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Does persistent organic pollutant PFOS (perfluorooctane sulfonate) negative impacts on the aquatic invertebrate organism, *Astacus leptodactylus* [Eschscholtz, 1823]

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Abstract

Highly persistent perfluorooctane sulfonate (PFOS) is an industrial fluorinated organic chemical with significant bioaccumulation and biomagnification properties. The purpose of this study was to determine the toxic effects of sublethal PFOS on the aquatic invertebrate organism, narrow-clawed crayfish [*Astacus leptodactylus* Eschscholtz, 1823]. The 96 h LC_{50} value was determined as 48.81 mg/L (34.19–63.68 mg/L) with probit analysis. The sublethal experimental design was formed into four groups solvent control (DMSO, dimethyl sulphoxide), non-treated control group, and 1/10 (5 mg/L) and 1/100 (0.5 mg/L) of 96 h LC_{50} of PFOS, and crayfish were exposed for 48 h, 7 d, and 21 d under laboratory conditions. Total haemocyte counts (THCs) decreased, while the haemolymph total antioxidant status (TAS) values increased ($p < 0.05$) after exposure to 0.5 and 5 mg/L PFOS for 48 h, 7 d, and 21 d. Haemolymph total oxidative stress (TOS) levels significantly increased at 5 mg/L PFOS concentration ($p < 0.05$). Catalase (CAT) activities increased at both concentrations after 48 h and 7 d and then returned to control levels after 21 d; whereas superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities did not change in muscle tissue ($p > 0.05$). GPX and CAT activities decreased, but SOD activity increased in hepatopancreas tissue ($p < 0.05$). SOD activity at both concentrations and CAT activity at 5 mg/L PFOS exposure decreased in gill tissue, while GPX activity increased at both concentrations of 48 h and 7 d and returned to control values on day 21 of exposure. Histopathological alterations were detected in hepatopancreas and gill tissues. Lamellar deformations, epithelial hyperplasia, and haemocytic infiltrations were observed in the gill tissues, whereas tubular degeneration, tubule loss, necrosis, and lesions in the hepatopancreas tissues were the major recorded alterations. As a result, the sublethal concentrations of PFOS have toxic effects on crayfish and histologically cause tissue damage. Our findings also support a better understanding of the early toxicological effects of PFOS in freshwater ecosystems. Also, it could be concluded that *A. leptodactylus* is a reliable model for examining histopathological alterations and differences in enzyme activities together with the haemolymph findings in toxicology studies amid aquatic species.

Keywords Perfluorooctane sulfonate · Haemolymph analysis · Histopathology · Oxidative stress · *Astacus leptodactylus*

Introduction

Persistent Organic Pollutants (POPs) are toxic chemicals that generally have lipophilic properties adversely affecting human and environmental health by accumulating in tissues, transferring from one species to the next through the food web, and being transported between countries (Sharma et al. 2014; Kissa 2001). Perfluorooctane sulfonate (PFOS), a water-soluble contaminant contrary to the classical apolar POPs, binds protein-rich tissues like the liver, circulates in the blood, and persists for years (Giesy and Kannan 2001; Jones et al. 2003; Olsen et al. 2007). The main causes of water PFOS contamination (surface, ground, and waste) and

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soil are the discharges from production facilities, fire-fighting activities, wastewater, sludge of sewage plants, and pesticide applications (Moody et al. 2003; Schultz et al. 2004; Huset et al. 2011; Liu et al. 2017). In addition, waste products containing PFOS are released via leachates from landfills due to their high solubility (Oliaei et al. 2013).

Depending on their accumulation capacity, PFOS is more toxic than the congener perfluorooctanoic acid (PFOA), a fully fluorinated anion (Cui et al. 2009). Generally, it is used as a salt or incorporated into larger polymers. Potassium, diethanolamine, ammonium, and lithium salts of PFOS are considerable examples of commercial use (UNEP 2006). In addition to those, PFOS can also be used in metal plating, photo imaging semi-conductor, medical device industries, sealing materials, aqueous film-forming foams, hydraulic fluids for aviation, and insecticide formulations (Chen et al. 2017; Kannan et al. 2002; Paul et al. 2009; Saikat et al. 2013; Stahl et al. 2011; Huang et al. 2020).

PFOS and its salts were added to Annex B of Persistent Organic Pollutants under the Stockholm Convention in 2009 (UNEP 2009). Even though production has halted, and applications limited to North America and Europe for its global elimination, there has been an increased demand in China (Liu et al. 2017). Although the usage of PFOS and its derivatives has been restricted since 2000, PFOS is still measured in aquatic environments due to its high persistent and lipophilic characteristics. The concentrations of PFOS in surface waters have been stated in the range from high pg/L to high ng/L (Kunacheva et al. 2012; Lindim et al. 2016). It has been measured at the highest levels in predatory and bottom-dwelling species (>3000 ng/g wet weight) due to its biomagnification attribute (Houde et al. 2006; Kannan et al. 2005; Martin et al. 2004). Laboratory and field studies have shown that the concentrations of PFOS ranging from µg/L to mg/L have the potential to induce sublethal toxicity including reproduction, development, and gene expression in various aquatic species (Shi et al. 2008; Jeong et al. 2016). Studies on the acute toxicity of freshwater invertebrates are very limited (Boudreau et al. 2003; Li 2009; Jeong and Simpson 2019; Logeshwaran et al. 2021). Li (2009) reported that PFOS is toxic to aquatic invertebrates, with acute toxicity values ranging from 10 to 300 mg/L and green neon shrimp (*Neocaridina denticulate*) was found to be the most sensitive freshwater species to PFOS with a 96 h LC_{50} of 10 mg/L. Hayman et al. (2021) reported the order of species sensitivity to PFOS and PFOA, starting with the most sensitive as Mediterranean mussel *Mytilus galloprovincialis*, the purple sea urchin *Strongylocentrotus purpuratus*, dinoflagellate *Pyrocystis lunula*, and opossum shrimp *Americamysis bahia*.

In previous studies on various animal species, immunotoxic and neurotoxic effects, developmental and reproductive toxicity, oxidative stress, endocrine disruption, and genetic toxicity have been reported for PFOS (Austin et al. 2003; Ankley et al. 2005; Abbott 2009; DeWitt et al. 2009; Wan et al. 2012; Chen et al. 2018). Additionally, various other toxic effects of PFOS have been reported in aquatic invertebrates. Logeshwaren et al. (2021) studied 48 h acute and 21 d chronic assays on water fleas (*Daphnia carinata*) exposure to PFOS and PFOA. Chronic exposure to PFOS exhibited mortality and reproductive defects in *D. carinata* at 0.001 mg/L. Using the comet assay, DNA damage was detected at 1 and 10 mg/L for 96 h. Seyoum et al. (2020) reported developmental and reproductive toxicity of PFOS on *Daphnia magna*. Downregulation of related genes and decline in fertility were observed. Also, the lifespan with early mortality after 5 d and hatching decreased after exposure to 25 µM PFOS. Embryotoxic effects of increasing concentrations of PFOS (ranging from 0.5 to 10 mg/L) have been reported for the sea urchin, *Paracentrotus lividus* by Gunduz et al. (2013). Larval malformations in the skeletal system at low concentration and developmental arrest induced by dose-depended exposure were recorded. The growth of embryos was affected highly by 10 mg/L PFOS in the early life stages. Extensive screening of biota samples worldwide showed PFOS to be a global pollutant, and that it bioaccumulates at higher trophic levels in the food chain (Bossi et al. 2005; Fromme et al. 2009).

Related to their location in the food chain and their sensitivity to environmental stress factors, crustaceans are excellent representatives as biomarkers for monitoring water quality (Issartel et al. 2010). Crayfish are the largest mobile macrobenthic detrivores with a relatively simple anatomy in temperate freshwater ecosystems (Brittle et al. 2016). *Astacus leptodactylus* is a crayfish species with commercial value, widely distributed in Eurasian fresh waters (Balık et al. 2005; Köksal 1988). Freshwater crayfish are such an ideal model organism for monitoring the ecotoxicological risks of freshwater bodies (Sepici Dinçel et al. 2013).

Previous studies presented some toxic effects of PFOS in different fish species (Oakes et al. 2005; Liu et al. 2007; Hagenaars et al. 2008; Ji et al. 2008; Dorts et al. 2011). Hyperactivity, locomotor activity, developmental toxicity, thyroid disruption, hepatotoxicity, alteration of gene expression, yolk sac utilization, induced cell death in the tail region, brain and eye of embryos, histologically induced hepatic steatosis, and disrupted lipid metabolism caused lesions in the muscle fibers and various malformations in embryos/larvae of zebrafish exposed to PFOS were shown (Shi et al. 2009; Huang et al. 2010; Cheng et al. 2016; Cui et al. 2017; Jantzen et al. 2016; Tse et al. 2016).

Touaylia et al. (2019) assessed oxidative stress caused by PFOS on *Gammarus insensibilis* by measuring malondialdehyde (MDA) and superoxide dismutase (SOD) activities and the activity of acetylcholinesterase (AChE). SOD activity increased at a low concentration of PFOS

while MDA increased in all treated groups (1, 1.6, and 3.1 mg/L of PFOS). AChE activity did not exhibit any significant changes, indicating no PFOS neurotoxicity. Immunotoxic effects of PFOS have been shown in Chinese mitten-handed crab (*Eriocheir sinensis*). Total haemocyte counts (THCs) were found low in PFOS exposed groups (0.1 mg/L, 1.0 mg/L, 10 mg/L), and the lowest count was measured after 21 d in the 10 mg/L treated group. Inhibition of phenoloxidase and superoxide dismutase activities were also significant in crabs exposed to 10 mg/L PFOS. Phenoloxidase and SOD increased initially, then decreased overtime at the other concentrations (Zhang et al. 2015).

In the present study, crayfish, an alternative bioindicator model organism for aquatic toxicology, was selected due to their key role in the freshwater aquatic food webs. The toxicity and the effects of PFOS have been examined on some crustacean species but are not available on crayfish, *A. leptodactylus* in the open literature. The aim of the present study is to focus on the effects of the persistent organic compound, PFOS in freshwater habitats using the bioindicator organism crayfish. We analyzed the biochemical and physiological responses of *A. leptodactylus* to the toxic effects of the sublethal PFOS exposure experiments at three time intervals, and at two concentrations. The total haemocyte counts, selected haemolymph parameters, and histopathological changes were evaluated in addition to direct toxicological data. The study also aimed to assess the antioxidant and oxidative status of the crayfish with the haemolymph total antioxidant status (TAS), total oxidative stress (TOS) levels, and the tissue levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities as biomarkers potentially affected by PFOS.

Materials and methods

Test organism and acclimation process

The freshwater crayfish (*Astacus leptodactylus* Eschscholtz, 1823), weight 29.10 ± 0.39 g (mean ± SEM) and length 10.27 ± 0.05 cm (mean ± SEM) were obtained from a local breeder (Lake Egirdir, Turkey) during the inter-moult stage. Crayfish were immediately transferred to the laboratory and stocked in glass aquariums during the acclimation period. Aquariums were filled with aerated dechlorinated tap water.

Crayfish were adapted for two weeks under laboratory conditions. Aquariums were constantly aerated with air pumps and regularly cleaned by siphoning. Water temperature was adjusted to 21 °C by thermostatic heaters. Experiments were carried out in the laboratory of the Biology Education Department at Gazi University. Experiments were performed according to the rules of "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996). The crayfish were fed daily with raw trout fish during the adaptation period.

Test chemical and experimental design

Heptadecafluorooctanesulfonic acid potassium salt (PFOS, CAS Number 2795-39-3, purity ≥ 98%, Sigma–Aldrich, USA) was used in the experiments. The substance was stored at +4 °C and brought to room temperature before use. To prepare the stock solution, PFOS in powder form was weighed in a volumetric glass flask, a certain volume was obtained by completing analytical purity with DMSO (dimethyl sulphoxide). Automatic pipettes with polypropylene tips were used for dosing, then glass mixing rods provided homogenization.

An acute toxicity test was carried out to estimate the median lethal concentration of PFOS on the selected organism to decide the experimental sublethal concentrations. After probit analysis (Supplementary Material A), the sublethal concentrations were selected as 1/10 and 1/100 of 96 h LC_{50} concentrations according to Sprague (1971) and decided as 5 mg/L and 0.5 mg/L PFOS. Applied concentrations in the aquarium water were in line with nominal and remained constant during the study.

Experimental aquariums containing 15 L of dechlorinated tap water were classified as exposure groups (0.5 and 5 mg/L PFOS concentrations) and control groups (DMSO control group and non-treated control group). Semi-static bioassay experimental method was used. Exposure media were renewed every 48 h. In the experiments, exposure results of 48 h, 7 d, and 21 d were investigated. All tests were repeated twice.

The water quality parameters were measured daily during the experiment (Supplementary Material B). The 18 h L: 6 h D cycle photoperiod was maintained during the experiments. Air pumps were on during the experiments and stopped only before the dosing instance. Crayfish were not fed for 48 h before and during the acute toxicity tests. The health status of crayfish was followed throughout the assays. No mortality was observed in the experiments.

Haemolymph sampling and analysis

The haemolymph samples were drawn from the base of the second walking legs of the crayfish using a disposable 2.5 cc syringe after exposure to PFOS for 48 h, 7 d, and 21 d. Prior to the sampling, crayfish were anesthetized on ice to slow down the animal activity. For haemocyte counting, the haemolymph samples were diluted with an equal volume of fixative (4% formalin). The other samples were placed in

Eppendorf tubes without anticoagulants and stored at −80 °C until haemolymph analysis.

Total haemocyte counts (THCs)

THCs of crayfish exposed to two different concentrations (0.5 mg/L, 5 mg/L) of PFOS for 48 h, 7 d, and 21 d were determined. The haemocytes were counted with Thoma under a light microscope according to Yavuzcan and Benli (2004).

Total antioxidant status (TAS)

Total antioxidant status was measured using a commercial assay kit (Rel Assay Diagnostics, TR). The principle of the assay is incubation of ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulphonated)] with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. $ABTS^+$ loses colour based on antioxidant status. The change of colour shows total antioxidant level of the sample, absorbance measured at 660 nm. The antioxidant status of the added samples and the concentration of the colour formed are proportional (Miller et al. 1993).

Total oxidative stress level (TOS)

Total oxidative stress levels were measured using a commercial assay kit (Rel Assay Diagnostics, TR). TOS test principle is based on the oxidative stress level of the environment, the transformation of N, N-dimethyl-p-phenylenediamine (DMPD) into its radical form, and determination of the formed coloured radical product ($DMPD^+$) colorimetrically. After 20 µL DMPD (100 mM) was added to 10 µL haemolymph sample taken into 2 ml acetate buffer (0.1 M, pH: 4.8), the sample was incubated at 37 °C for 75 min. $DMPD^+$ formation was determined spectrophotometrically at 505 nm. The assay is calibrated with hydrogen peroxide prepared in 2.52 mM $FeCl_2$ and PBS as standard (Verde et al. 2002). The results are expressed in micromolar H_2O_2 equivalents per liter (µM H_2O_2 Equiv/ L).

Tissue processing for histopathology

After haemolymph sampling, gills, hepatopancreas, nephridium, heart, gonads, and muscle tissues were dissected and removed immediately for histological examination; fixed in Davidson's fixative (330 mL- 95% ethyl alcohol; 220 mL- formalin; 115 mL glacial acetic acid; 335 mL distilled water) (Bell and Lightner 1988). Tissues were placed in 70% ethyl alcohol after 24 h fixation.

Tissue samples were rinsed in tap water, dehydrated in graded alcohol series and embedded in paraffin. Paraffin blocks of tissue samples were cut at 5–6 µm thickness using rotary ThermoShandon microtome. After deparaffinization, slides were stained with Hematoxylin and Eosin according to routine procedures (Luna 1968). The slides were examined under a light microscope.

Biochemical analyses

For biochemical analyses, dissected gill, hepatopancreas, and muscle tissues were immediately frozen in liquid nitrogen, afterward stored at −80 °C until enzyme activity measurements. Superoxide dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPX) activities were measured using commercial assay kits (Cayman Chemical, USA). The biochemical analysis of the tissues were carried out individually with seven crayfish for each exposure concentration in two replicates.

Hepatopancreas, gill, and muscle tissue samples were rinsed with phosphate-buffered saline, pH: 7.4 to determine superoxide dismutase activity. Tissues were homogenized in 5–10 mL cold 20 mM HEPES buffer (at pH 7.2 containing 1 mM EDTA, 210 mM mannitol, and 70 mM sucrose) per gram. The homogenates were centrifuged at 4 °C for 5 min at 1500 × *g*, and the removed supernatants were kept at −80 °C until the assay. SOD assay was performed according to the method of Sun et al. (1988). SOD activity was inhibited by decreasing NBT, with xanthine–xanthine oxidase used as a superoxide generator, and one IU was defined as the quantity of SOD required to produce 50% inhibition. Tissue SOD activity was expressed as U/100 mg tissue.

CAT activity was measured according to the manufacturer's directions. It was done by the dismutation of H_2O_2 at 240 nm based on the method of Aebi (1984). The tissues were homogenized on ice with 5–10 mL cold buffer (50 mM phosphate buffer at pH: 7.0 with 1 mM EDTA). Tissues were centrifuged at 4 °C for 15 min at 10,000 × *g*; the supernatants were removed and kept on ice until the enzyme assay.

GPX activity was measured according to the protocol of the Glutathione Peroxidase Assay Kit (Cayman Chemical, the USA). Tissue samples were homogenized in Tris-HCl containing EDTA (1/10; w/v). Tissue glutathione peroxidase activity results were stated as nmol/min/100 mg.

Statistical analysis

The LC_{50} value with 95% confidence limits was calculated by a computer program (CEAM 1999) using Finney's Probit Analysis. The data were tested for normality and homogeneity of variances using the Shapiro–Wilks and Levene test. To compare continuous variables between multiple outcome variables, two-way ANOVA was used.

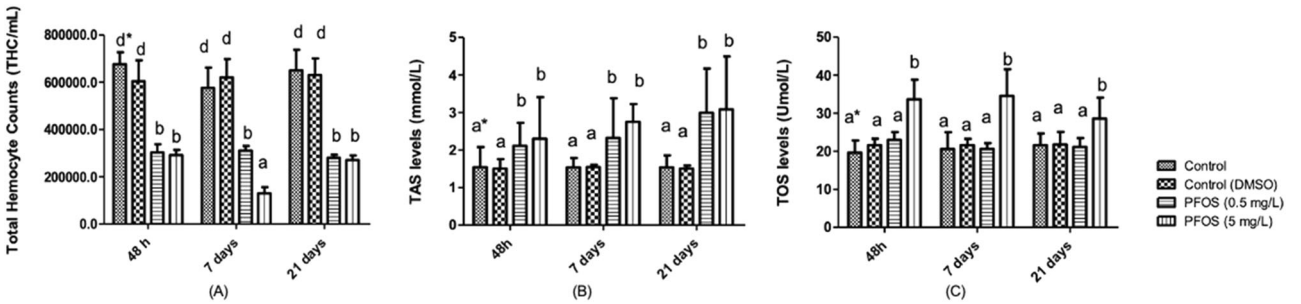

Fig. 1 **A–C** THCs, TAS and TOS values (M ± SE) after exposure to 0.5 and 5 mg/L of PFOS for 48 h, 7 days and 21 days in crayfish. *Different lowercase letters indicate statistically significance difference ($P < 0.05$)

Bonferroni test was used for multiple comparisons of groups. The statistical level of significance for all tests was considered to be 0.05. The results were expressed in the form of mean ± standard error (SEM).

Results

Acute toxicity

The determined 96 h LC_{50} values with 95% confidence limits of PFOS on crayfish was 48.81 mg/L (34.19–63.68 mg/L). There was no mortality in the control groups. The probit results of PFOS on crayfish are depicted in Supplementary Material A. The toxicity of PFOS is found in the Category: Acute 3 in crayfish. The Category: Acute 3 means 96 hr LC_{50} values are calculated as >10 – ≤100 mg/L (GHS, 2011).

Total haemocyte counts (THCs)

THCs decreased significantly after exposure to two different sublethal PFOS concentrations in groups ($p < 0.05$). The difference was not statistically significant in groups exposed to PFOS except 5 mg/L for 7 d, and no significant differences were observed between the control groups ($p > 0.05$). The results are shown in Fig. 1A.

Total antioxidant status (TAS)

Haemolymph TAS levels increased significantly in all groups exposed to PFOS and reached the highest level after 21 d ($p < 0.05$). There were no significant differences between control groups. The results are shown in Fig. 1B.

Total oxidative stress (TOS)

Haemolymph TOS levels of crayfish were increased after 48 h and 7 d exposure to 5 mg/L PFOS. Although a decrease was observed after 21 d, it was still higher than the control statistically ($p < 0.05$). There was no significant difference of haemolymph TOS levels between 0.5 mg/L PFOS exposed, and the control groups. The results are shown in Fig. 1C.

Histopathological alterations

After exposure to sublethal PFOS, the muscle, heart, gonads, and nephridium tissues of the crayfish did not reveal any significant histopathological findings compared to the control groups. However, the gill and the hepatopancreas tissues were affected histologically from the sublethal PFOS exposure. The main histopathological lesions were observed in the hepatopancreatic tissues. All results of the histopathological findings are shown in Table 1. Gill tissues of the crayfish following exposure to PFOS exhibited lamellar deformations, epithelial hyperplasia, and haemocytic infiltrations (Fig. 2). Tubular degeneration, tubular loss, necrosis, and lesions were observed in the hepatopancreas tissues depending on the duration and the concentration of the PFOS (Fig. 3).

Biochemical analyses

SOD activities decreased in gill tissues at both concentrations. The highest decline was observed in the group exposed to 5 mg/L PFOS for 48 h, but the decrease was not statistically significant ($p > 0.05$). SOD activities in the gill tissue of crayfish are described in Fig. 4A. In hepatopancreas tissues, SOD activities increased in all groups significantly except the group exposed to 0.5 mg/L for 48 h ($p < 0.05$) (Fig. 4B). It was detected that there was no significant difference in the SOD activities between the control group and the groups exposed to PFOS in muscle tissue. ($p > 0.05$) (Fig. 4C).

CAT activities of gill tissues in crayfish exposed to 0.5 mg/L for 21 d and 5 mg/L PFOS during all exposure period decreased significantly ($p < 0.05$). There were no differences between the control groups and the groups exposed to 0.5 mg/L PFOS considerably for 48 h and 7 d ($p > 0.05$). The CAT activity results of gill tissues are shown in Fig. 5A. CAT activities of hepatopancreas tissues did not

Table 1 Histopathological findings of the crayfish after exposure to PFOS concentrations

Tissue/Histopathology	Control			Control (DMSO)			0.5 mg/L PFOS			5 mg/L PFOS		
	2 d	7 d	21 d	2 d	7 d	21 d	2 d	7 d	21 d	2 d	7 d	21 d
Gill												
-Haemocytic infiltration in the vessels	–*	–	–	–	–	–	+	+	++	+	++	+++
-Deformations of the lamella	–	–	–	–	–	–	+	+	++	+	++	+++
-Epithelial hyperplasia of lamella	–	–	–	–	–	–	+	+	+++	+++	+++	+++
Hepatopancreas												
-Degenerations of the tubules (tubule lumen degeneration, enlargement of tubules)	–	–	–	–	–	–	+	+	+++	++	++	+++
- Tubule loss	–	–	–	–	–	–	+	+	++	+	+++	+++
-Tubule necrosis	–	–	–	–	–	–			–	–	–	+

*(-) none (no histological alterations), which representing normal histological structure; (+) mild; (++) moderate and (+++) severe histopathological alterations in the tissues

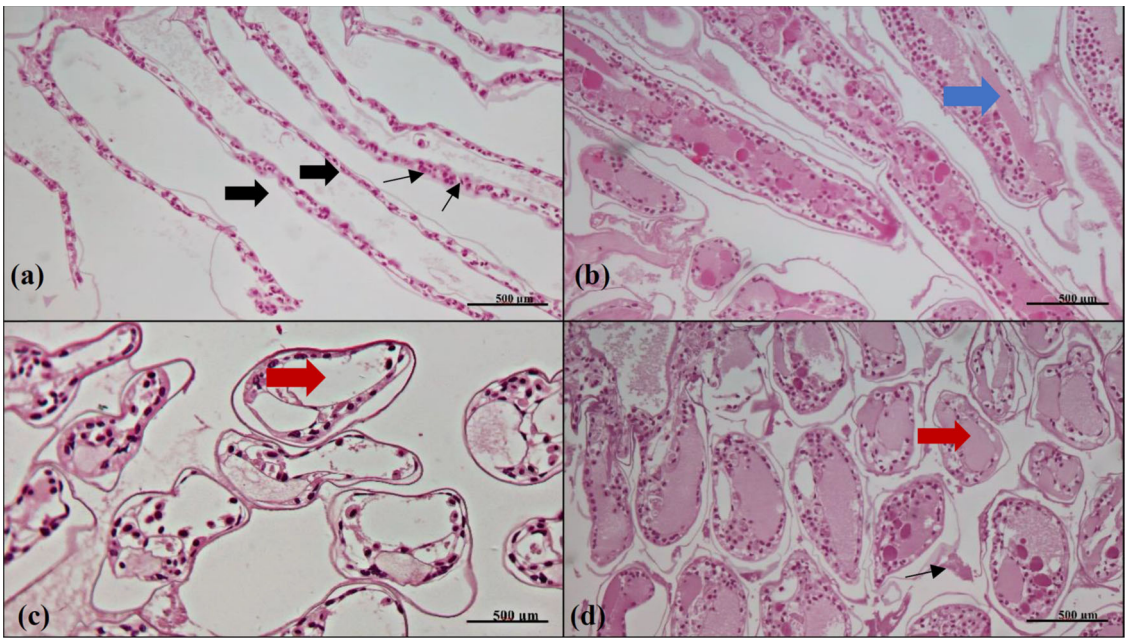

Fig. 2 Gill tissue of crayfish (**a**) longitudinal section of normal gill lamella (black thick arrow) with epithelial cells (black thin arrow), (**b**) epithelial hyperplasia (black thin arrow) and hemocytic infiltration (blue arrow) after exposed to 5 mg/L PFOS for 21 days, (**c**) cross section of the normal gill tissue with afferent and efferent vessels (red arrow), (**d**) hemocytic infiltration (red arrow) of afferent and efferent vessels after exposed to 5 mg/L PFOS for 21 days

change in the groups exposed to 0.5 mg/L of PFOS following 48 h and 7 d compared to control groups; however, a sharp decrease was observed in the group after 21 d and reached the lowest level compared to other groups ($p < 0.05$). CAT activity in the groups exposed to 5 mg/L PFOS decreased significantly for all test periods and reached the lowest level after 21 d in hepatopancreas (Fig. 5B). In muscle tissues, CAT activities increased significantly after 48 h and 7 d in both groups exposed to 0.5 mg/L and 5 mg/L PFOS. However, there was no significant difference between the groups after 21 d ($p > 0.05$). CAT activity results of the muscle are shown in Fig. 5C.

Muscle tissue GPX activities did not alter after PFOS exposure ($p > 0.05$) (Fig. 6A). GPX activities significantly increased after 48 h and 7 d at both concentrations ($p < 0.05$), but returned to control groups' levels after 21 d in gill tissues (Fig. 6B). In hepatopancreas, GPX activities significantly decreased after 48 h and 7 d when compared to control groups at both concentrations (Fig. 6C). Although GPX activity increased in the groups exposed to PFOS after 21 d in hepatopancreas, the results were still statistically lower compared to control groups ($p < 0.05$).

Discussion

This study presents the toxicological data of persistent organic pollutant PFOS on the crayfish, an aquatic model

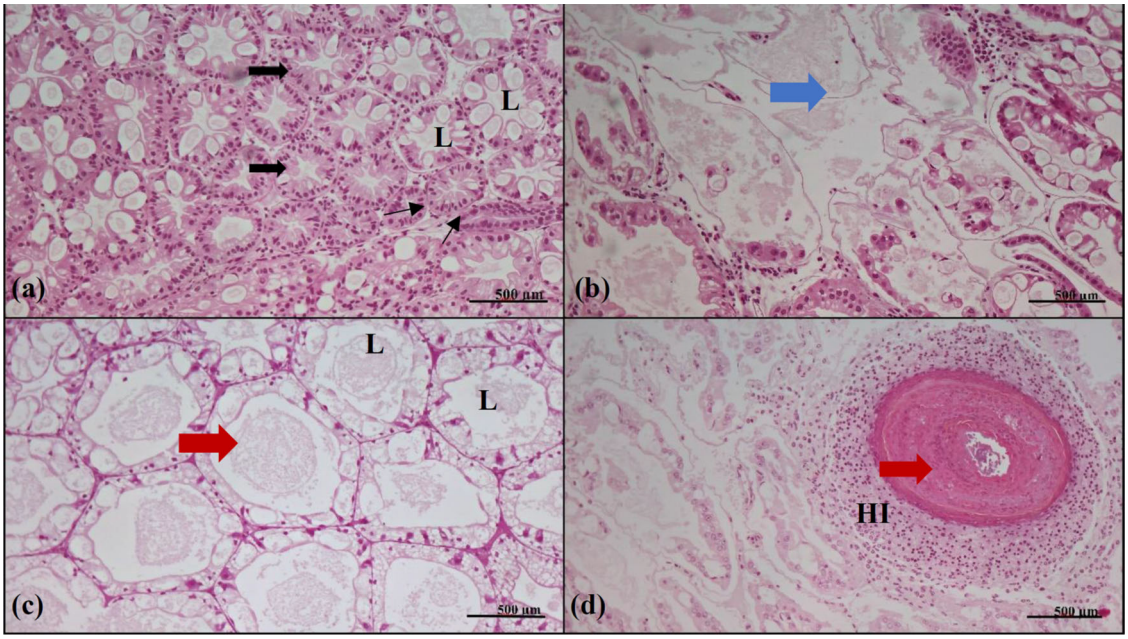

Fig. 3 Hepatopancreas tissue of crayfish (**a**) histological appearance of normal tubules (black thick arrow) with lumens (L) and blister like B cells (black thin arrow), (**b**) degeneration of tubules (blue arrow) after exposed to 5 mg/L PFOS for 21 days, (**c**) enlargement of tubule lumen (red arrow), (**d**) necrosis of tubule (red arrow) with hemocytic infiltration (HI) after exposed to 5 mg/L PFOS for 21 days.

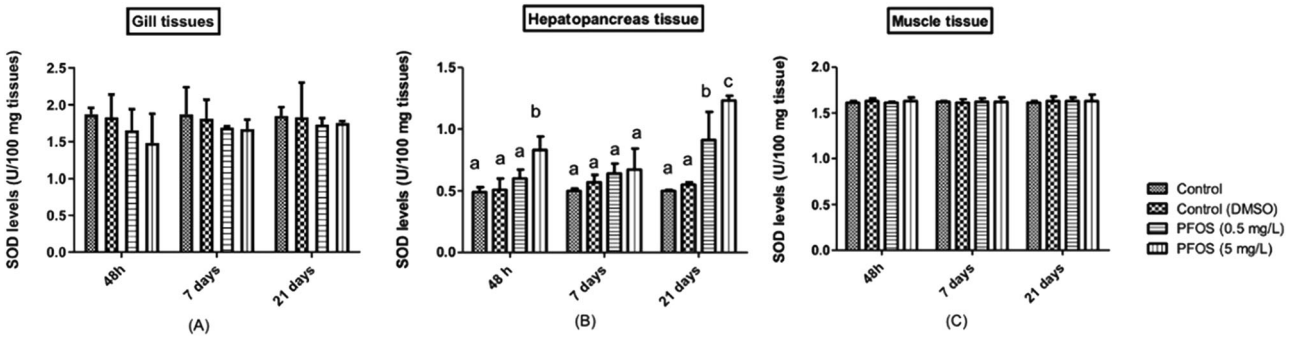

Fig. 4 **A**–**C** SOD (superoxide dismutase) activity (M ± SE) in gill (**A**), hepatopancreas (**B**) and muscle (**C**) tissues after exposure to 0.5 and 5 mg/L of PFOS for 48 h, 7 days and 21 days in crayfish. *Different lowercase letters indicate statistically significance difference ($P < 0.05$)

and bioindicator invertebrate organism. Haemocytes are quite reliable biomarkers for monitoring humoral and cellular immunologic status of invertebrates. Their number would increase or decrease when the species is affected by environmental stress factors including pollutants (Jussila et al. 1997; Perazzolo et al. 2002; Smith and Johnston 1992; Le Moullac and Haffner 2000; Liu et al. 2021). The total haemocyte counts (THCs) of *A. leptodactylus* decreased significantly after exposure to two different sublethal PFOS concentrations for all time periods. This decrease in the THCs in the freshwater crayfish may be a reflection of the stress on organisms due to the deterioration of the water quality of the pollutant (PFOS). These results may also indicate the immunosuppressive effects of PFOS on freshwater crayfish immunity, which could make them susceptible to disease (Yavuzcan and Benli 2004; Günal et al. 2021; Arslan 2022; Khoei 2022). Decreases in haemocytes have been recorded in various crustaceans under stress conditions caused by environmental contaminants or changes in water parameters. Zhang et al. (2015) indicated similar significant decrease of *Eriocheir sinensis* THCs after PFOS exposure (0.1, 1.0, and 10 mg/L). Qiu et al. (2011) recorded a remarkable reduction in THCs in the first three and sixth hours when temperature dropped in the shrimp *Litopenaeus vannamei*. Qin et al. (2012) observed a decrease in THCs of the freshwater crab *Sinopotamon henanense* after exposure to cadmium (58 and 116 mg/L) for 96 h. Similarly, Mello et al. (2011) reported a decline in

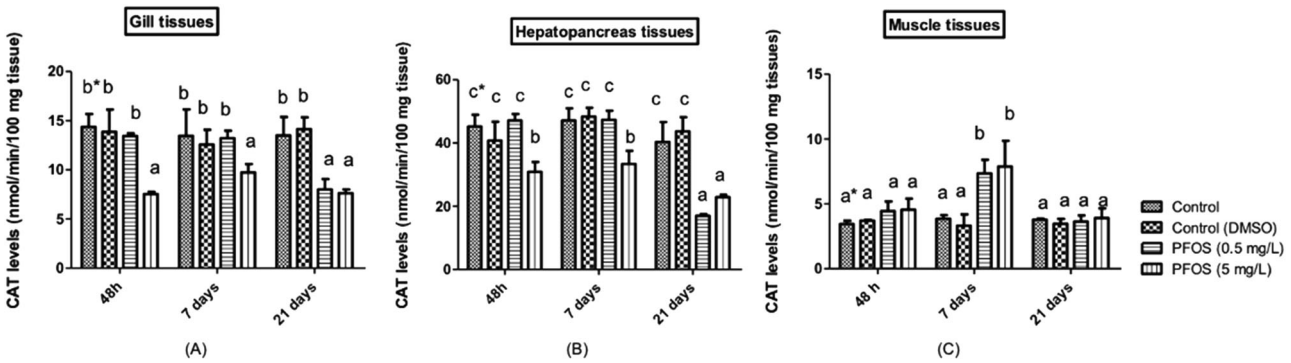

Fig. 5 **A**–**C** CAT (catalase) activity (M ± SE) in gill (**A**), hepatopancreas (**B**) and muscle (**C**) tissues after exposure to 0.5 and 5 mg/L of PFOS for 48 h, 7 days and 21 days in crayfish. *Different lowercase letters indicate statistically significance difference ($P < 0.05$)

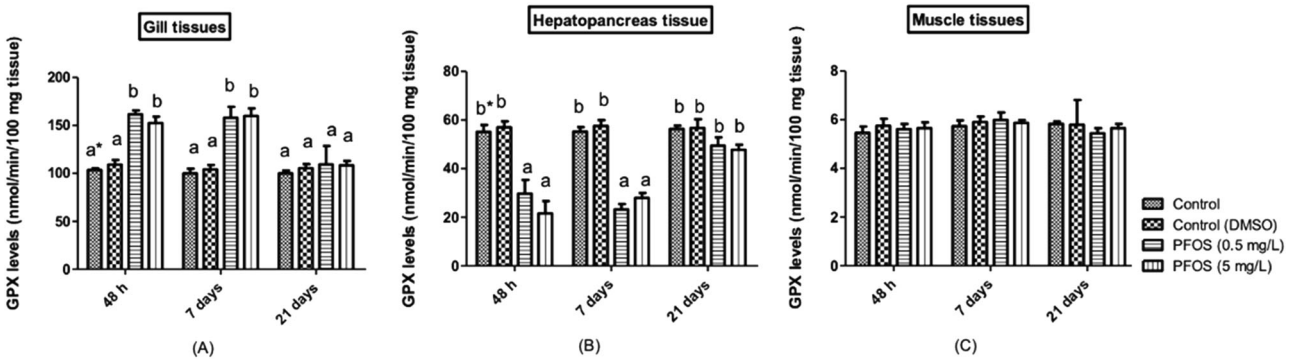

Fig. 6 **A**–**C** GPx (glutathione peroxidase) activity (M ± SE) in gill (**A**), hepatopancreas (**B**) and muscle (**C**) tissues after exposure to 0.5 and 5 mg/L of PFOS for 48 h, 7 days and 21 days in crayfish. *Different lowercase letters indicate statistically significance difference ($P < 0.05$)

THCs after exposure to 250 SC Sirus herbicide of pyrazosulfuron-ethyl (0.1–1000 µg/L) for 96 h in *Litopenaus vannamei*. Contrary to our results, Gunal et al. (2021) determined an increase in THCs of *A. leptodactylus* exposed to 0.09 µg/L permethrin for 48 and 96 h.

TAS represents the full spectrum of antioxidant activity against various nitrogen and ROS species (Yağan et al. 2014). The measurement of individual antioxidant biomarkers leads to loss of labour time and cost; necessitates qualified staff and complex techniques. Nonetheless, the measurement of the total antioxidant status (TAS) is the preferred, easy to perform and rapid method by researchers. Previous studies have indicated that plasma or haemolymph TAS levels of aquatic animals are reliable biomarkers of immune reactions against stressors (Livingstone 2003; Valavanidis et al. 2006; Sepici Dincel et al. 2009). In the present study, haemolymph TAS levels of PFOS exposed crayfish exhibited significant rise dependent on exposure time for both concentrations. Hoff et al. (2003) did not observe any changes in serum TAS of juvenile carp, *Cyprinus carpio* exposed to PFOS after 1 and 5 d. Similarly, our findings, Yucel Isıldar et al. (2020) determined increased haemolymph TAS levels of crayfish *A. leptodactylus* exposed to 0.05 µg/L deltamethrin but the increase was not significant. Franco-Martinez et al. (2016) observed an increase in TAS levels of the gill tissues of mussels *Mytilus galloprovincialis* exposed to Pb, Cd, and mixture of Cu+Pb +Cd, but results were not statistically significant. The increase in TAS, especially its increase with time, reveals the defensive response of the antioxidant system against increased reactive oxygen species (ROS) (Selvi et al. 2011). Contrary to these, significant reduction in haemolymph TAS of *M. galloprovincialis* was observed by Kaloyianni et al. (2009). They firstly recorded unaltered haemolymph TAS levels of mussels after 6 d of exposure to Cd and Zn, but a certain decrease revealed after exposure to PAHs mixture and lindane for 12 d. Patetsini et al. (2013) observed a reduction in haemolymph TAS levels of mussels, *M. galloprovincialis* after exposure to 0.05 µg/L chlorpyrifos and penoxsulam from the initial day to the 30th day. A decrease was reported in plasma TAS of *Cyprinus carpio* exposed to 24 h and 48 h to esbiothrin (5 and 10 µg/L), but TAS levels increased significantly after 72 h in both exposure doses (Selvi et al. 2011). Low TAS levels mostly indicate

oxidative stress or increased susceptibility to oxidative damage (Young 2001).

Haemolymph TOS levels in the present study did not change in groups exposed to 0.5 mg/L PFOS, whereas it was higher in groups exposed to 5 mg/L PFOS compared to controls. Although TOS level decreased after 21 d when compared to 48 h and 7 d in 5 mg/L PFOS exposed groups, it was still higher than that of the control groups. Knowledge gaps exist in evaluation of TOS as biomarker in aquatic invertebrates in the open literature. Our findings are valuable as the first report about TOS levels determination of *A. leptodactylus*. A similar increase was observed by Franco-Martinez et al. (2016) in TOS and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to Pb, but a rise in TAC was not statistically significant.

Crayfish exposed to PFOS revealed evident alterations in the gills and hepatopancreas tissues in the present study. Control groups did not show any histopathological changes in the tissues. In addition, no significant histopathological changes were observed in the muscle, heart, gonads, and nephridium tissues of crayfish exposed to PFOS. Concentrations of 0.5 mg/L and 5 mg/L PFOS caused obvious lamellar deformations and haemocytic infiltration in the gill tissues. Presently no study reporting PFOS toxicity damage in crayfish gill histology is in the open literature. Similar to our results, Desouky et al. (2013) reported haemocytic infiltration in the haemocoelic space and swelling of the gill lamellae after 24 h exposure to 0.36 mg/L of ethion on gills of *Procambarus clarkii*. Benli et al. (2016) reported hyperemia and melanisation on the gill lamella after being exposed to 9 mg/L (2,4-dichlorophenoxy) acetic acid (2,4-D) for one week on the same crayfish species, narrow-clawed crayfish (*A. leptodactylus*). Yu et al. (2018) found 44.29% gill cuticula vagueness, degeneration, and 33.27% epithelial cell lesions at 96 h after 0.24 mg/L pymetrozine exposure on gills of the crayfish *Procambarus clarkii*.

In this study, tubular degeneration, tubular loss, necrosis, and lesions were observed in the hepatopancreas of the crayfish exposed to PFOS. Few studies are showing the hepatotoxic effect of PFOS in aquatic organisms. Tse et al. (2016) indicated that exposure to PFOS can cause liver pathogenesis and non-alcoholic fatty liver disease changes in different metabolic processes in the liver in zebrafish. Fang et al. (2013) determined that the fish larvae of marine medaka (*Oryzias melastigma*) after exposure to 16 mg/L PFOS at 17 days' post-hatch showed a marked enlargement on the cell nuclei of the liver. Similar to histopathological findings in the present study, histological alterations were observed in hepatopancreas tissues of crayfish exposed to various types of toxicants. Benli (2015) determined necrosis on tubule cells in the hepatopancreas of *A. leptodactylus* in the treated groups with etofenprox (0.04 and 0.1 µg/L) for 96 h. The lesions in hepatopancreas tissue were more severe at the 0.1 µg/L etofenprox exposed group. Gunal et al. (2021) reported degenerative tubules in hepatopancreas tissues and haemocytic infiltrations in the afferent and efferent vessels in gills of *A. leptodactylus* after being exposed to 0.09 µg/L permethrin for 48 and 96 h. Zhang et al. (2019) found tubule lumen dilatation in the hepatopancreas tissue of adult male *P. clarkii* after exposure to Cd. The hepatopancreas exposed to 10 mg/L Cd for 72 h showed evident epithelium vacuolization. Chabera et al. (2021) observed focal dilatation of tubules, increased number of fibrillar cells, and haemocyte infiltration in the interstitium *of Pacifastacus leniusculus* exposed to 0.45 µg/L and 2.7 µg/L chloridazon-desphenyl (Ch metabolite) and 2.7 µg/L chloridazon (Ch) for 30 days.

ROS are produced as a normal product of cellular metabolism in healthy organisms. The level of ROS can be stabilized by an antioxidant defense system including SOD, CAT and GPX. However, if the balance is broken and shifts in the direction of oxidants, that is, if the cell is exposed to more ROS, it results in oxidative stress (Halliwell 1999). ROS are produced in mitochondria by leakage electrons in the complex I and III enzyme systems in the electron transport chain. Due to leakage of electrons into the cell, the concentration of molecular oxygen is reduced. Therefore, the cell protects itself via antioxidant enzyme systems like SOD, CAT, GPX, and small non-protein antioxidants such as reduced glutathione (Bal et al. 2021). Some environmental pollutants may induce ROS and cause oxidative stress, thus altering antioxidant activities (Stara et al. 2012). Several studies have reported oxidative stress responses to PFOS in aquatic organisms (Shi and Zhou 2010; Feng et al. 2015; Lu et al. 2015). In previous studies, antioxidant enzyme activities have been observed as biochemical biomarkers for environmental monitoring in crustacean species (Hossain et al. 2021; Kim et al. 2018; Pan and Zhang 2006). However, data on the antioxidant enzyme activities of *A. leptodactylus* exposed to PFOS are still lacking.

The results of the present study revealed different responses of enzyme activities in selected tissues of crayfish. This might be explained as the different metabolic activities of the examined tissues and their different responses to environmental conditions (Borkovic et al. 2008). The SOD activity showed a tissue-dependent response in the present study after exposure to PFOS. The SOD activity decreased in gill tissues after exposure to all concentrations of PFOS whereas it significantly increased in hepatopancreas. There was no significant change recorded in muscle tissues. Similar to our results, Amraoui et al. (2018) observed a decrease in SOD activity in the gill tissues after 7 d of exposure to 10 mg/L PFOS in *Unio ravoisieri*. Lu et al. (2015) reported that SOD activity reduced by 0.2, 1, and 5 mg/L PFOS exposure in *Daphnia magna*. Liu et al. (2007) studied SOD activities in the

hepatocytes of *Oreochromis niloticus*. SOD activity did not change in 1, 5, and 30 mg/L PFOS treated groups whereas there was an increase in the 15 mg/L treated group after 24 h. Contrary to our results, increased SOD activity in the gill tissues was determined in *A. leptodactylus* after exposure to carbaryl for 48 h and 7 d (Benli et al. 2012).

CAT activity decreased in the gill and hepatopancreas tissues at 0.5 and 5 mg/L PFOS for 21 d. In particular, a marked decline was observed in hepatopancreas tissues at both concentrations at high exposure time (21 d). In contrast, an increase was seen in muscle tissues after 48 h and 7 d at both concentrations, and no change was observed between control after 21 d in muscle tissues. The decreased CAT activity induces the accumulation of hydrogen peroxide and increases lipid peroxidation (Halliwell 1999). Similar to our findings, Lu et al. (2015) found decreased CAT activity in all PFOS treated groups; furthermore, maximum reduction was recorded in the 5 mg/L PFOS exposed group after 7 d. Kim et al. (2010) reported a decrease in serum of *C. carpio* 864 ng/g treated group, but it was not significant. Contrarily, a concentration-dependent increase in CAT activity (0.2, 0.4, 1 mg/L) was observed in zebrafish embryos (96 hpf) by Shi and Zhou (2010). Amraoui et al. (2018) determined high CAT activity in the gill and digestive gland after exposure to 10 mg/L PFOS. CAT activity increase in the gill and hepatopancreas tissues was also determined in different pesticide exposure of *A. leptodactylus* (Benli et al. 2012; Yucel Isildar et al. 2020). Contrasting results of the CAT activities in the literature may be explained with the changes in potential antioxidants depending on the habitat, the species, the tissues, and chemicals in contaminated environments (Pala 2019).

GPX activities of gill tissues significantly increased in both concentrations of PFOS after 48 h and 7 d, but it returns to control group levels after 21 d. On the other hand, GPX decreased in hepatopancreas tissues. Despite the increase after 21 d in both concentrations, GPX activity was still statistically lower than in the control groups. Also, there was no alteration observed in muscle tissues. Feng et al. (2015) reported a decline in GPX levels in the liver of *Carassius auratus exposed to* 10 µmol/L PFOS after 4 d. Liu et al. (2007) reported a dose-dependent reduction of GPX activity in primary cultured hepatocytes of *Oreochromis niloticus*. Antioxidant enzyme activities facilitate organisms' partial or complete stress defense induced under unsafe environmental conditions. However, high level toxicity may have inhibited these enzymes by causing excessive ROS production (Xu et al. 2013a), which leads to oxidative cell damage in tissues (Xu et al. 2013b).

In the 0.5 mg/L PFOS exposed group, the TAS in the haemolymph, SOD activities in the hepatopancreas and GPX activities in the gill tissues were higher, while the TOS in the haemolymph, SOD activities in the gill, GPx activities in the hepatopancreas, and CAT activities in the gill tissues decreased. Compared to the 0.5 mg/L PFOS exposed group, the SOD activities in the gill tissues, GPX activities in the hepatopancreas and CAT activities in the gill and hepatopancreas tissues were lower, the TAS and TOS in the haemolymph, SOD activities in the hepatopancreas, GPX activities in the gill tissues and CAT activities in the muscle tissues increased in the 5 mg/L PFOS exposed group.

Conclusion

The current study clearly showed that the persistent organic pollutant PFOS reduces the THCs, alters the antioxidant enzyme activities, and produces histopathological alterations in gill and hepatopancreas tissues of the freshwater crayfish even at low doses. While total antioxidant status levels increased depending on the doses and exposure time, total oxidant status levels increased only 5 mg/L at 48 h and 7 d but decreased at 21 d. While catalase and glutathione peroxidase activities decreased, superoxide dismutase activity increased in hepatopancreatic tissues. The superoxide dismutase activity decreased in the gill tissues. The obtained results of this study indicated that the sublethal concentrations of PFOS are toxic even at the cellular, biochemical and histopathological levels for aquatic organisms. The biomarkers used in the current study are proper for determining the PFOS toxicity, and the freshwater crayfish can be used as a bioindicator species to exhibit early PFOS toxicity.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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