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Effects of chlorpyrifos on the freshwater mussel *Unio elongatulus eucirrus* (Bourguignat, 1860), with a focus on neurotoxicity and oxidative stress responses

Efectos del clorpirifos en el mejillón de agua dulce *Unio elongatulus eucirrus* (Bourguignat, 1860), con especial atención a la neurotoxicidad y las respuestas al estrés oxidativo

Faruk Zorlar, Engin Şeker, Ayşegül Pala*

Munzur University, Faculty of Fisheries. Tunceli, Türkiye.
**Corresponding author: aysegulpala@munzur.edu.tr*

ABSTRACT

This study set out to investigate the effects of the organophosphate insecticide chlorpyrifos (CPF) on certain biochemical parameters of the freshwater mussel *Unio elongatulus eucirrus.* Mussels were exposed to varying concentrations of CPF (10, 20, and 40 µg·L^{-1}) for duration of 96 hours. Samples were collected from the mussels at the 24th and 96th hours of the experiment. The results indicated that exposure to CPF concentrations led to a decline in the activities of acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities and, accompanied by an increase in malondialdehyde (MDA), and decreased reduced glutathione (GSH) levels in mussels. In conclusion, the present study demonstrates that CPF caused AChE inhibition, formation of oxidative stress, and negative effects on certain antioxidant parameters in the freshwater mussel *U. elongatulus eucirrus*.

Key words: Antioxidants; chlorpyrifos; *Unio elongatulus eucirrus*; oxidative stress

RESUMEN

Este estudio se propuso investigar los efectos del insecticida organofosforado clorpirifos (CPF) sobre ciertos parámetros bioquímicos del mejillón de agua dulce *Unio elongatulus eucirrus*. Los mejillones estuvieron expuestos a concentraciones variables de CPF (10, 20 y 40 µg·L^{-1}) durante 96 horas. Se recogieron muestras de los mejillónes a las 24 y 96 horas del experimento. Los resultados indicaron que la exposición a concentraciones de CPF provocó una disminución de las actividades de la acetilcolinesterasa (AChE), la superóxido dismutasa (SOD), la catalasa (CAT) y la glutatión peroxidasa (GPx) y niveles de glutatión reducido (GSH) un aumento en los niveles de malondialdehído (MDA) y en mejillones. En conclusión,el presente estudio demuestra que el CPF provocó la inhibición de la AChE, la formación de estrés oxidativo y efectos negativos sobre ciertos parámetros antioxidantes en el mejillón de agua dulce *U. elongatulus eucirrus*

Palabras clave: Clorpirifos; mejillón de agua dulce; *Unio elongatulus eucirrus;* estrés oxidativo; antioxidantes

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INTRODUCTION

The pervasive utilization of pesticides facilitates their dispersal to diverse aquatic ecosystems via surface runoff and bioaccumulation [1]. The distribution of pesticides in water has deleterious consequences for numerous non–target species inhabiting freshwater ecosystems [2]. Organophosphorus (OP) pesticides, which constitute over 50% of global pesticide usage, find application in diverse contexts (e.g. weed and insect control, sterilisation, etc.). These neurotoxic compounds, have been demonstrated to induce neural dysfunction in non–target organisms, result in paralysis due to overstimulation, and can even lead to the death of the organism [3].

Pesticides have been shown to inhibit the action of key enzymes in response to xenobiotic–induced stress, thereby increasing oxidative stress and neurotoxicity and exerting unrepairable effects [4]. CPF is an insecticide belonging to the OP group, which is poorly soluble in water, non–volatile, and insensible to UV. Due to its extensive utilisation and prolonged half–life, ranging from 10 to 120 days, CPF has frequently been detected in various countries in surface water, groundwater, and even rainwater [5]. The toxicant CPF is transmitted to the aquatic ecosystem through various means and is taken up by aquatic organisms, causing harmful effects [6]. CPF's action mechanism is to inhibit the acetylcholinesterase (AChE) enzyme, which causes acute and chronic toxicity to non–target organisms [7]. AChE is required for neurotransmission in the cholinergic synapses and is an often–used biomarker for determining the specific neurotoxicity of OPs in aquatic invertebrates [8]. Previous studies have noted that CPF, as to its inhibitory effect on AChE activity, also causes oxidative stress and affects the antioxidant system [1, 9, 10, 11, 12].

Freshwater mussels represent a significant component of aquatic ecosystems and contribute to a variety of ecosystem functions, including nutrient cycling and water quality maintenance [13]. Their filtration features and extensive geographical distribution have led to their utilization as bioindicator species in freshwater biomonitoring for a considerable duration [14]. The experimental organism in this study is *Unio elongatulus eucirrus*, a freshwater mussel species that is extensively distributed in freshwater reservoirs in Türkiye [15]. This present study aims to examine the effects of CPF on certain biochemical parameters in *U. elongatulus eucirrus*. To this end, the activity of acetylcholinesterase (AChE) was determined as a neurotoxicity marker, alongside malondialdehyde (MDA) level, reduced glutathione (GSH) level, and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, which were evaluated as oxidative stress biomarkers were in *U. elongatulus eucirrus* exposed to CPF.

MATERIAL AND METHODS

Chlorpyrifos

In the study was used a 48% form of chlorpyrifos (CPF) which purchased from a commercial enterprise (active ingredient CPF, 480 g·L^{-1}).

Freshwater mussel (*Unio elongatulus eucirrus*)

Freshwater mussels (*U. elongatulus eucirrus*) (FIG. 1), the living material of the study, were obtained from the local fishers in the Keban Dam Lake Çemişgezek aquaculture hunting area. The mussels were collected by scraping the areas with bubbles near the shore and brought alive to the Munzur University Fisheries Faculty Laboratory in clean 5 L plastic containers in the water of their environment. The creatures were placed in pre–prepared glass aquariums with oxygen provided by air motors (Sobo, SB-948, China) and kept in these aquariums for a month, allowing their adaptation to laboratory conditions. The mussels were fed with phytoplankton (mostly green algae) twice a day (d) during the adaptation period, and the aquarium water was changed every other d. A total of 120 mussels with an average width of 11.32 ± 1.09 mm, a length of 23.39 ± 1.74 mm, and a height of 10.95 ± 1.16 mm (GBS, 150mm Digital Caliper, Türkiye) were used in the study.

FIGURE 1. ***Unio elongatulus eucirrus*** **morphological view**

Application of CPF concentrations to *Unio elongatulus eucirrus*

Following a comprehensive review of the extant literature, the sublethal concentrations of CPF to be utilized in the experimental study were determined to be 10, 20, and 40 µg·L^{-1}, respectively [16, 17]. The experimental study comprised four distinct groups: Control, 10 µg·L^{-1} CPF, 20 µg·L^{-1} CPF, and 40 µg·L^{-1} CPF.

Ten mussels have been placed in each of the four experimental groups. All groups except the control group were exposed to CPF concentrations for 96 hours (h), and all experiments were performed in triple repeat. During the experiments, oxygen content, temperature, and pH values of the water were measured regularly (YSI, 556 MPS, multi–parameter device, USA)

Sample collection and preparation of supernatants

Five mussels were sampled from all groups at 24 and 96 h of the experiment. The soft tissue parts of the mussels were separated from their shells using forceps and scalpels, transferred to Eppendorf tubes, and storaged at -20°C (Vestel, BZP–M3203W, Türkiye) until analyzed.

To measure AChE activity, the tissue samples taken from all groups were weighed (AND, FX-300i, China) and diluted with 1/10 of 0.05 M sodium–phosphate buffer (containing 0.25 M sucrose, pH 7.4), and homogenized with a Teflon–glass homogenizer (IKA,

T10, Basic Ultra Turrax).The homogenates were centrifuged (NÜVE, NF 800 R, Türkiye) at 2223 G for 15 min, and supernatants were obtained. Soft tissue samples of 0.5 g were taken from the mussel samples and weighed, then PBS buffer (phosphate buffered saline solution) was added at a rate of 1/5 w/v and homogenized with a Teflon–glass homogenizer in the presence of ice. The homogenates were centrifuged (NÜVE, NF 800 R, Türkiye) at 588 G for 15 min in a refrigerated centrifuge, and the supernatants were used to determine oxidative stress parameters.

Determination of biochemical parameters

AChE activity was measured following the method of Elman *et al.* [18]. Oxidative stress parameters and antioxidant activities used kits (Cayman) with catalog numbers 10009055 (MDA), 703002 (GSH), 706002 (SOD), 707002 (CAT), 703102 (GPx), respectively, and measured on an ELISA reader. The protein level used to calculate specific AChE enzyme activity was determined according to Lowry *et al.* [19].

Statistical analysis

In statistical analysis of the data using the SPSS 24.0 statistics program. To this end, one–way variance analysis and Duncan tests were used for data.

RESULTS AND DISCUSSION

During the trials, the average oxygen content of the water was 8.50 ± 0.42 mg·L^{-1}, the temperature was 17.5 ± 1.6°C, and the pH value was 8.2 ± 0.1. No significant differences were recorded in the oxygen, temperature, and pH values of water throughout the study.

Changes in AChE activity

Effects of CPF on AChE level in *U. elongatulus eucirrus* are given in FIG.2.

At the 24th and 96th h of the study, AChE activity was statistically lower in all groups where CPF concentrations (10, 20, and 40 µg·L^{-1}) were applied than in the control group (P<0.05).

The AChE activity inhibition rates (%) in the control and experimental groups are given in FIG. 3.

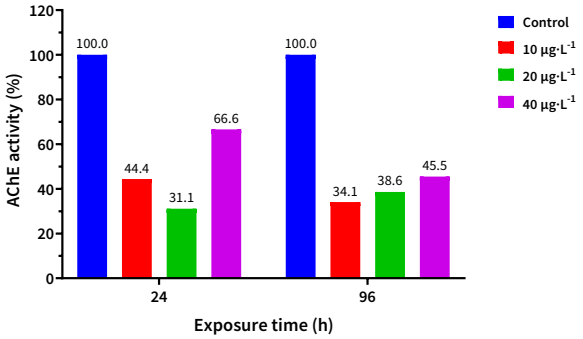

FIGURE 3. Inhibition rates (%) of AChE activity in *Unio elongatulus eucirrus*

In the experimental groups, the lowest AChE inhibition was 31.1% in the 20 µg·L^{-1} CPF group (24th h), and the highest inhibition rate was observed as 66.6% in the 40 µg·L^{-1} CPF group (24th h).

AChE is an important enzyme used as a biomarker in biomonitoring research of aquatic invertebrates and is specifically inhibited by OPs and carbamate pesticides [8]. CPF is known as a good AChE inhibitor [20]. This study was determined that CPF significantly inhibited the AChE enzyme activity in the soft tissue of *U. elongatulus eucirrus* (inhibition rate; min. 31.1 max. 66.6%). Similar results were obtained by Hanna *et al.* [1] that AChE activity in the gills of *U. tigridis* exposed to CPF decreased significantly with increasing concentration, and this decrease occurred gradually from 24 to 96 h. In addition, in freshwater mussel (*Amblema plicata*) exposed to CPF, AChE activity was found to be similar to control after 24 h of exposure, but decreased significantly after 96 h [21]. This result was compatible with the findings of Stalin *et al.* [22]. Also, Stalin *et al.* [22], noted that 73% inhibition of hepatopancreatic AChE activity occurred in freshwater mussel Lamellidens marginalis that 5 ppm CPF for 30 d. These findings are consistent with studies reporting that AChE inhibition occurs in different species of freshwater mussels exposed to CPF. The observed AChE inhibition is due to the neurotoxic effect of CPF.

Changes in MDA level

Effects of CPF on MDA level in *U. elongatulus eucirrus* are given in FIG. 4.

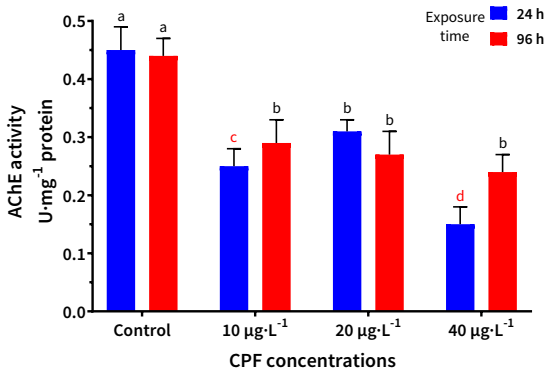

FIGURE 2. Effects of CPF on AChE activity in *Unio elongatulus eucirrus*

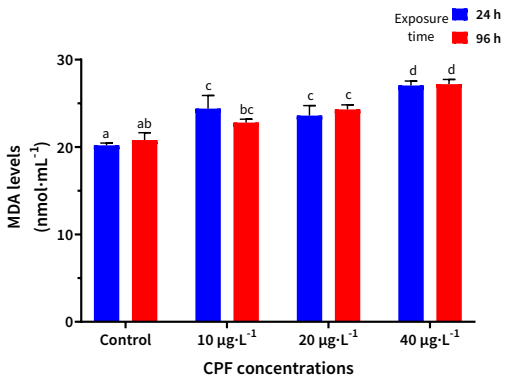

FIGURE 4. Effects of CPF on MDA level in *Unio elongatulus eucirrus*

The MDA level of the 10 µg·L^{-1} CPF group, was found to increase compared to the control group at the 24th h (P<0.05) and close to the control at the 96th h (P>0.05). The MDA levels of other CPF groups were higher than the control group throughout the experiment (P<0.05).

Oxidative stress occurs when antioxidants are depleted or excessive accumulation of ROS, or both, are out of balance. Lipid peroxidation (LPO) is a cellular damage mechanism and indicator of oxidative stress [9]. MDA is one of the end aldehyde products of LPO, and its level in organisms reflects the degree of free radical–induced LPO damage [23, 24]. In this study, the MDA levels of mussels in all groups given CPF were higher than the control group. (P<0.05). Similar to the findings of this study, Al–Fanharawi *et al.* [25] reported that *Unio tigridis* exposed to three different CPF concentrations for 21 d caused a significant increase in MDA with the highest CPF concentration (65 mg·L^{-1}). A similar result was noted by Hanna *et al.* [1]. They observed a gradual increase in MDA levels with increasing CPF concentration over the 96 h experimental period. This increase in MDA levels indicates that CPF causes an increase in ROS production.

Changes in GSH level

Effects of CPF on GSH level in *U. elongatulus eucirrus* are given in FIG. 5.

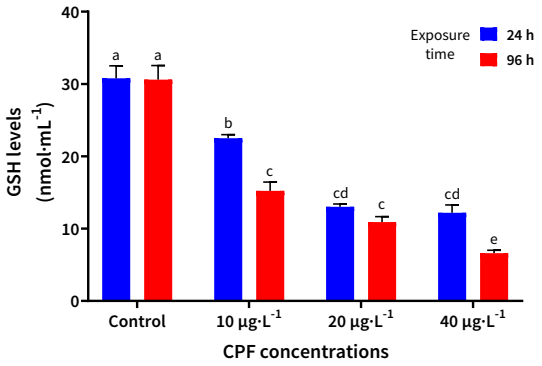

FIGURE 5. Effects of CPF on GSH level in *Unio elongatulus eucirrus*

At the 24th and 96th hours of the study, the GSH levels of all groups treated with CPF were lower compared to the control group. (P<0.05).

GSH protects cells from cellular redox cycle exchanges caused by toxic substances. Its decrease or depletion may increase the risk of oxidative stress [26]. In this study, the GSH level of mussels in all experimental groups given to CPF was detected to be lower than the control group throughout the experiment (P<0.05). Köprücü *et al.* [27] noted that in the GSH level was an important decrease in the digestive glands and gills of *U. elongatulus eucirrus*, in which sublethal concentrations of cypermethrin were administered for 96 h. Additionally, was reported a reduction (approximately 39%) in GSH levels of the gills of the freshwater mussel *Unio ravoisieri* exposed to 50 and 100 µg·L^{-1} concentrations of permethrin for seven d [28].

The present study results were similar to the findings of studies with other pesticides on freshwater mussels. The reason for the decrease in GSH levels may be the increase in the use of GSH converted to oxidized glutathione and inadequate GSH regeneration [9].

Changes in CAT activity

Effects of CPF on CAT activity in *U. elongatulus eucirrus* are given in FIG. 6.

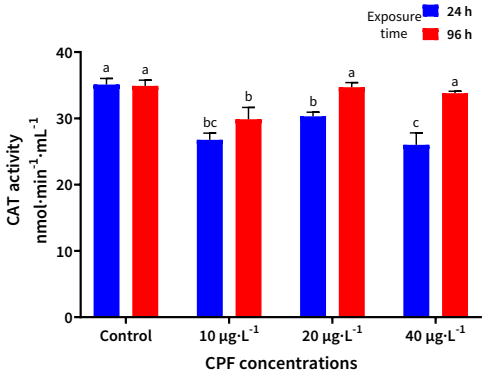

FIGURE 6. Effects of CPF on CAT activity in *Unio elongatulus eucirrus*

The CAT activity of the 10 µg·L^{-1} CPF group was lower than the control group at 24 and 96 h (P<0.05). The CAT activity of the 20 µg·L^{-1} CPF and 40 µg·L^{-1} CPF groups was lower than the control group at 24 h of the experiment and close to the control group at 96 h (P>0.05).

CAT has a protective role against free radicals and converter H_2O_2 to H_2O an important antioxidant enzyme [29]. In this research, CPF sublethal concentrations induced decreases in CAT activity in soft tissues of *U. elongatulus eucirrus*. These findings corroborate previous studies with CPF and other pesticides in the mussels. For example; it has been reported that CAT activity in freshwater mussel *U. tigridis*, where 22, 32, and 65 g·L^{-1} concentrations of CPF were applied for 21 d, decreased with the highest CPF concentration [25]. Similarly, a decrease in CAT activity (at the highest concentration) was submitted in the gills of *U. ravoisieri* exposed to 50 and 100 ug·L^{-1} permethrin for seven days [28]. However, in the current study, CAT activity increased at the 96th h of the experiment and approached the control group. This increase in CAT activity may be due to the neutralization of superoxide radicals increased by CPF stress.

Changes in SOD activity

Effects of CPF on SOD activity in *U. elongatulus eucirrus* are given in FIG.7.

At the 24th h of the study, the SOD activity of all groups administered CPF was close to the control (P>0.05). At the 96th h, a significant decrease was in the SOD activity of other groups except the 20 ug·L^{-1} CPF group compared to the control group (P<0.05).

SOD, CAT, and the GSH–dependent enzymes (GSH–Px, etc.) have an important role in the process of detoxification of ROS as

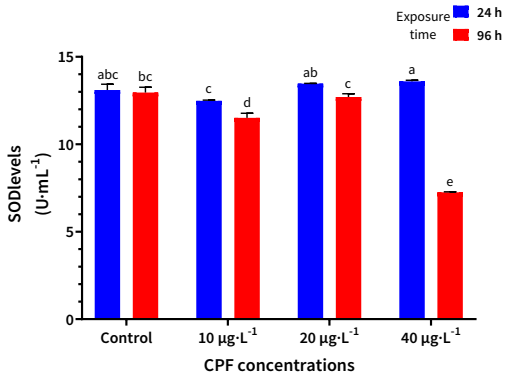

FIGURE 7. Effects of CPF on SOD activity in *Unio elongatulus eucirrus*

antioxidants [30]. The SOD enzyme has a major mission in the defense mechanism of biological cells. It catalyses the degradation of oxygen radicals (O_2) and produces oxygen and hydrogen peroxide (H_2O_2) molecules [31]. In this study, there was a decrease in the SOD activity of mussels that concentrations of CPF applied for 96 hours compared to the control (excluding 20 µg·L^{-1} concentration). Similar observations were reported by Fanharawi *et al*. [25]. They exposed the mussel *Unio tigridis* to three different CPF concentrations (22, 32, and 65 mg·L^{-1}) for 21 d. And they revealed that except for the group given the lowest CPF concentration, SOD activity in the *U. tigridis* was reduced. in the other groups. These findings also coincide with the reported by Caccitore *et al*. [30], who recorded a significant decrease in the SOD activity of freshwater gastropod *Planorbarius corneus* exposed to 7.5 µg·L^{-1} CPF concentrations for 48 h.

Changes in GPx Activity

Effects of CPF on GPx activity in *U. elongatulus eucirrus* are given in FIG. 8.

It was detected that the GPx activity of all groups treated with CPF was significantly lower than the control group at the 24th and 96th h of the study (P<0.05). GPx plays a mean role in glutathione metabolism and has a much higher affinity for H_2O_2 than CAT can remove H_2O_2 even at low concentrations [32]. In this study, GPx activity was reduced in the mussels to which CPF was applied in all groups. This result agrees with the previous observation

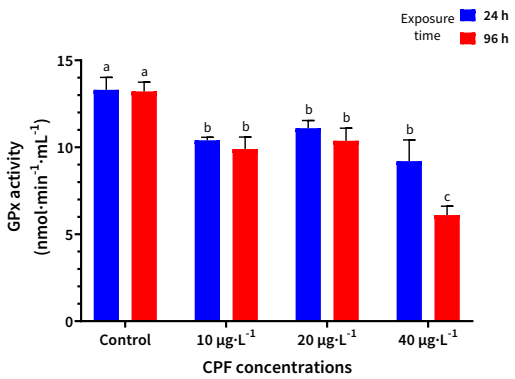

FIGURE 8. Effects of CPF on GPx activity in *Unio elongatulus eucirrus*

by Janaki Devi *et al.* [33], who demonstrated that sea mussels (*Donax faba*) exposed to sublethal concentrations of CPF for 96 h decreased GPx activity in all tissues compared to the control group. It has been reported that decreased GPx activity may be associated with a reduction in GSH levels, which is needed to reduce the effect of ROS [34].

CONCLUSIONS

In conclusion, this study showed that CPF exerts neurotoxic effects by inhibiting AChE activity up to 66% in freshwater mussel *U. elongatulus eucirrus*, causing oxidative stress and changes in antioxidant enzymes.

Conflict of Interest

The authors declared no conflict of interest.

ACKNOWLEDGEMENT

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