with 1 ml of the same buffer and 5 µl of 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA). The resulting mixtures were incubated at 37°C for 30 min (Lab. Companion SI-600 incubator shaker, Jelio Tech., Korea). After incubation, measurements were taken by reading the absorbance at Ex 485 nm/Em 525 nm with fluorescent spectrophotometer LS55 (PerkinElmer, USA). The glutathione (GSH) concentration was measured using the dithionitrobenzoic acid recycling method applied by Ellman (1959). In this method, the chromophoric product resulting from the reaction of reactive DTNB

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(5,5'-dithiobis-2-nitrobenzoic acid) and sulfhydryl groups has a molar absorption at 412 nm. 1 ml of the prepared hemolysate was added to the solution prepared with distilled water and containing 1.67 g metaphosphoric acid, 0.2 g Na_2EDTA + 30 g NaCl, and the sample was cleansed of protein. 2.4 ml of Na_2HPO_4 and 0.3 ml of DTNB were added to the supernatants and cleaned by centrifugation (3000 g 10 min^{-1}). The formation of 5-thio-2-nitrobenzoic acid, proportional to the GSH concentration, was monitored at 412 nm and 25°C versus reagent controls (Yonar 2012).

The level of lipid peroxidation was obtained by determining the level of malondialdehyde (MDA), a product of lipid peroxidation (Alak et al. 2021). 200 μl of the prepared homogenate was mixed for a short time with 800 μl of phosphate buffer, 25 μl of a synthetic antioxidant (BHT), and 500 μl of 30% TCA. After incubation at -20°C for 2 hours, the samples were centrifuged (15 min 460 g). 1 ml of the supernatant was transferred to another Eppendorf tube, and 75 μl of EDTA-Na_2H_2O and 250 μl of TBA were added. After a gentle vortexing process, the mixture was kept in a water bath (90°C) for 15 min. MDA levels were calculated by reading the absorbance of samples at 532 nm after have been cooled to room temperature (Alak et al. 2017, 2018, 2019a, b, c, d; Atamanalp et al. 2021b).

Measurement of DNA damage (8-OHdG): an ELISA kit was used to determine 8-Hydroxy-2-Deoxyguanosine (8-OHdG) levels. The prepared standards were added to the Microelisa Strip Plate at 50 μl, then the manufacturer's protocol was applied and the measurement was made in the ELISA (Plate Reader) device by reading the absorbance at 450 nm. The 8-OHdG level in the samples was calculated according to the formula created by drawing the standard graph suitable for the optical densities and concentrations of the 8-OHdG standards (Alak et al. 2018). CASP3 ELISA Kit was used for the measurement of apoptosis parameters (Caspase-3). The prepared standards were added to the Microelisa Strip Plate at 50 μl, and the measurement was made in the ELISA (Plate Reader) device by reading the absorbance at 450 nm according to the manufacturer's protocol. The Caspase-3 level in the samples was calculated according to the formula created by drawing the standard graph suitable for the optical densities and concentrations of the Caspase-3 standards (Alak et al. 2018).

2.6. Determination of the neurotoxicity level in brain tissues

Acetylcholinesterase (AChE) activity of homogenates used in enzyme activities was determined by reading the absorbance at 412 nm according to Ellman et al. (1961) (Ucar et al. 2022).

Statistical analysis of data: The analysis was performed with SPSS statistical analysis package software (version 24.0) and the statistical significance level was set at 0.05. Since the groups showed normal distribution, one-way ANOVA from parametric tests and Duncan from Post Hoc tests were used to compare values. Data are given as mean ± SD. Microplastic size distribution and FTIR data graphs were prepared using the OriginLab software program.
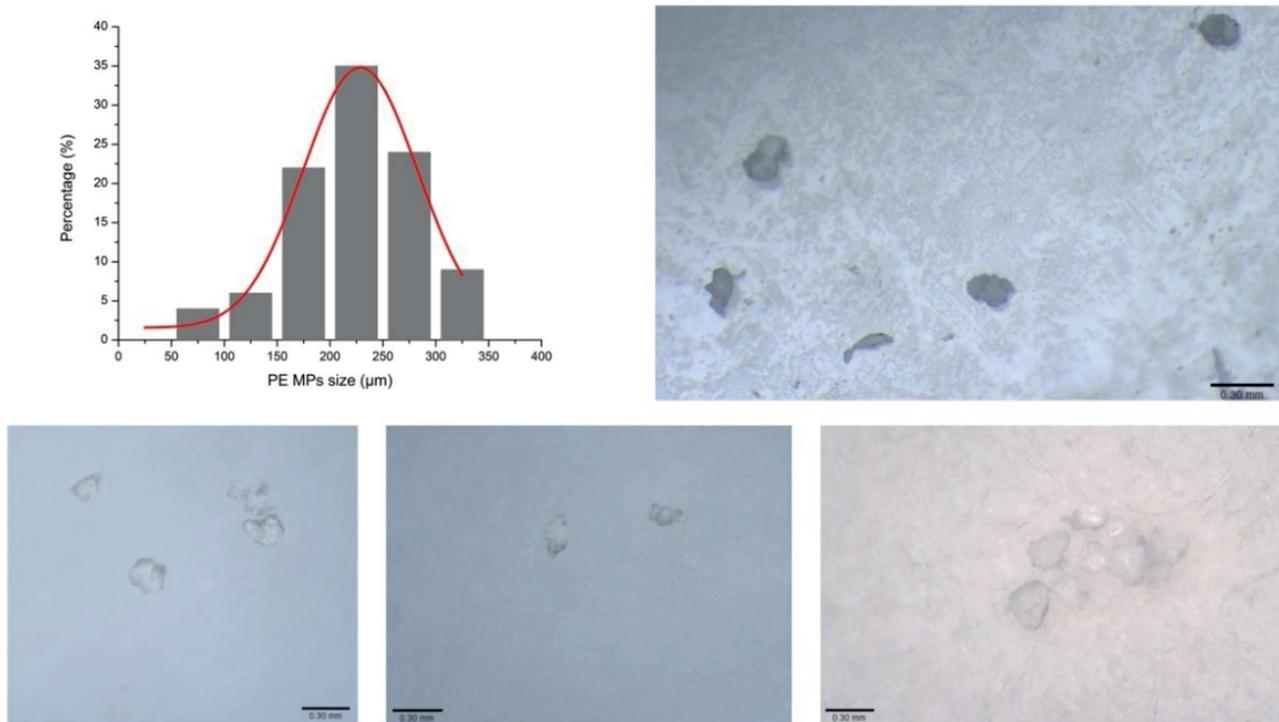
3. Results and discussion

Although depuration has been considered a promising detoxification strategy for organisms exposed to any chemical to combat exogenous stressors, a specific mechanism for the effect of depuration on the MP toxicity has not been identified (Iheanacho and Odo 2020, Sun et al. 2023). Therefore, it should be supported by studies involving other aquatic organisms, biomarkers and MP derivatives. In the literature review, no scientific article was found on rainbow trout in which the processes up to DNA damage and apoptosis were evaluated with the multi-biomarker approach, as well as important biomarkers and hematological indices in detoxification of plastic pollution, detoxification and neurotoxicity in gill, liver, muscle and brain tissues. Therefore, this study is the first pilot study to evaluate the ROS/GSH/MDA pathway with all parameters.

### 3.1. Characterisation of PP and PE MPs

The microscope images of the MPs used in the dietary exposure are presented in Figs. 1 and 2. The average size ± standard deviation (SD) of the particles was 142 ± 83 μm (min.-max: 35-357 μm) for PP, and 220 ± 59 μm (min.-max: 52-344 μm) for PE.

The characteristic wave numbers of two types of MPs (PP and PE) were analysed using FTIR to determine the toxic effects of MPs and the curative effects of HRW application in rainbow trout. The obtained spectra are presented in Figures 3A-B. A few peak spectra were determined, of which the significant increases in wave numbers of 717 cm^{-1}, 1465 cm^{-1}, and 2914 cm^{-1} can be estimated to be due to PE as indicated in previous studies (Gulmine et al. 2002, Fan et al. 2021). Again, the peaks around 1464 cm^{-1} and 2915^{-1} in our results may be due to PP microplastic (Fan et al. 2021, Morgado et al. 2021).

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Figure 1

Microscope images of polyethylene microplastics used in dietary exposure of *O. mykiss* and size distribution of polyethylene microplastics (PE MPs) analysed through particle counting
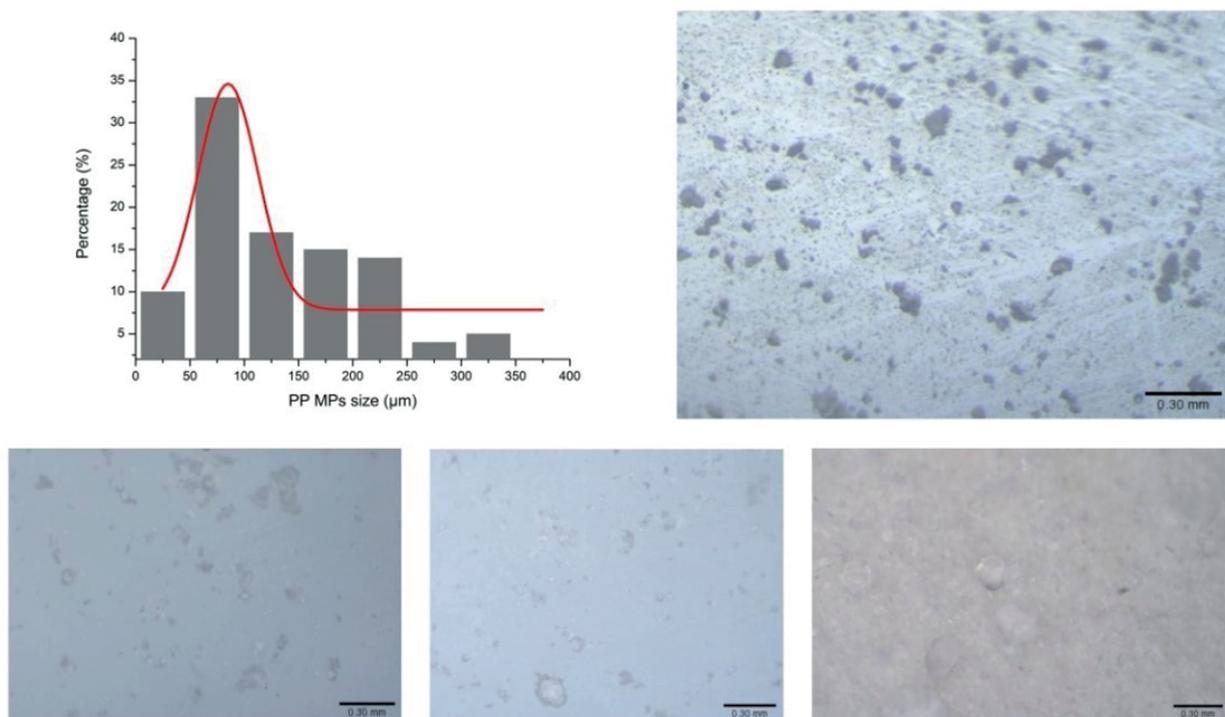
Figure 2

Microscope images of polypropylene microplastics used in dietary exposure of *O. mykiss* and size distribution of polypropylene microplastics (PP MPs) analysed through particle counting
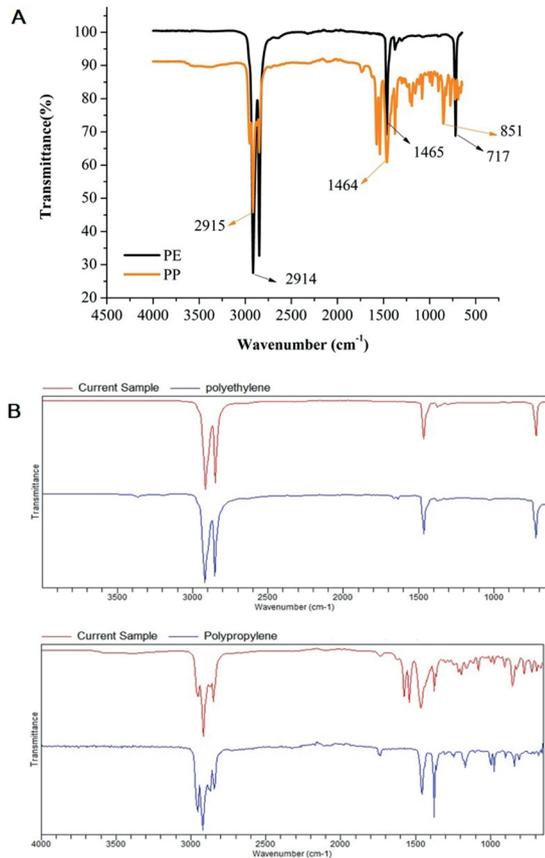
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Figure 3

(A-B) FTIR spectra of microplastics used in dietary exposure of *O. mykiss*

3.2. Amount of MPs in fish tissues

The amount of PP and PE microplastics in different tissues of fish after having been exposed to PP and PE microplastics included in the diet is given in Table 2. The highest MP amount was determined as 3.8 ± 1.3 and 3.8 ± 0.8 MPs/individual in the gills and gastrointestinal tract in the PP+PE application group, respectively. The whitish/gray-coloured and fragmented PP and PE microplastics observed especially in the gastrointestinal tract and gills are shown in Figure 4.

3.3. Blood indexes

Table 3 shows the hematological indices (RBC, Hb, Hct, MCHC, MCV, Plt, WBC count) of the different fish groups. A significant decrease in these indices could be observed for the PP+PE group compared to the other groups, while MCH levels increased ($p < 0.05$) (Table 3). These alterations have been evaluated as a defense mechanism response to MP toxicity (Hamed et al. 2019).

In particular, MPs can affect blood physiology by acting as a chemical or a physical effect in the blood. Changes in various hematological features after MP exposure have been confirmed by several studies where hematological parameters were used as potentially reliable indicators for assessing toxicity in fish. After MP enters the fish circulatory system it can cause metabolic disorders and fatal reactions such

Table 1

Total length, total weight, tissue and organ weights of the fish used in the experimental groups

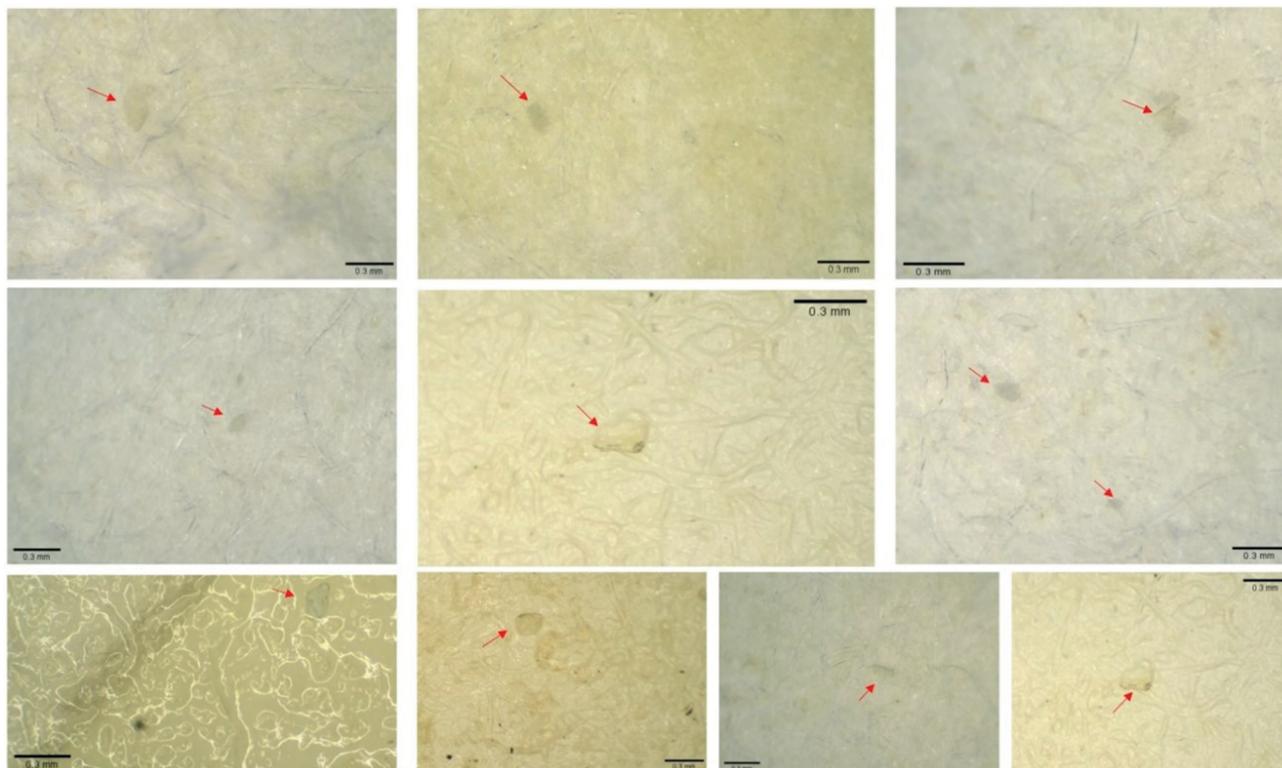| Groups (n = 15)	Total weight (g)	Total length (cm)	Liver (g)	Gills (g)	Gastrointestinal system (g)	Muscle (g)
Control	207.5 ± 54.6	24.8 ± 1.0	3.5 ± 0.5	5.1 ± 0.8	18.2 ± 0.6	~5
HRW	267.2 ± 54.6	27.2 ± 1.3	3.8 ± 0.3	6.2 ± 0.7	20.7 ± 4.0	~5
PP+PE	235.0 ± 86.8	26.6 ± 2.6	3.6 ± 0.6	4.9 ± 1.0	17.6 ± 3.3	~5
PP+PE+HRW	159.5 ± 15.2	23.0 ± 0.6	2.5 ± 0.4	3.7 ± 0.3	16.2 ± 2.5	~5

Data are represented as means ± SD

Table 2

Amount, dimensions, and polymer type of PP and PE MPs in muscle, liver, gill, and gastrointestinal tract tissues of *O. mykiss* after dietary exposure

Fish tissues (n=5)	Treatment groups											
	Control			HRW			PP+PE			PP+PE+HRW		
	Average MPs	Size range (µm)	Microplastic type (%)	Average MPs	Size range (µm)	Microplastic type (%)	Average MPs	Size range (µm)	Microplastic type (%)	Average MPs	Size range (µm)	Microplastic type (%)
Muscle (MPs g⁻¹)	0	-	-	0	-	-	0.2 ± 0.4	0-40	100% PP	0	-	-
Liver (MPs ind.⁻¹)	0	-	-	0	-	-	0.4 ± 0.5	41-51	100%PP	0	-	-
Gills (MPs ind.⁻¹)	0.8 ± 0.4	129-229	75% PE, 25% PP	0.6 ± 0.5	111-255	66.7% PP, 33.3% PE	3.8 ± 1.3	44-255	52.6% PE, 47.4% PP	3.2 ± 0.8	66-200	56.3% PE, 43.8% PP
Gastrointestinal system (MPs ind.⁻¹)	0.4 ± 0.5	92-107	%50 PE, %50 PP	0.8 ± 0.4	95-274	75% PP, 25% PE	3.8 ± 0.8	67-325	63.2% PE, 36.8% PP	1.2 ± 0.8	125-279	66.7% PE, 33.3 %PP

Values are presented as mean MP particle count ± standard deviation

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Figure 4

Microscopic images of PE and PP MPs in the tissues of dieted fish

Table 3

Hematological index alterations

Hematological index	Treatment groups	Values (Mean ± SD)
Hemoglobin (g dL)	Control	9.3 ± 0.3[b]
	PP+PE	8.5 ± 0.3[b]
	PP+PE+HRW	11.9 ± 0.8[a]
	HRW	9.0 ± 0.02[b]
RBC (10^6 mm^{-3})	Control	1.3 ± 0.1[b]
	PP+PE	1.1 ± 0.04[c]
	PP+PE+HRW	1.8 ± 0.08[a]
	HRW	1.0 ± 0.1[d]
WBC (10^4 mm^{-3})	Control	13.9 ± 1.7[b]
	PP+PE	9.8 ± 0.9[c]
	PP+PE+HRW	16.7 ± 0.9[a]
	HRW	9.6 ± 0.6[c]
PLT (10^4 mm^{-3})	Control	445.7 ± 26.1[a]
	PP+PE	357.7 ± 13.7[ab]
	PP+PE+HRW	545.3 ± 22.7[a]
	HRW	248.0 ± 98.8[b]
HCT (%)	Control	34.1 ± 1.3[b]
	PP+PE	29.0 ± 1.7[c]
	PP+PE+HRW	37.9 ± 0.4[a]
	HRW	34.3 ± 1.15[b]
MCV (μm^{-3})	Control	157.7 ± 4.3[b]
	PP+PE	155.3 ± 6.2[b]
	PP+PE+HRW	157.5 ± 6.4[b]
	HRW	284.3 ± 14.2[a]
MCH (pg)	Control	54.1 ± 2.1[b]
	PP+PE	52.7 ± 0.6[b]
	PP+PE+HRW	52.5 ± 2.1[b]
	HRW	74.9 ± 4.3[a]
MCHC (g 100 ml^{-1})	Control	27.3 ± 1.5[bc]
	PP+PE	29.3 ± 1.7[ab]
	PP+PE+HRW	31.3 ± 1.9[a]
	HRW	26.23 ± 0.4[c]

Lowercase superscripts (a, b, c) indicate significant differences among the same colon within each experimental treatment group; *$p < 0.05$

as immune responses, endocrine disorders, oxidative stress, and gene expression (Alak et al. 2022). The toxic substance exposures negatively change the blood's electrolyte balance and oxygen-carrying capacity and causes a reduction in cell size due to RBC exosmosis (Alak et al. 2022b, Uçar et al. 2022). In addition, MPs can be internalised in the blood affecting hematological properties by damaging RBCs through mechanical, osmotic, and oxidative stress (Kim et al. 2021). MP exposure also decreases the hematological properties of fish such as RBC, Ht, and Hb, which is believed to be due to the decrease in Hb content and synthesis rate caused by stress and consequently tissue oxygenation disorder (Kim et al. 2021).

Anemia may develop in association with suppression of erythropoiesis and/or erythrocyte lysis, usually due to hematopoietic tissue damage resulting from increased cell wall mechanical fragility (Ucar et al. 2022). The fluctuations (increase and decrease) in erythrocyte and hematocrit content may be part of the defensive response to stress, especially environmental or toxic substance-induced stress. However, the purification experiment showed that homeostasis in fish returned to normal physiological levels after erythrocyte counts were reduced.

The increase in Hb content in the recovery group

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(PP+PE+HRW) suggested that homeostasis was restored. Mitochondria and other redox-active heme prosthetic groups may be important targets for molecular hydrogen (Kura et al. 2022). The decreases in PP+PE values at which self-purification was attempted can be attributed to possible disruption of the hematopoietic process caused by the MPs of these materials (Iheanacho and Odo 2020). H_2 would preferentially scavenge •OH before other ROS under Hb catalysis possibly due to its higher oxidisability and smaller size. The small size is considered suitable for ROS and H_2 to enter the porous channel of the Hb protein (Hancock et al. 2022). This can explain the phenomenon that H_2 selectively scavenges highly cytotoxic •OH rather than other ROS (Jin et al. 2022).

WBCs play an immunological role in the organism's system for defense purposes. In the obtained findings, PP+PE regulated the WBC production's inhibition in the self-healing group, leading to a phenomenon known as leukopenia (Jeney 2017).

Erythrocyte indices (MCV, MCH, and MCHC) are important biomarkers for providing useful information about hemoglobin content and the size of erythrocytes (Alak et al. 2022b), therefore they are used to diagnose the anemic state in animals (Parlak et al. 2021, Atamanalp et al. 2022b). Changes in erythrocyte parameters (elevation and/or decrease) outside the normal physiological range in most cases indicate microcytic or macrocytic anemic conditions. In this study, the results observed in the PP+PE group showing a reduction in MCH and MCV contents are similar to the symptoms of microcytic anemia (Iheanacho and Odo 2020).

3.4. Oxidative stress response

Figure 5 shows the levels of CAT, SOD, GPx, GR, and antioxidant enzyme activities of HRW, PP+PE, and PP+PE+HRW groups.

The obtained results showed that CAT activity in the brain (↓46% PP+PE, ↓45% PP+PE+HRW, and ↓31% HRW), gill (↓54.3% PP+PE, ↓54.1% PP+PE+HRW, and ↓35% HRW), and liver (↓41% PP+PE, ↓40% PP+PE+HRW, and ↓25% HRW) tissues were significant ($p < 0.05$).

SOD activity in the brain (↓64% PP+PE, ↓63% PP+PE+HRW, and ↓45% HRW), gill (↓18% PP+PE, ↓17%
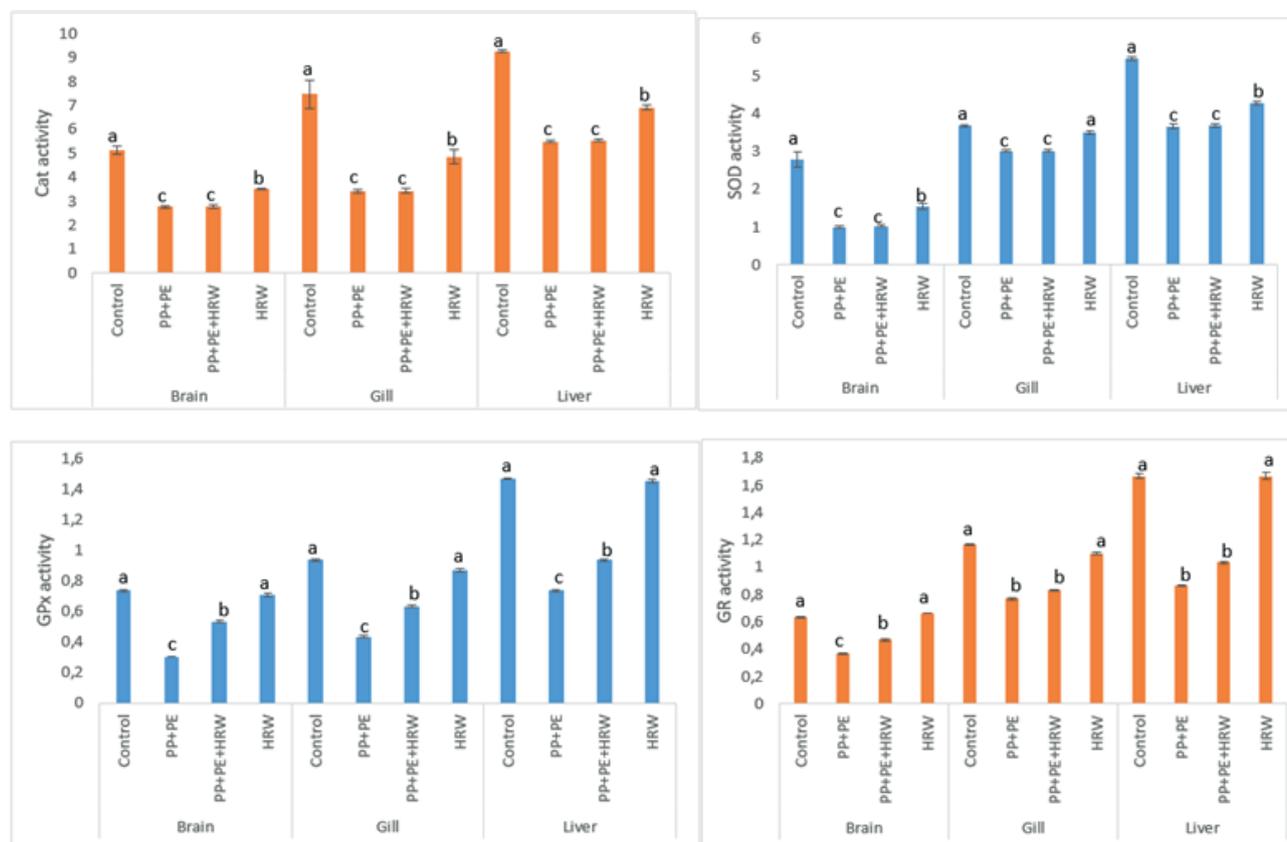

Figure 5

Antioxidant enzyme activity of fish tissues after the application of different recovery treatments. Lowercase superscripts (a, b, c) indicate significant differences among the same colon within each experimental treatment group, *$p < 0.05$

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PP+PE+HRW, and ↓4% HRW), and liver (↓33% PP+PE, ↓32% PP+PE+HRW, and ↓21% HRW) tissues were significant ($p < 0.05$).

GPx activity in the brain (↓58% PP+PE, ↓26% PP+PE+HRW, and ↓3% HRW), gill (↓53% PP+PE, ↓31% PP+PE+HRW, and ↓6% HRW) and liver (↓49% PP+PE, ↓36% PP+PE+HRW, and ↓0.6% HRW) tissues were significant ($p < 0.05$).

The levels of GR activity in the brain (↓41% PP+PE, ↓25% PP+PE+HRW, and ↑5%HRW), gill (↓34% PP+PE, ↓28% PP+PE+HRW, and ↓5% HRW), and liver (↓47% PP+PE, ↓37% PP+PE+HRW, and ↑0.4% HRW) tissues were significant ($p < 0.05$).

Excessive ROS production and reduction of antioxidant defense systems have important roles in the toxic damage developments in tissues. SOD converts the superoxide anion radical to H_2O_2, which is detoxified to H_2O by GSH-Px (glutathione peroxidase) or CAT. Accordingly, their deficiency can cause excessive oxidative stress. In this study, we found that HRW treatment significantly improved CAT and SOD activities of the brain, liver, and gill origin exposed

to microplastic damage. Many reports revealed the up-regulation of SOD in the presence of molecular hydrogen in animals (Nogueira et al. 2018; Qiu et al. 2019), plants (Zulfiqar et al. 2021) and humans (Ohta 2014).

Carnovali et al. (2021) reported that HRW levels below 15% were safe for zebrafish embryos and did not affect growth rate or vitality, but toxicity in zebrafish embryos was found at levels starting from 25% HRW. However, in our study, we used an H_2/O_2 mixture (2/1, v/v), which provides an additional advantage over the previous study because dissolved oxygen is necessary for the respiration process.

Levels of GSH, MDA, and ROS in tissues compared to those of control were, respectively, follows:

In the PP+PE group, brain: ↓54%, ↑76%, ↑73.3%, gill: ↓53%, ↑101.3, ↑50%, and liver: ↓45%, ↑42%, ↑53% ($p < 0.05$). In the PP+PE + HRW group, the brain: ↓38%, ↑29%, ↑47%, the gill: ↓46%, ↑41%, ↑20%, and the liver: ↓36%, ↑21%, ↑43%, and for the HRW group, the brain: ↓24%, ↑9%, ↑3%, gill: ↓34%, ↑7%, ↑13%, and liver: ↓25%, ↑5%, ↑10% ($p < 0.05$) (Figure 6).
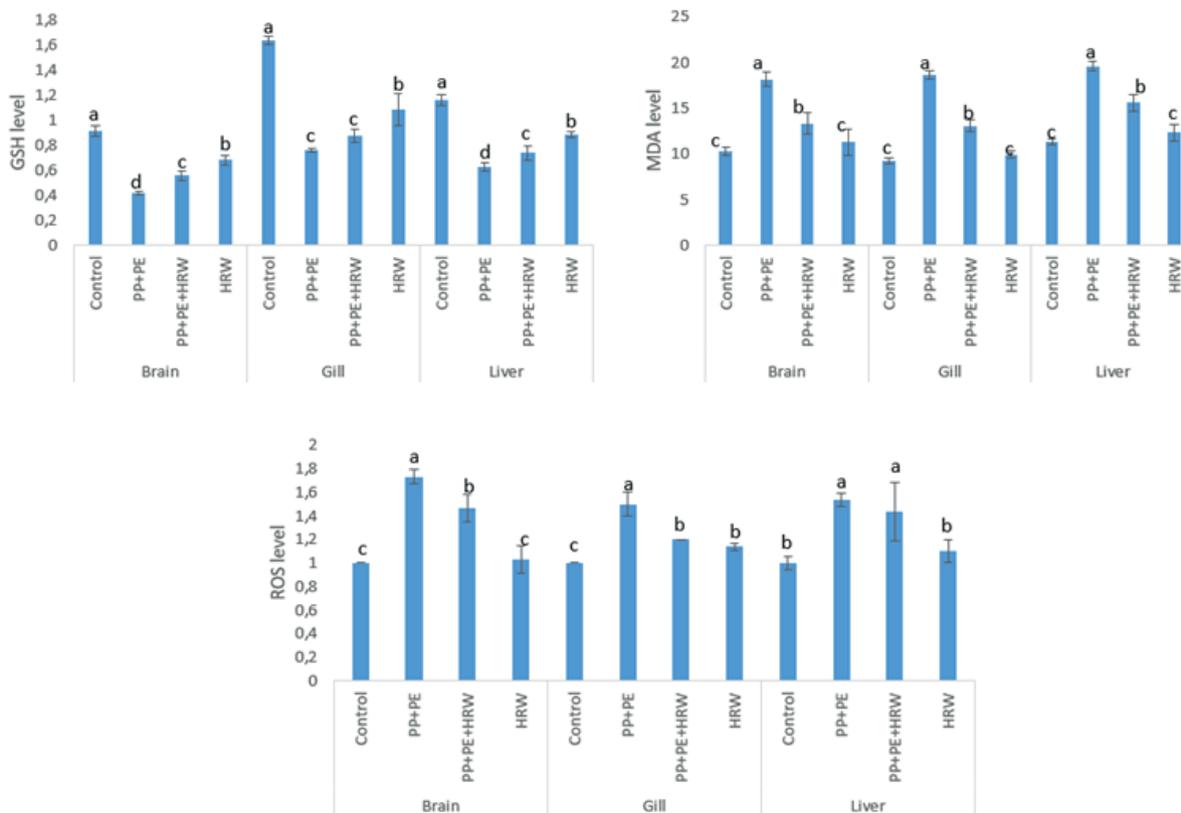

Figure 6

Antioxidant and oxidation status of fish tissues after the application of different recovery treatments. Lowercase superscripts (a, b, c) indicate significant differences among the same colon within each experimental treatment group, *$p < 0.05$

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It has been reported that molecular hydrogen has antioxidant effects. Inhibitions in enzyme activities were determined in both treatment groups to be different than control. Also, these enzyme (SOD, CAT, GPx, and GR) activities were increased in the PE+PP+HRW group with the effect of HRW. Our findings are consistent with those of Kura et al. (2022), who found that HRW application exhibited a significant decrease in MDA and ROS levels (markers of oxidative stress) in MP toxicity (Kura et al. 2022). Regarding the relationship between molecular hydrogen and oxidative stress, it has been reported that molecular hydrogen selectively scavenges harmful ROS such as the hydroxyl radical and peroxynitrite Nucleic acid oxidation and lipid peroxidation are thereby reduced, and cells/tissues are protected from oxidant stress and apoptotic damage (Ohsawa et al. 2007). In addition, it has been stated that molecular hydrogen increases the gene expression of antioxidant enzymes (SOD and CAT) and reduces oxidative stress (Saitoh et al. 2022). Moreover, as a new antioxidant, hydrogen gas has been used in animal models to protect against different injuries (cerebral, hepatic and myocardial, I/R injury, and neonatal cerebral hypoxia-ischemia) and to improve glucose and lipid metabolism (Wang et al. 2011, Kokturk et al. 2022).

This shows the potential therapeutic effect of HRW. These studies suggest that molecular hydrogen found in HRW not only directly captures the most reactive radicals of ROS but also activates the cellular detoxification system by stimulating the specific antioxidant enzyme gene's expression. Lipid peroxidation produced by reactive oxygen species is one of the most critical mechanisms for cellular damage and death. Membrane-associated polyunsaturated fatty acids readily bind to the hydroxyl radical in a process that leads to the peroxidation of lipids, which can impair membrane fluidity and cell division, resulting in cell lysis (Wang et al. 2011). Although HRW has been reported to have antioxidant and anti-inflammatory effects, few studies have determined its effects on the molecular mechanisms by which it may protect mitochondrial function and regulate endogenous antioxidant enzymes (Lin et al. 2015). The mechanism underlying the mechanistic mode of action for molecular hydrogen may be related to the hormesis model (Lin et al. 2015, Kura et al. 2022). Molecular hydrogen can scavenge free radicals, especially hydroxyl radicals ($\cdot$OH) and peroxynitrite anion (ONOO-), which are the strongest oxidants and react indiscriminately with nucleic acids, lipids, and proteins, resulting in DNA fragments, lipid peroxidation, and protein inactivation (Wang et al. 2011, Wang et al. 2022). However, it is likely that molecular hydrogen cannot scavenge most amounts/types of ROS, but it can activate endogenous antioxidant abilities rather than directly scavenge hydroxyl radicals (Takade and Miwa 2022).

As a biomarker of oxidative DNA damage, 8-OHdG in tissue or body fluid is known as a sensitive indicator. 8-hydroxydeoxyguanosine, an important product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA (Alak et al. 2017). When our findings were evaluated in terms of 8-OHdG levels, it was found that PP+PE and PP+PE+HRW groups compared to the control group showed induction levels, respectively, as follows: ↑71% and ↑34% in brain tissue, ↑138% and ↑72% in gill tissue, and ↑90%, and ↑68%, in liver tissue ($p < 0.05$). However, for HRW treatment, these DNA damage levels were in the brain: ↑17%; gill ↑29%, and liver ↑13% tissues ($p < 0.05$) (Figure 7). HRW treatment showed an inhibiting effect of molecular hydrogen found in HRW on oxidative stress by reducing MDA and 8-OHdG levels in the brain, liver, and blood tissues after having been exposed to PP+PE.

Similarly, the values of caspase 3 activity in different fish groups compared to control were as follows: for the PP+PE self-healing group, in the brain ↑103%, gill ↑104%, and liver ↑82.0; and for the PP+PE+HRW group, in the brain ↑70%, gill ↑65%, and liver ↑76% ($p < 0.05$). For HRW treatment, this enzyme activity was found, compared to control, in the brain at ↑25%, gill at ↑16%, and liver at ↑31% ($p < 0.05$) (Figure 7).

The results show that HRW attenuated the increase in 8-OHdG and caspase 3 levels caused by MP toxicity in all tissues. Our observations are in line with recent studies (Lin et al. 2015) showing molecular hydrogen protective effect against neuronal apoptosis by
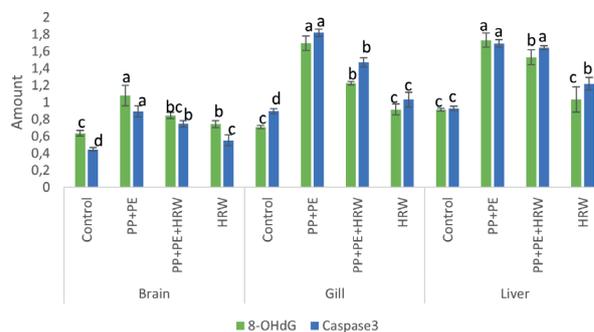

Figure 7

DNA damage level and apoptosis activity of fish tissues after the application of different recovery treatments. Lowercase superscripts (a, b, c) indicate significant differences among the same colon within each experimental treatment group, *$p < 0.05$

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decreasing mitochondrial dysfunction by suppressing ROS-activated caspase signaling (Lin et al. 2015).

The increase in some oxidative biomarkers found in the HRW group compared to the control may be related to the extra amounts of oxygen in water provided by the oxy-hydrogen gas machine allowing to increase the oxidative reactions in cells/tissues. Although dissolved oxygen is important for the respiration of fish, extra amounts may exhibit negative effects. A further comparative study using both oxy-hydrogen gas and pure hydrogen gas should be performed to distinguish the effect of only hydrogen-rich water without an extra oxygen addition.

Regarding the AChE activity that is considered a neurotoxicity marker, inhibitions were detected at the levels of ↓51%, ↓35.8%, and ↓13.86% in the PP+PE, PP+PE+HRW, and HRW groups, respectively, compared to the control group ($p < 0.05$) (Figure 8). Our results of PP+PE+HRW show that HRW effectively scavenges ROS and thus may contribute to AChE activation according to PP+PE. It is thought that molecular hydrogen has an important neuroprotective property that is mediated by its effects on oxidative stress (Lin et al. 2015). Our findings reveal a potential curative effect of HRW as a neuroprotective agent against PP+PE-induced oxidative stress damage.
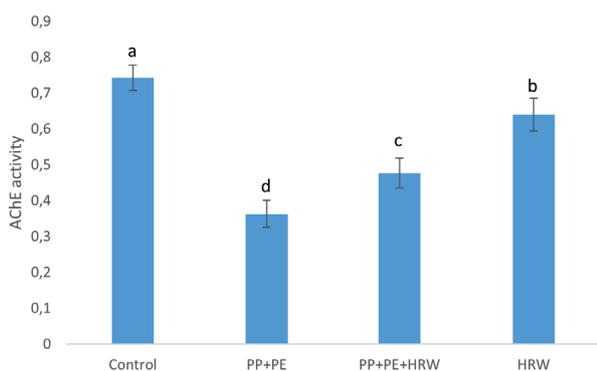

Figure 8

AChE activity after the application of different recovery treatments. Lowercase superscripts (a, b, c) indicate significant differences among the same colon within each experimental treatment group, *$p < 0.05$

4. Conclusion

In the present study, the curative effects of HRW prepared by oxy-hydrogen gas machine on the MP toxicity in *O. mykiss'* brain, liver, and blood tissue and its various biomarkers were evaluated. Beneficial trends of HRW treatment towards mitigation of PP+PE

toxicity were observed. Importantly, we demonstrated in our study that HRW application can alleviate the PP+PE toxicity-induced increases in MDA and ROS levels, DNA damage and apoptosis levels, and promote antioxidant enzyme expression. However, additional and/or longer-term studies are needed to confirm the amendatory effect of molecular hydrogen found in other studies. The present study shows for the first time that oxy-hydrogen gas produced by a water electrolysis machine can be suggested as a cost-effectively and eco-friendly method for the protection of fish from the oxidative damage produced by the ingestion of microplastics.

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