alterations in biochemical parameters of the freshwater fish, *Alburnus mossulensis*, exposed to sub-lethal concentrations of Fenpropathrin

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**Abstract:** Fenpropathrin is a new pyrethroid insecticide used to control crop pests. The aim of this study was to evidence fenpropathrin-induced oxidative stress and alterations in biochemical parameters in the freshwater fish, *Alburnus mossulensis*. Total antioxidant capacity, malondialdehyde (MDA), catalase activity (CAT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CK), acetylcholinesterase (AChE) in the whole body extract were measured in *A. mossulensis* after exposure to sub-lethal concentrations of fenpropathrin (approximately equal to 1, 2, 3, 5 and 10% of 96 h LC50) for 15 days. The 24, 48, 72 and 96 h LC50 of the fenpropathrin for *A. mossulensis* was 562.28±45.19, 218.18±18.75, 136.18±11.90 and 121.38±11.84 µg/L at 24±2 °C. Exposure to 2.75, 5.50 and 12.6 µg/L fenpropathrin significantly increased AST activity in fish. A significant increase in the ALP and LDH activities was observed in fish after a 15 day exposure to 1.25, 5.50 and 12.60 µg/L fenpropathrin. Fenpropatrin significantly induced lipid peroxidation and increased MDA levels in fish. Compared with the control group, total protein levels in fish decreased after exposure to 2.75, 5.50 and 12.60 µg/L fenpropathrin on day 15. Total antioxidant capacity, AChE and CPK activities in fish exposed to fenproparthin were significantly lower than control group. There was a significant increase in the CAT and ALT activities in fish after exposure to 5.50 and 12.60 µg/L fenpropathrin. In conclusion, fenpropathrin has the potential to disrupt biochemical parameters in *A. mossulensis* and to induce oxidative stress.

**Introduction**

The use of pyrethroid insecticides has recently increased compared to organochlorine pesticides due to their high selectivity and lower persistence in the environment (Goulding et al., 2013). Fenpropathrin [2, 2, 3, 3- tetrachlorocyclopropanecarboxylic acid cyano (3-phenoxyphenyl) methyl ester] is a new pyrethroid insecticide used to control crop pests in tomatoes, tobacco, cotton farms and fruit orchards. Pyrethroid insecticides enter surface water via air drift, leaching from agricultural land and surface runoff during or after the application of pesticides (Gan et al., 2005; Woudneh and Oros, 2006; Weston et al., 2009; Goulding et al., 2013). Although there is no report indicating the presence of fenproparthin in surface water and groundwater, the presence of other pyrethroid pesticides in sediment, surface water (Amweg et al., 2006; Holmes et al., 2008; Feo et al., 2010), deltaic regions of big rivers (Feo et al., 2010; Weston and Lydy, 2010) and estuaries (Woudneh and Oros, 2006) and agriculture wastewater (You et al., 2004) have been reported. Pyrthroid pesticides are lipophilic compounds, which may be absorbed through gills, skin or
alimentary ducts. Food poisoning with pyrethroids is another influential pathway of these pesticides on fish (Macneale et al., 2010). After entering the body, pyrethroid pesticides can be accumulated in fat tissues (Banaee, 2013). Pyrethroid pesticides in animal's body are rapidly hydrolyzed in the body of the exposed animals and their metabolites excreted. Carboxy esterases play an important role in the detoxification of these pesticides (Sogorb and Vilanova, 2002). Reactive oxygen species (ROS) produced during the detoxification process of pyrethroid pesticides are known as important agents that cause oxidative stress (Ansari et al., 2011; El-Demerdash, 2011). In addition, alterations in biochemical parameters (Das and Mukherjee, 2003; Parvez and Raisuddin, 2005), and hematologic factors (Saxena and Seth, 2002; Muranli and Güner, 2011), reproductive disorders (Moore and Waring, 2001), neurological disorders (Soderlund et al., 2002), histopathological changes (Velmurugan et al., 2007; Korkmaz et al., 2009; Kan et al., 2012), and mutations (Ansari et al., 2011; Begel et al., 2011), and reduced survival rate of larvae and embryos (Köprücü and Aydin, 2004; Ural and Sağlam, 2005), are reported in several fish species exposed to various pyrethroid pesticides.

Werner and Moran (2008) suggested that acute toxicity of pyrethroids to fish is usually higher than 200 ng/L. However, synthetic pyrethroid insecticides are highly toxic to fish and aquatic invertebrates (Polat et al., 2002; Datta, A. Kaviraj, 2003; Ural and Sağlam, 2005; Feo et al., 2010). There is little information on the toxicity of fenpropatrin to aquatic organisms. *Alburnus* species belong to the Cyprinidae family that is found in most rivers in Iran. The wide distribution of the freshwater fish *Alburnus* sp. makes this species a potential and useful biomarker for monitoring aquatic ecosystems. Therefore, the aim of the present study was to evaluate the changes in some biochemical parameters of the freshwater fish *Alburnus mossulensis* after exposure to sub-lethal concentrations of fenpropatrin.

**Materials and Methods**

**Fish:** Freshwater fish, *Alburnus mossulensis*, 7.36±1.60 g were netted from the Maroon River, Khuzestan Province, Iran. Fishes were kept in glass aquaria with dechlorinated water to acclimatize them to laboratory conditions (24±2°C, pH: 7.4±0.2, hardness 355±25 mg/L of CaCO₃ and 16 L: 8 D photoperiods) for two weeks prior to use. Specimens were fed with live food and commercial diet for ornamental fish.

**Acute toxicity test:** The acute toxicity test was performed according to semi-static methods described in the OECD procedure. Fishes were not fed 24 h before the experiments and during the acute toxicity test. The experiments consisted of a control group and five experimental groups. Acute test was performed to determine the appropriate toxicity range for the sub-lethal assay. 10 fish per group were exposed to different fenpropathrin concentrations (0.0, 10, 50, 100, 250, 500 and 1000 µg/L fenpropathrin, purity 30%) in 85 L aquarium. During the 96 h acute toxicity experiment, water in each aquarium was aerated and had the same conditions as the acclimation period. Test solutions were renewed every 24 h to maintain the chemical and the water quality. Every 24 h the dead fish were removed and the number of survivals was recorded. The experiment was repeated in triplicate. LC₅₀ values were calculated by the Probit Analysis test (Banaee et al., 2011).

**Sub-lethal toxicity test:** For sub-lethal toxicity tests, the concentrations of fenpropatrin in water were maintained modestly below the 96 h LC₅₀ value. Based on this value, five sub-lethal concentrations 1, 2, 3, 5 and 10% of 96 h LC₅₀ were chosen for the freshwater fish (*Alburnus mossulensis*). The fish were divided into five treatments and a control group by triplicate (10 specimens per each aquarium). Test solutions in each aquarium were renewed every 24 h. On the other hand, the twenty percent of water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of fenpropatrin near the nominal level. During the experiment, fishes
were fed twice a day with commercial food, and the fish mortality was recorded.

At the end of the experimental period, on day 15, all fish per treatment were captured and anaesthetized with a clove powder extract. Then, they were sacrificed and washed with buffered normal saline. Fish were homogenized during two minutes in ice cold phosphate buffer (pH 7.4; 1:10, w/v) using a glass homogenizer and then centrifuged for 15 min at 15000 g at 4°C with a refrigerated centrifuge. Supernatants were immediately used to measure biochemical parameters by using spectrophotometric assays.

**Biochemical Analysis:** Lactate dehydrogenase (LDH) activity determination is based on measuring the conversion of pyruvate to L-lactate by monitoring the NADH oxidation. Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. In the alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to form glutamate and pyruvate. LDH converts pyruvate to lactate and NAD⁺. In the determination of creatine phosphokinase (CK) activity, the enzyme reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH. All these activities were monitored by measuring the change in absorbance at 340 nm. Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm (Moss and Henderson, 1999). Acetylcholinesterase (AChE) activity was determined by adding an adequate volume of the sample into a cuvette containing 0.1 M phosphate pH 8.0, and acetylcholine iodide (0.015 M) and dithiobisnitrobenzoic acid (0.01 M) as substrates. AChE activity was recorded during 180 s at 405 nm (Thomas, 1998). Levels of protein in liver tissue were determined by standard procedures used in clinical biochemistry laboratories based on manual biochemical kits (ParsAzemon Co, Iran) (Johnson et al., 1999).

Total antioxidant capacity was assayed according to the ferric reducing ability of plasma (FRAP) method. Briefly, the FRAP reagent contained 5 mL of a (10 mmol/L) TPTZ (2,4,6-triprydyl- s- triazine) solution in 40 mmol/L HCL plus 5 mL of FeCl₃ (20 mmol/L) and 50 mL of acetate buffer, (0.3 mol/L, pH=3.6) and was prepared freshly. Aliquots of 100 μL supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-triazine (Fe³⁺-TPTZ) complex to ferrous tripyridyl-s-triazine (Fe²⁺-TPTZ) at pH 3.6 and temperature 25°C is directly proportional to the concentration of total antioxidant in the sample. Fe²⁺-TPTZ has an intensive blue color that can be monitored up to 5 min at 593 nm by a UV/VIS spectrophotometer.
Calculations were performed using a calibration curve of FeSO₄•7H₂O (100 to 1000 µM/L) (Iris et al., 1996). Malondialdehyde (MDA) content was assessed by a modification of a thiobarbituric acid assay and was expressed as µmol/g tissue (Placer et al., 1966). 500 µl of the supernatant was transferred to a Pyrex tube and mixed with 2500 µl trichloroacetic acid (20%) and 1000 µmL triclorthiobarbituric acid (67%). The tubes were then placed in boiling water (100°C) for 15 min. After cooling, the mixtures were extracted with a solution containing 1000 µL of distilled water and 5000 µL n-butanol: pyridine (15: 1). The mixture was then centrifuged with a solution containing 1000 µL of distilled water and 5000 µL n-butanol: pyridine (15: 1). The mixture was then centrifuged at 2000 g for 15 min at 4°C. The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels. MDA concentration was calculated with an external standard of MDA. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards. Concentrations of MDA in whole body samples are expressed in µM per g protein.

Catalase activity was measured by an assay with hydrogen peroxide based on formation of its stable complex with ammonia molybdate. 0.2 ml of supernatant was incubated in 1 ml reaction mixture containing 65 mM hydrogen peroxide in 60 mM sodium-potassium phosphate buffer, pH 7.4 at 25°C for 4 min. The enzymatic reaction was stopped with 1 ml of 32.4 mM ammonium molybdate and concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm. All chemical materials were obtained from Merck Co, Germany. All biochemical parameters were measured in duplicate by UV/Vis Unico spectrophotometer (Model 2100).

Statistical analysis: Statistical analyses were performed using SPSS (Release 19 IBM) software. Data are presented as mean ± SD. All the data were tested for normality (Kolmogorov-Smirnov test). Data were analyzed by one-way of variance analysis (ANOVA). The significant means were compared by Duncan test and a P<0.05 was considered statistically significant.

Results

Numerical value of LC₅₀ of fenpropathrin at 24, 48, 72 and 96 hours are presented in Table 1. Fish mortality was progressively elevated with increasing the concentration of fenpropathrin. Sub-lethal concentrations of fenpropathrin were determined according to 96h LC₅₀.

No mortality was observed in fish exposed to sub-lethal concentrations of fenpropathrin and control group during experimental periods. Loss of appetite, increased mucus secretion, increase in abnormal behavior, swimming in the surface of water and swimming vertically were the major changes observed in fish after exposure to the higher concentrations (5.50 and 12.60 µg/L) of fenpropathrin.

Changes in the enzymatic activities determined in the whole body of fish exposed to different concentrations of fenpropathrin are presented in Table 2.

AST activity in the whole body of fish exposed to 2.75, 5.50 and 12.60 µg/L fenpropathrin was significantly higher than in the control group. A
A significant increase in ALT activity was observed in the whole body of fish exposed to 5.50 and 12.60 µg/L fenpropathrin when compared with control group. AChE and CPK activities significantly decreased in the whole body of fish after exposure to all concentrations of fenpropathrin. There was a significant increase in the ALP and LDH activities in the whole body of fish exposed to 1.25, 5.50 and 12.60 µg/L fenpropathrin when compared with control group.

Alterations in the malondialdehyde, total protein, total antioxidant levels and catalase activity in the whole body of fish exposed to different concentrations of fenpropathrin are presented in Figures 1 to 4.

A significant increase in MDA levels in the whole body of fish exposed to fenproparthin was observed in all treated groups when compared with control group (Fig. 1).

Total antioxidant capacity in the whole body of fish exposed to fenpropathrin progressively decreased with increased concentrations of the pesticide (Fig. 2).

There was a significant increase in the CAT activity in the whole body of fish after exposure to 5.50 and 12.60 µg/L Fenpropathrin respect to the control group (Fig. 3).

The results of this study showed that total protein levels in the whole body of fish exposed to 2.75, 5.50
and 12.60 µg/L fenpropathrin significantly decreased when compared with control group (Fig. 4).

**Discussion**
The present results show that fenpropathrin is highly toxic to freshwater fish, *A. mossulensis*. The toxicity of fenpropathrin on *A. mossulensis* increased with increasing the concentration and exposure time. When fishes were exposed to 10 µg/L fenpropathrin, only 3.34% died after 96 h, whereas all the fishes (100%) died after 48 h when were exposed to a concentration of 1000 µg/L fenpropathrin. In addition, the 24, 48, 72 and 96 h LC$_{50}$ values of fenpropathrin of *A. mossulensis* were calculated as 562.3±45.2, 218.2±18.8, and 136.2±11.9 and 121.4±11.8 µg/L, respectively.

Imbalance, vertical swimming and swimming in the water, loss of appetite, bleeding at the base of the fins and eye balls were important clinical symptoms observed in fish exposed to high concentrations of fenpropathrin. Behavior disorders in fish exposed to fenpropathrin maybe associated to the neurotoxicity potential of pyrethroid pesticides. Neurological disorders in animals are often attributed to a dysfunction in ion channels of neurons, particularly sodium ions after exposure to pyrethroid pesticides (Sogorb and Vilanova, 2002; Banaee, 2013). However, a decrease in AChE activity may also play a role on the behavioral changes in fish after exposure to fenpropathrin. Reduced AChE activity in the whole body of *A. mossulensis* exposed to fenpropathrin may be due to changes in this enzyme function (Tayebati et al., 2009; El-Demerdash, 2011). Decreases in the AChE activity levels in different tissues of fish were observed after exposure to chlorpyrifos (Halappa and David, 2009; Sharbidre et al., 2011), diazinon (Banaee et al., 2011), methyl parathion (Sharbidre et al., 2011), monocrotophos (Rao, 2006), and atrazine (Santos and Martinez, 2012), permethrin and deltamethrin (Goulding et al., 2013). Although, there is no information on the detoxification mechanism of fenpropathrin in fish, free radicals that are produced during the biotransformation of this pesticide in liver tissue may be the most important cause of oxidative stress in *A. mossulensis*. Increases in MDA levels in the whole body extract of fish may be an important bio-indicator of lipid peroxidation in different tissues of freshwater fish, after exposure to fenpropathrin. An increase in MDA levels were reported in different tissues of fish exposed to diazinon (Oruç and Usta, 2007; Isik and Celik, 2008), deltamethrin (Yonar and Sakin, 2011), methyl parathion (Monteiro et al., 2006; Isik and Celik, 2008; Sharbidre et al., 2011), chlorpyrifos (Sharbidre et al., 2011), carbamazepine (Li et al., 2010), and trazine (Paulino et al., 2012). A decrease in total antioxidant capacity was an important detrimental response of the fish antioxidant defense system to increased free radicals after exposure to fenpaprothrin. Similar results were observed in rainbow trout and carp after exposure to diazinon and cyfluthrin (Sepici-Dinçel et al., 2009; Banaee et al., 2013). The overproduction of free radicals during pesticide detoxification may be associated with a decrease in the hepatic total antioxidant capacity (Monteiro et al., 2006; Banaee et al., 2013). Impairment in the synthesis of enzymatic and non-enzymatic antioxidants may be the most important factor in reducing the levels of cellular total antioxidant. Therefore, the decline in the cellular antioxidant capacity makes the cells more vulnerable to oxidative stress damage. A decrease in non-enzymatic antioxidant levels were observed in different tissue of fish exposed to chlorpyrifos (Sharbidre et al., 2011) and andatrazine (Santos and Martinez, 2012). The elevated MDA levels and total antioxidant capacity are indicative of increased oxidative stress in fish. The results in the present study indicated that the imbalance between oxidants and antioxidants in cells was the most important factor in causing oxidative stress in fish after exposure to fenpropathrin. Similar results were observed in the sheepshead minnow (*Cyprinodon variegatus*) (Harper et al., 2008), *Channa punctata* (Sayed et al., 2003; Atif et al., 2005), medaka (*Oryzias latipes*) (Ingeborg et al., 2002) exposed to bifenthrin esfenvalerate, respectively.
Catalase plays an important role in the elimination of hydrogen peroxide in cells (Banaee et al., 2013). Hydrogen peroxide may play a role in increasing MDA levels in the fish exposed to fenpropatrin. Moreover, an increase in catalase activity in the whole body of fish after exposure to high concentrations of fenpropatrin may be effective in the removal of hydrogen peroxide produced in cells during detoxification process. An increase in CAT activity in different tissues of fish exposed to atrazine (Paulino et al., 2012) was reported.

AST and ALT enzyme activities are important in cellular nitrogen metabolism, oxidation of amino acids, and liver gluconeogenesis (Banaee, 2013). In stress situations, increased activity of liver enzymes such as AST and ALT has stimulatory effects on gluconeogenic mechanism (Banaee, 2012; 2013). Thus, increased levels of these aminotransferase activities may have played an important role in energy supply for fish exposed to fenpropatrin. Similar changes were observed in Labeorohita after exposure to fenvalerate (Prusty et al., 2011). Increase in ALT and AST activities were observed in plasma, liver and kidney of Oreochromis mossambicus, Cyprinus carpio and Korean rockfish (Sebates schlegeli) after exposure to monocrotophos (Rao, 2006), bifenthrin (Velisek et al., 2009) and cypermethrin (Jee et al., 2005).

The results showed that the protein of the whole body was reduced, which may be related with the increased transaminase activities. The increased activity of these enzymes may lead to protein breakdown to provide energy and regeneration of tissue damages. Fish muscle breakdown under stress situation may be one of the main reasons for decreasing tissue protein. Decreased total protein levels were reported in Oreochromis mossambicus, O. niloticus, Cyprinus carpio, and Oncorhynchus mykiss exposed to endosulfan (Kumar et al., 2011), malathion (Patil and David, 2008), diazinon (Banaee et al., 2008; Banaee et al., 2011; Banaee et al., 2013), lindane (Saravanan et al., 2011), and cypermethrin (Korkmaz et al., 2009), bifenthrin (Velisek et al., 2009) and cypermethrin (Jee et al., 2005).

Reduction of muscle mass of fish may decrease CPK activity in fish after exposure to fenpropatrin. Rosalki (1998) believed that damage to connective tissues and a reduction of muscle mass are the main reasons for the decrease in the activity of the CPK enzyme.

After fish exposure to pesticides, LDH may increase to supply energy to fish. LDH is an enzyme that participates in the anaerobic pathway of carbohydrate metabolism. The increase of LDH activity is a diagnostic index widely used to recognize increases of anaerobic metabolism resulting from depletion of energy under anaerobic and environmental stress conditions (Banaee, 2012; 2013). The increase of LDH activity in the whole body extract of fish was a physiological mechanism to provide more energy to deal with the effects fenpropatrin on the freshwater fish, A. mossulensis. Similar changes in LDH activity were observed in crayfish exposed to endosulfan (Banaee and Ahmadi, 2011). Increased LDH activity in the gill and brain of tilapia, Oreochromis mossambicus after exposure to monocrotophos was reported by Rao (2006). In contrast, Tripathi and Shasmal (2011) showed an inhibitory effect of chlorpyriphos on LDH activities in different tissues of the fish.

ALP plays a significant role in phosphate hydrolysis and in membrane transport and it also acts as a good bio-indicator of stress in biological systems. Increased ALP activity in the whole body extract of fish may be due to the effects of fenpropatrin on transphosphorylation activity. Increases in ALP activity were reported in the whole body extract of crayfish after exposure to endosulfan (Banaee and Ahmadi, 2011). Rao (2006) reported that the ALP activities were increased in plasma, gills and kidneys of tilapia exposed to monocrotophos.

It can be concluded that fenpropatrin was highly toxic to A. mossulensis. Exposure to sub-lethal concentrations of fenpropatrin resulted in significant biochemical alterations and behavioral changes which may be potentially disruptive for the survivability of A. mossulensis. This fact should be taken into consideration when this pesticide is used.
for pest control in agriculture fields surrounding freshwater ecosystems. In conclusion, measuring oxidative stress biomarker and other biochemical parameters in the present study was useful for monitoring the sub-lethal effects of fenpropathrin on freshwater fish.

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