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Research Article

Effects of combined polystyrene microplastics and chlorpyrifos pesticide on enzymatic activities of different tissues in rainbow trout (*Oncorhynchus mykiss*)

Hanachi P.[1]*; Malaki M.[1]; Karbalaei S.[2]

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Abstract

Microplastics (MPs), mainly smaller than 5 mm, are vectors for various chemical compounds in the environment, such as pesticides and pollutants. Rainbow trout (*Oncorhynchus mykiss*) were exposed to two concentrations of chlorpyrifos (CPF; 2 and 6 μg/L), polystyrene microplastics (PS-MPs; 30 and 300 μg/L), and their combination. Significant changes were observed in the activities of catalase (CAT) and glutathione peroxidase (GPx) in mixed MPs and pesticide treatments. Microplastics increased the bioavailability of pesticides in kidney tissue samples for glutathione peroxidase (GPx) and catalase (CAT) enzymes. The activity of glutathione S-transferase (GST) in the liver showed a significant (p<0.05) increase between the treatments of 30 and 300 μg/L MPs compared to the control group. However, for GPx and CAT enzymes in the kidney showed increased activity compared to MPs and pesticide treatments. In conclusion, a mixed concentration of MPs and CPF showed significant effects on CAT, GPx, and GST activities in different tissue samples of *O. mykiss*.

Keywords: Antioxidative pathway, Chlorpyrifos, Antioxidant enzymes, Microplastic, Polystyrene

1-Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
2- Research and Development Division, Arian Saeed Industrial Group, Tehran, Iran
* Corresponding author's E-mail: p.hanachi@alzahra.ac.ir

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Introduction

Microplastics (MPs) are plastic particles smaller than 5 mm (Karbalaei *et al.*, 2018), which are widely found in aquatic environments. In terms of source, these small particles can be divided into two types, primary and secondary (Karami *et al*., 2016; Auta *et al*., 2017). Primary MPs are produced in various industries, such as the cosmetics industry in which MPs are used in the manufacture such as toothpaste and shampoos. Secondary MPs are formed by the physical, chemical, or biological decomposition of plastics released into the environment (Prokić *et al*., 2019). These particles enter the aquatic environment through various ways such as industrial and domestic wastewater. Activated sludge from wastewater treatment is a source of MPs pollution which introduces the largest number of MPs into the aquatic environments (Karbalaei *et al.*, 2018; Hanachi *et al*., 2019). Previous studies showed adverse effects of MPs on living organisms, especially aquatic animals (Khoshnamvand *et al.*, 2021*;* Karbalaei *et al.*, 2018). These particles can accidentally enter the digestive tract of organisms through ingestion and block the passage of food (Garrido *et al*., 2019). MP can also enter the body of living organisms through respiration and skin absorption (Prokić *et al*., 2019). MPs are able to become carriers of hydrophobic pollutants such as heavy metals and pesticides (Kavitha and Venkateswara 2008*;* Giacomo *et al.*, 2015; Chen *et al*., 2017; Oliveira *et al*., 2018). MPs are mainly derived from terrestrial sources and can be transported long distances due to being light-weight, durable and buoyant (Karbalaei *et al*., 2018).

The ingestion of MP by humans through the consumption of microplastic-polluted seafood is associated with potential risks to human health (Wang *et al*., 2019). Various enzymatic and non-enzymatic factors maintain the balance of removal and production of free radicals such as H_2O_2 (source of oxygen-derived free radicals), $O_2^{\bullet}$, $OH^{\bullet}$, and $NO^{\bullet}$ in the antioxidant pathway present in all living organisms. Oxygenation of cell membrane lipids may also occur as a result of reactive oxygen species. This pathway inhibits lipid oxidation and may damage by affecting antioxidant enzymes activity (*e.g.* glutathione S-transferase) and non-enzymatic (e.g. glutathione) (Ho *et al*., 2018; Hanachi *et al*., 2021).

Chlorpyrifos (CPF) (O, O-diethyl O-3,5,6-Trichloro-2-pyridylphosphorothioate) toxin is released into aquatic environments through agricultural or domestic wastewater and can bioaccumulate in aquatic organisms, especially fish. In addition, CPF is transmitted through the food chain to the human body raising health concerns (Hanachi *et al*., 2007; Hanachi *et al*., 2020). Therefore, this study aimed to investigate the effect of CPF and polystyrene microplastics (PS-MPs) individually and in combination on the activities of catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) in the liver, muscle, and kidney of rainbow trout (*Oncorhynchus mykiss*).

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Materials and methods

Microplastic preparation

Polystyrene was purchased from Takhtjamshid petrochemical Co, Iran. Microplastics were crushed by Ultra Centrifugal Mill ZM 200 (Germany) through a 0.5 mm sieve. The morphology of gold-coated MPs was analyzed in a previous study by scanning electron microscopy (SEM; VEGA3 TESCAN; Czech Republic). The particle size distributions of MPs were calculated by Image J software. The size was in the range of 21.89 to 466.7 μm, with 60% of the particles sized below 100 μm, 34% between 100-250 μm, 4% between 250-400 μm, and 2% greater than 400 μm. The model MPs were confirmed through Fourier transform infrared spectroscopy (FTIR, Bruker tensor 27, Germany) with irregularly shaped virgin polystyrene fragments. Infrared (IR) spectra of PS-MPs were shown in our recent study (Hanachi *et al*., 2021).

Fish samples

100 healthy *O. mykiss* were purchased from a private fish farm and transferred to the Aquatic Laboratory after three weeks of acclimatization in dechlorinated aerated water (temperature: 16-17˚C) in a 200 L fiberglass tank. The fish were fed with a pellet (containing 42% protein and 15% lipid) until they were used in the experiment and no mortality was observed during acclimatization. This study was carried out when the fish were in the juvenile development stage, because changes at the molecular levels in adult fish may trigger a series of physiological responses (Amiri *et al*., 2018; Hanachi *et al*., 2020).

Exposure of fish to MPs and CPF

Juvenile fish with a mean total weight of 25.1±8.1 g and mean total length of 9.2±2.2 cm (mean±SD) were randomly distributed into fiberglass aquaria (200 L) filled with dechlorinated aerated water (5 fish per aquarium) and acclimatized for one week before the experiment. The experiment was conducted in three replicates. The exposure was conducted based on OECD method (OECD, 1992) with some changes by Karami *et al*. (2016). The pollutant model was CPF (purity 99.8%), purchased by Gharda Chemicals Ltd from India. In this study, CPF concentration was below the reported median lethal concentration (LC50) for *O. mykiss*, which was 9 μg/L. (U.S. Environmental Protection Agency, 1996). The standard stock of CPF (5 mg/mL in ethanol) was used to prepare different CPF concentrations. The concentrations of MPs (30 and 300 μg/L) used in this study were within the range that was reported in earlier research in aquatic environments. Microplastic fragments (0.1 g) were added to 1 L of Milli-Q water in a bottle. Then an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) was used for 20 min to homogenize suspension and finally suspended MPs were kept in a dark place at 4˚C to avoid microbial growth. A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 μg/L)

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with MPs (30 and 300 µg/L) in a sterile glass tube, then the tubes were incubated for 28 h in an orbital shaker (Amiri *et al*., 2018; Hanachi *et al.*, 2020). The experimental treatments were negative control (without MPs, CPF, or solvent), solvent control (included ethanol <0.01%); PS-MPs treatments (30 and 300 µg/L), CPF treatments (2 and 6 µg/L), and CPF-loaded MPs treatments (2 µg/L CPF +30 µg/L MPs, 6 µg/L CPF+30 µg/L MPs, 2 µg/L CPF +300 µg/L MPs, and 6 µg/L CPF+300 µg/L MPs). Two air stones up and down parts of each aquarium were aerated to reduce the aggregation of PS-MPs. The water quality parameters were as follows: temperature 17.1±1.2˚C, dissolved oxygen 6.6±0.3 mg/L, pH 7.4±0.2, alkalinity 43.7±4.07 mg $CaCO_3$, and hardness 167.2±6.3 mg $CaCO_3$. The fish were fed two times at 2% of body weight. Theaquarium water was replaced with dechlorinated aerated water every 24 h, and then spiked with CPF/MPs concentrations. No mortality was recorded throughout exposure time. The measured CPF concentrations in PS-MPs (µg/mg) and CPF concentrations in water (µg/L) and their results have been explained in our previous study (Karbalaei *et al*., 2021). The fish were euthanized with an overdose of clove oil after 96 h of experimental exposure, washed two times with dechlorinated tap water, and the tissues were removed and kept at -30˚C.

CAT enzyme assay

Tissue samples (100 mg) were homogenized with 1 mL of lysis buffer. The resulting mixture was centrifuged at 4°C for 8 min at 8000 ×g the supernatant was kept to measure enzyme activity. In 2cc microtubes, 50 µL Assay buffer, 50 µL of solution 1 of assay kit (methanol), and 10 µL of R2 solution (hydrogen peroxide) were mixed and finally, 100 µL samples or different concentrations of standard solutions were added. The microtubes were gently shaken for 20 minutes at a temperature below 20°C. Then 100 µL of solution 4 (chromogen) was added to the test mixture, and after mixing, 40 µL of solution 3 (potassium hydroxide) was added to stop the reaction. The microtubes were incubated at room temperature for 10 minutes after 10 minutes shaking below 20°C. Finally, 50 µL of solution 5 (potassium periodate) was added to oxidize the compound resulting from the reaction of formaldehyde and chromogen and change its purple color. The microtubes were centrifuged at 4000 ×g at 4°C for 5 minutes and the supernatant was separated. 200 µL of each solution was added to the microplate wells and the adsorption was read at 550 nm (by using plate reader Epoch, USA) in triplicates. The experiment was conducted in triplicate. Finally, the activity of the enzyme was calculated (Hanachi *et al*., 2020).

GPX Enzyme Assay

100 mg was weighed from the sample tissue and homogenized with 200 µL of Assay buffer solution. The resulting

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mixture was centrifuged at 4 °C for 15 min at 9000 ×g. The supernatant is used to evaluate the activity of glutathione peroxidase. First, 50 µL of the sample or different concentrations of the standard was placed in plate wells and 40 µL of solution 1 [(40 mM GR, GSH, and nicotinamide adenine dinucleotide phosphate (NADPH)] was added. At this stage, all glutathione oxide (GSSH) is reduced by the activity of the enzyme glutathione reductase in solution 1. After 15 min of incubation at room temperature, the adsorption was read at 340 nm (by using a plate reader Epoch, USA). The adsorption was measured at 340 nm at 0 and 10 min after the start of the reaction and then enzyme activity was calculated (Amiri *et al*., 2018).

GST Enzyme Assay

100 mg of tissue was weighed and after washing with PBS buffer pH=7.2 homogenized with PBS buffer (pH=5.6). The resulting mixture was centrifuged at 8000 ×g for 4 min at 4°C and the supernatant was kept to measure enzyme activity. In the test tubes, 980 µL of PBS buffer (pH = 6.5), 100 µL of 1-chloro-2,4-dinitrobenzene (CDNB) (100 mM), and 10 µL of 100 mM glutathione were added. After mixing, 100 µL of the sample was added to the test solution. The adsorption of the solution at 340 nm was measured twice at 3 min intervals (by using a plate reader Epoch, USA) (Hanachi *et al*., 2020).

Statistical analysis

Data were expressed as means ± standard deviation (SD). All analyses were performed by Tukey multiple comparison test, if the ANOVA test showed a significant difference (p<0.05). Two-way ANOVA with interaction was applied to compare the impacts of CPF in the absence and existence of MPs. SPSS software version 24 was used to conduct all the statistical analyzes.

Results

CAT activity in the liver tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). No significant difference was observed in CAT activity among treatments (Fig. 1). However, the CAT activity in the liver was higher than in other studied organs.

CAT activity in the muscle tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). The CAT activity was increased by higher concentrations of MPs and CPF (30 µg/L MPs + 6 µg/L CPF and 300 µg/L MPs+ 6 µg/L CPF) in muscle tissue samples (p<0.05). Similarly, the CAT activity was increased in 30 µg/L MPs + 2 µg/L CPF treatments. There is no significant difference in the CAT activity in MPs treatments (30 and 300 µg/L MPs) (Fig. 2).

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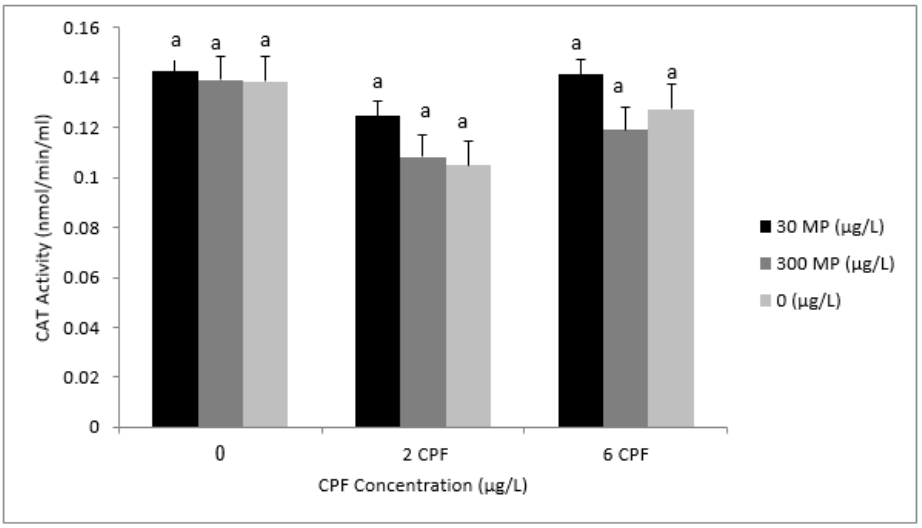

Figure 1: Catalase (CAT) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) liver tissue. Letters indicate a significant difference between groups (p<0.05).

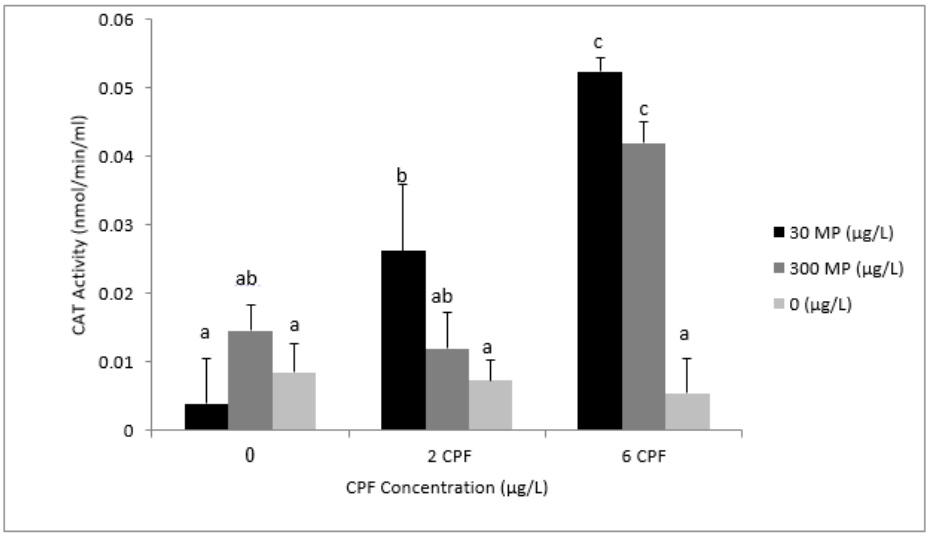

Figure 2: Catalase (CAT) in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) muscle tissue. Letters indicate a significant difference between groups (p<0.05).

CAT activity in the kidney tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). The CAT activity was increased by a high concentration of CPF and MPs at 30 µg/L (p<0.05). There is no significant difference in the CAT activity of MPs treatments and the control group (Fig. 3). In the absence of MPs, the amount of the enzyme has no significant difference in 2 and 6 µgL concentrations of CPF. No significant differences were observed in the CAT activity between 30 µg/L MPs + 2 µg/L CPF and 300 µg/L MPs+ 2 µg/L CPF treatments compared to the control group.

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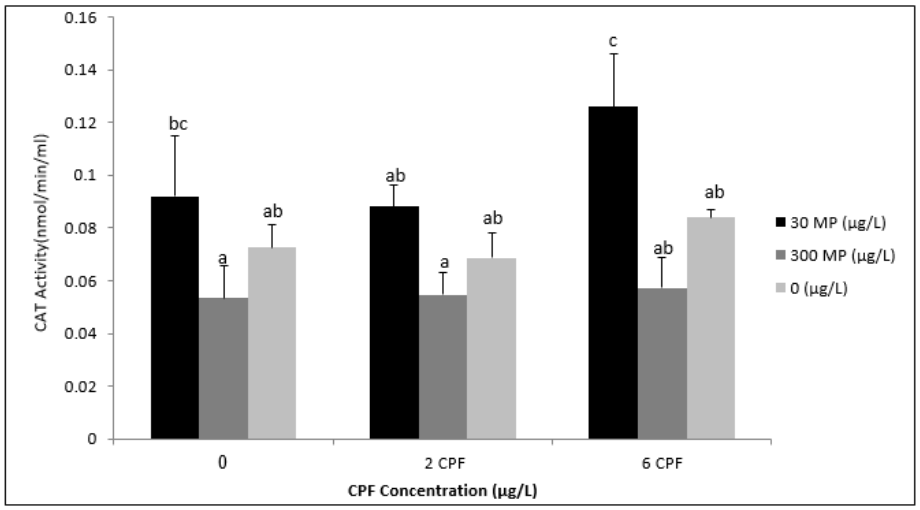

Figure 3: Catalase (CAT) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) kidney tissue. Letters indicate a significant difference between groups (p<0.05).

GPx activity in the liver tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). The GPx activity in the liver was decreased in MPs treatments (30 and 300 µg/L) compared to the control group. In contrast, the GPx activity was increased in 300 µg/L MP and 2 µg/L CPF treatments. In higher concentrations of CPF mixed with 30 µg/L MPs, the GPx activity significantly increased compared to the individuals in 30 µg/L MPs (p<0.05). The GPx activity significantly decreased in the liver tissue sample of 2 µg/L CPF (p<0.05; Fig. 4).

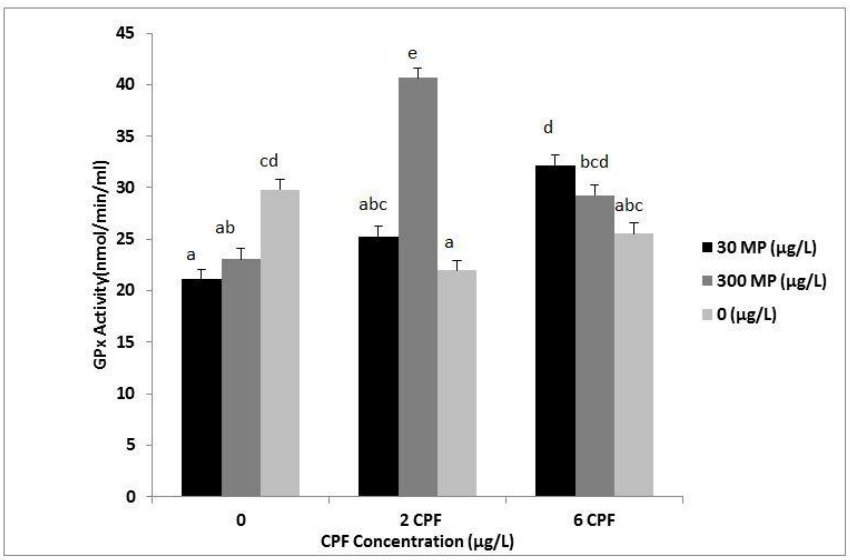

Figure 4: Glutathione peroxidase (GPx) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) liver tissue. Letters indicate a significant difference between groups (p<0.05).

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GPx activity in the muscle tissue

The GPx activity significantly decreased in 300 µg/L MPs compared to the control group (p<0.05). A significant increase was observed in mixed 300 µg/L MPs and 6 µg/L CPF (Fig. 5). The GPx activity significantly decreased in mixed 2 µg/L CPF with 30 and 300 µg/L MPs compared to the control group (p<0.05). A significant decrease was observed in 2 and 6 µg/L CPF compared to the control group (p<0.05).

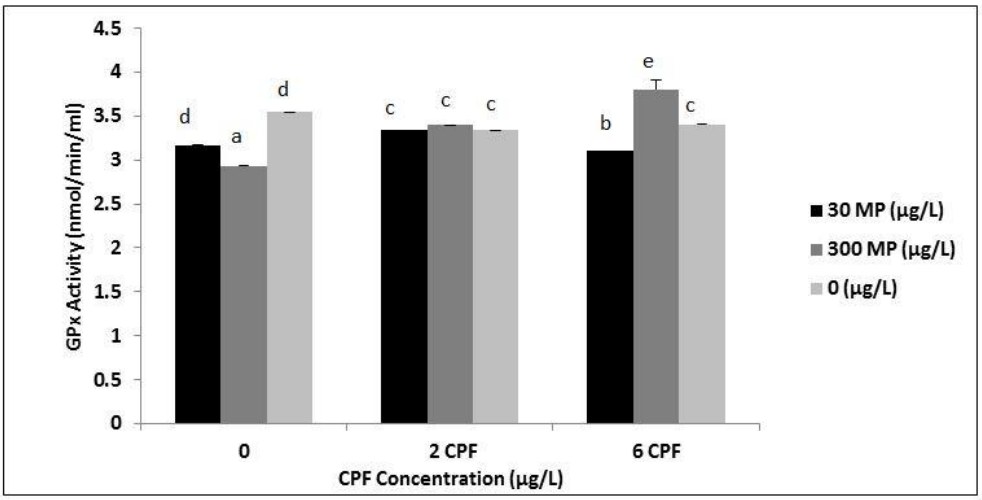

Figure 5: Glutathione peroxidase (GPx) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) muscle tissue. Letters indicate a significant difference between groups (p<0.05).

GPx activity in the kidney tissue

MPs and CPF were combined by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). The results of GPx activity in the kidney tissue are shown in Figure 6. There was a significant difference (p<0.05) among some treatments. The GPx activity significantly decreased in 30 µg/L MPs and increased in 300 µg/L MPs (p<0.05). A significant decrease was observed in mixed CPF with 30 and 300 µg/L MPs compared to the control group. Similarly, a significant decrease was observed in CPF concentrations (2 and 6 µg/L) compared to the control group (p<0.05) (Fig. 6).

GSTs activity in the liver tissue

Figure 7 shows the results of the GSTs activity in the liver tissue. There was a significant (p<0.05) increase between 30 µg/L MPs and 300 µg/L MPs treatments compared to the control group. The GST activity in 2 and 6 µg/L CPF groups treated with 30 and 300 µg/L MPS significantly (p<0.05) increased compared to the control group.

GSTs activity in the muscle tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). No significant relationship was observed among any of the treatments (Fig. 8).

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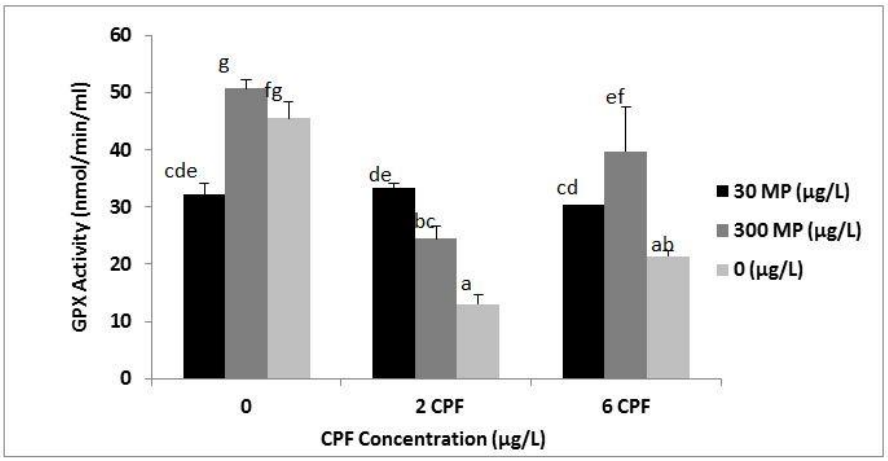

Figure 6: Glutathione peroxidase (GPx) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) kidney tissue. Letters indicate a significant difference between groups (p<0.05).

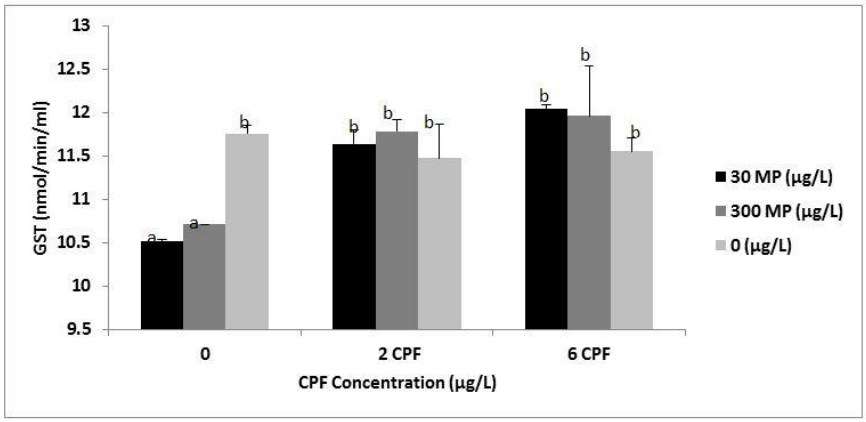

Figure 7: Glutathione transferase (GST) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) liver tissue. Letters indicate a significant difference between groups (p<0.05).

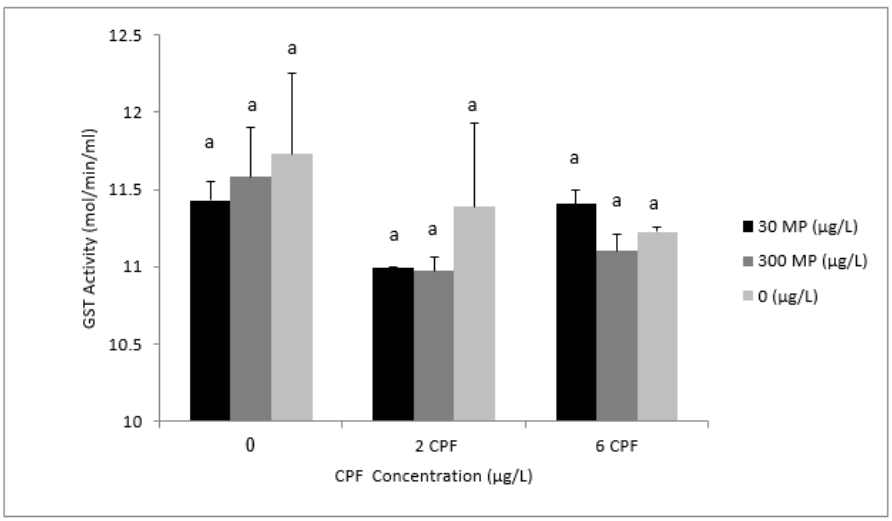

Figure 8: GST activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) muscle tissue. Letters indicate a significant difference between groups (p<0.05).

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GSTs activity in the kidney tissue

A combination of MPs and CPF was prepared by mixing target CPF concentrations (2 and 6 µg/L) with MPs (30 and 300 µg/L). As we can see in Figure 9, no significant difference was observed among any of the treatments in the kidney tissue.

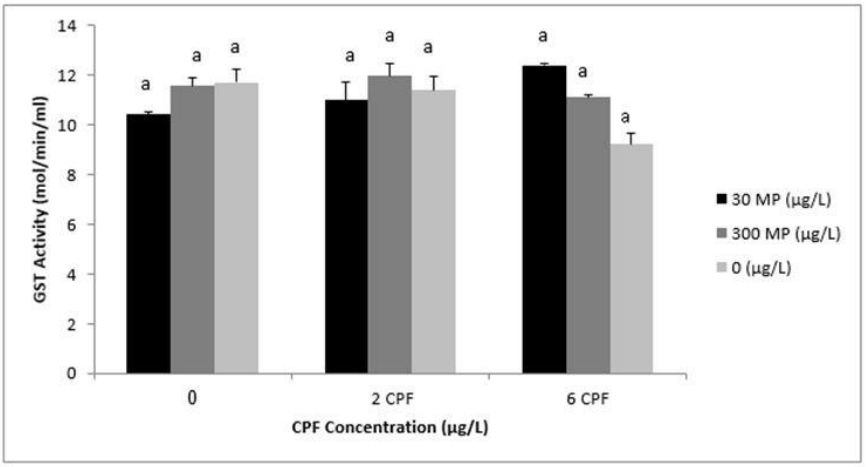

Figure 9: Glutathione transferase (GST) activity in different microplastic treatments of polystyrene microplastics (PS-MPs) and chlorpyrifos (CPF) in rainbow trout (*Oncorhynchus mykiss*) kidney tissue. Letters indicate a significant difference between groups (p<0.05).

Discussion

Catalase is one of the sensitive biomarkers for evaluating oxidative damage caused by chemicals in the environment. Based on other results, we expected the enzyme activity to be inhibited by CPF. Significant changes in the enzyme activities of *O. mykiss* were observed in mixed MPs and CPF treatments. A study on the microplastic effect of polyethylene on the toxicity of CPF in a microalgae (*Isochrysis galbana*) indicated a reduction in the bioavailability of pesticides by MPs (Garrido *et al*., 2019). In the kidney tissue, the CAT activity was inhibited by higher concentrations of MPs due to higher toxicity. In the liver and kidney tissue samples, the combination of pesticide and microplastic increased the activity of the enzyme, while these compounds alone inhibited the activity of the enzyme. Microplastics appear to reduce pesticide toxicity or stimulate enzyme activity. In a study, it was observed that GPx activity in saline oysters was decreased after 3 and 6 days of exposure to the microplastic combination of polystyrene and polyethylene with pyrene (Oliveira *et al*., 2013). Another study showed the combination of MPs and mercury inhibited the activity of GPx enzyme in the freshwater oyster (*Corbicula fluminea*) for 8 and 14 days (Oliveira *et al*., 2018). A previous study showed that SOD, GSH, and GSH-Px activities were decreased in Chinese mitten crab (*Eriocheir sinensis*) in response to the higher concentrations of PS-MPs (Prokić *et al*., 2019). As a result, MPs at low concentrations are more toxic to the kidney and at high concentrations in muscle tissue inhibited the GPx activity.

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As a result, the combination of pesticide and microplastic at low concentrations of MPs increased enzyme inhibition and toxicity of xenobiotic compounds, but at high MPs concentrations the enzyme activity was increased and the toxicity was decreased. However, in the liver and kidney tissues, the presence of MPs has reduced the toxicity and thus reduced the bioavailability of CPF. GST activity is one of the biomarkers of antioxidant pathways and it requires the NADPH cofactor to function, which is supplied by another enzyme called glutathione reductase (Hanachi *et al.,* 2007). The results of this study showed that MPs inhibited GST activity in the liver tissue, consistent with the inhibition of GST activity in the reef coral *Pocillopora damicornis* by MPs after 24 h. In contrast, another study found that MPs alone and in combination with mercury did not affect GST activity in *Corbicula fluminea* oysters (Oliveira *et al*., 2018). In this study, GST activity under the influence of MPs and pesticides alone and in combination with each other in the liver and muscle tissues did not change significantly. In a study, researchers observed that MPs alone and in combination with a contaminant called Pyrene had no effect on GST activity in *Pomatoschistus microps* (Oliveira *et al*., 2013). In this study, the exposure of MPs and CPF caused minimal toxic effects for 96 h, however, chronic exposures should be performed to assess the long-term impacts of the combined effects of various types of MP and chemical pollutants on this species.

In conclusion, few studies have been conducted on the combined effects of MPs and various contaminants, especially organophosphate pesticides. This study highlights the impact of combined MP and pesticides on antioxidant enzymes activity in *O. mykiss*. Although the toxic effects of residual pesticides and MP on aquatic biota may not be properly understood. Further studies are needed to elucidate the effects of different MP types alone and in combination with other types of pesticides. In addition, chronic exposures should be performed to evaluate the long-term effects of PS-MPs on *O. mykiss*.

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