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How Does Cyphenothrin Affect the Freshwater Mussel as In Vitro and In Vivo Models?

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Abstract Cyphenothrin, one of the synthetic pyrethroids, developed as an alternative to organophosphorus and carbamate pesticides. It is used in veterinary medicine and household application against insects. Due to the contamination of the aquatic ecosystems, non-target aquatic organisms are affected. The current study aimed to evaluate the cyphenothrin effects on in vitro and in vivo models of freshwater mussels *Unio delicatus* Lea, 1863. While antioxidant enzyme (glutathione) was measured in both models, the total hemocyte counts were only detected in vivo models after exposure to cyphenothrin (1 and 10 µg/L) for 24-h and 48-h exposure times. A decrease in total hemocyte count occurred depending on the dose and duration ($p < 0.001$). In both in vitro and in vivo models of gill and digestive gland tissues, higher glutathione levels were obtained at a dose of 10 µg/L compared to the control groups in both exposure times ($p < 0.001$). The results of the study suggest that the antioxidant parameters could represent biomarkers to evaluate the effects of pollutants on in vitro and in vivo models of freshwater mussels.

Keywords Cyphenothrin · Glutathione · Total hemocyte counts

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1 Introduction

Anthropogenic activities cause pollution of aquatic ecosystem with pesticides. The pesticides in the aquatic ecosystems result in various effects on aquatic organisms (Timpano et al., 2022). The main types of pesticides found in the marine and freshwaters are herbicides, insecticides, and fungicides (Staley et al., 2015). Among insecticides, synthetic pyrethroids are widely used chemicals. They are derived from pyrethrin extracts from the flowers of *Chrysanthemum cinerariaefolium* (Ensley, 2018). Many pyrethroid varieties show high efficacy as insecticides for agricultural and domestic use. Due to their low toxicity to mammals and their short-term persistence in the environment, they are highly used in insecticide species (Feng et al., 2009; Lutnicka et al., 1999). Synthetic pyrethroids are classified into two types. While type I compounds that lack an alpha-cyano moiety include permethrin, resmethrin, and tetramethrin, type II compounds contain an alpha-cyano-3-phenoxybenzyl substituent including cypermethrin and cyphenothrin (Ensley, 2018).

Cyphenothrin (CAS No. 39515–40-7) is commonly used in veterinary medicine against ectoparasites and household application against flies, mosquitoes, and cockroaches due to its lower toxicity and effective insecticidal potential (Huang et al., 2020; Yücel & Özkul, 2016). However, this widespread use of cyphenothrin contaminates terrestrial and aquatic ecosystems, affecting non-target species as

well (Huang et al., 2020; Zhan et al., 2020). Lethal concentration values (LC_{50}) of cyphenothrin were investigated in studies with different aquatic species. The LC_{50} values are found for *Daphnia magna* as 0.92 µg/L and 0.43 µg/L for 48 h (USEPA/OPP, EFED, 2020), for *Barbus macrops* as 15 µg/L for 24 h, 12 µg/L for 48 h, and 10 µg/L for 72 h (Yameogo et al., 1991), for *Chrysichthys longidorsalis* as 630 µg/L for 24 h and 220 µg/L for 48 h (Yameogo et al., 1991), for *Oncorhynchus mykiss* as 0.34 µg/L and 0.37 µg/L for 96 h (USEPA/OPP, EFED, 2020), and for *Lebistes reticulatus* as 48.7 µg/L for 96 h (Erkmen et al., 2000). In addition, in a study conducted with common carp *Cyprinus carpio* exposed to cyphenothrin at a dose of 5 µg/L for 75 days, it was shown that histopathological findings were obtained in the gill, liver, kidney, and brain tissues (Yücel & Özkul, 2016).

Water pollutants have toxic effects on aquatic organisms and a wide range of biomarkers are used to investigate their effects on the cellular, biochemical, and physiological parameters (Wu & Wang, 2010). These methods measure the specific responses of organisms such as the alteration of total hemocyte counts to reflect the species' health/immune system (Andreyeva et al., 2019) or the glutathione values to show for oxidative stress (Almeida et al., 2005).

Mussels, filter-feeding organisms, are used as sentinels of aquatic toxicology studies and considered a non-target group to water pollutants toxicity (Wang et al., 2019). There are many studies conducted on marine and freshwater mussels that reported the alterations of cellular, biochemical, and physiological parameters of the organisms exposed to pollutants (Almeida et al., 2005; Verlecar et al., 2008; Wu & Wang, 2010; Wang et al., 2019; Stara et al., 2020; Katalay et al., 2022). One of the first physiological responses that points to environmental stresses in mussels is the change in the number of cells in the hemolymph; hemolymph is a tissue responsible for substance transport and the immune system in the organisms (Andreyeva et al., 2019). Abiotic or biotic-induced changes in the aquatic ecosystem may affect the normal metabolic activities of mussels. The oxidative stress mechanism is activated due to the production of reactive oxygen species (ROS) in organisms. The antioxidant defense system develops a defense system against this oxidative stress. These antioxidant systems, both enzymatic and non-enzymatic, are biomarkers used in environmental monitoring studies (Verlecar et al., 2008).

The use of mussels in scientific studies is increasing day by day both in the development of mussel farming areas and in basic research and analysis of pollutants. Studies with mussels are starting to leave their place to alternative models with the development of the 3R rule (refinement, reduction, and replacement) over time (Barrick et al., 2019). In vitro studies have been developed as an alternative to the investigation of toxic substances in in vivo animal experiments, providing rapid results and keeping the number and cost of experimental animals low. These models reveal the toxic effects of pollutants with great precision and reproducibility (Gómez-Mendikute et al., 2005). In vitro systems developed from different tissues of mussels are used to investigate the effects of aquatic pollutants (Yurdakök-Dikmen et al., 2018; Gómez-Mendikute et al., 2005; Arslan et al., 2021).

It is observed that studies comparing in vitro and in vivo applications are generally used in fields such as biomedical applications (Guzmán-Soto et al., 2021; Samadian et al., 2021), cancer research (Zhu et al., 2022), and pharmacological (Xiao et al., 2021) studies. The number of studies in the field of aquatic toxicology is quite low. Besides, the comparison of the effects of pollutants on the marine or freshwater mussels in vitro and in vivo models is limited (Panfoli et al., 2020; Barrick et al., 2019). Besides, when the studies of cyphenothrin on aquatic organisms are examined, no studies have been found on physiological or biochemical studies with freshwater mussels. The aim of this study is (1) to determine the first response of cyphenothrin on total hemocyte counts in vivo and (2) to investigate the effects of cyphenothrin on mussel gill and digestive gland cells in vivo and in vitro by using the glutathione parameter end-point.

2 Materials and Methods

2.1 Experiment Organisms

The model organism of this study is *Unio delicatus* Lea, 1863. Due to habituating a wide range area including river basins of the southwest to the east of Anatolia, the species is used for biomonitoring of the

aquatic ecosystems to investigate the water quality and pollution (Lopes-Lima et al., 2021). Gölbaşı Lake located south Anatolia (Adıyaman, Turkey) has rich biodiversity including *Unio delicatus* (Alkan Uçkun, 2018). The freshwater mussels were obtained from local fishermen from Gölbaşı Lake and were brought to the laboratory in the aerated water. To adapt to laboratory conditions, they were placed in 15 L aquariums containing 10 L of dechlorinated tap water for 2 weeks. The water was changed every 2 days by siphoning. During the adaptation period, the mussels were fed by Cyanobacteria *Spirulina* sp.

2.2 Chemical Preparation

The stock concentration of cyphenothrin (purity 94%) was prepared via dimethylsulfoxide (DMSO) as 10 mg/L. Before the experiments, the sublethal concentrations of the insecticide were determined by the preliminary experiments as 1 and 10 µg/L. The stock concentration was diluted with water for in vivo experiments while it was diluted with cell culture medium (Leibovitz 15) for in vitro experiments.

2.3 In Vivo Exposure Experiments

For 24 h and 48 h exposure times, there were two controls (non-treated and solvent) and two cyphenothrin groups (1 and 10 µg/L CP) in the experiments ($n=80$). Each groups contained 10 mussels which were selected randomly from stock aquariums. After each exposure time, ten mussels were sampled and taken their weight (34.35 ± 6.06 g) and length (5.05 ± 0.41 cm) parameters. With the help of 2.5 mL injection, the hemolymph tissues were taken from mussels. The total hemocyte counts (THCs) were evaluated according to Yavuzcan and Benli (2004). Then, the mussels were dissected, and their gill and digestive gland tissues were taken. The tissues were kept in − 80 °C until the biochemical analysis.

2.4 In Vitro Exposure Experiments

Three mussels, mean length of 4.98 ± 0.07 cm and mean weight of 33.09 ± 1.57 g, were chosen randomly from stock aquariums. The primary cell culture from gill and digestive gland tissues was made according to Yurdakök-Dikmen et al. (2018). To eliminate the contamination sources, the mussels were kept in absolute ethanol 1 min and the shells were flamed for 3 s. Then, the mussels were dissected with sterile scissors and pens under sterile conditions. The gill and digestive gland tissues were taken into sterile Petri dishes. After cutting into 3–5 mm pieces, the tissues were kept with trypsin solution (Capricorn Scientific, Germany) containing penicillin–streptomycin (Capricorn Scientific, Germany), amphotericin B (Capricorn Scientific, Germany), and fetal bovine serum (Capricorn Scientific, Germany) for 4 h. The cell culture medium Leibovitz 15 (Sigma Aldrich, USA) was added and the Petri dishes were incubated for 24 h. Then, the solution of Petri dishes was pipetted and filtered with 200 µm cell strainers. The filtered solutions containing cells were centrifugated for 10 min at 400 rpm and the supernatant was removed. The cell pellets were ready to exposure experiments. For the cyphenothrin exposure, the cells were placed into 24-well plate at the density of 10^5 cells/well. The cells were incubated for 24 h to attach to the well surface. The cell medium was removed and cyphenothrin concentrations (1 and 10 µg/L CP) were applied three replicates on each plate. The exposure durations were 24 h and 48 h. In each plate, there were two control groups: non-treated cells and solvent control group (DMSO).

2.5 Glutathione Assay

Glutathione analysis was performed according to the Ellman (1959). For this, cell and tissue samples were homogenized with metaphosphoric acid and then centrifuged at 3500 rpm + 4 °C for 10 min. Samples whose supernatants were taken were mixed with Ellman reagent and measured spectrophotometrically at wavelengths of 410 (A1) and 420 (A2) nm. After the determination of cell and tissue protein amounts by the Bradford (1976), glutathione values were obtained as (A1-A2)/protein.

2.6 Statistical Information

All the parameters in this study were distributed normally according to the Kolmogorov–Smirnov normality test. Data were expressed as mean ± standard deviation in the graphs. Differences were tested by one-way ANOVA test. The values $P<0.05$ were considered statistically significant. Statistical analysis was done using GraphPad Prism 5 program.

3 Results

In this study, in vitro and in vivo systems of freshwater mussels were exposed to cyphenothrin concentrations of 1 and 10 µg/L for 24 h and 48 h. At the end of the exposure times, the total hemocyte count was examined in in vivo systems, while the glutathione parameter was examined in in vitro and in vivo systems.

As in vivo study with freshwater mussels, the total hemocyte counts were evaluated for determination of the physiological effects of cyphenothrin. The changes of the total hemocyte counts of mussels exposed to two different cyphenothrin concentrations for 24-h and 48-h exposure periods is shown in Fig. 1. At the end of the 24-h exposure, the total hemocyte count was higher in the control groups. In 1 and 10 µg/L dose groups, 1.66 and 1.22 times less THCs were detected compared to the control group, respectively ($p = 0.018$). Similarly, after 48 h of exposure, the total hemocyte count was higher in the control groups. While 1.45 times less THCs were detected in the 1 µg/L dose group, 0.5 times less THCs were found in the 10 µg/L dose group compared to the control group ($p = 0.002$). When 24-h and 48-h exposure conditions were compared, high THC values were observed in the control, solvent control, and dose groups during the 24-h exposure period compared to 48-h exposure groups. Thus, there is a significant difference between the two periods ($p < 0.001$). The trend of change in the total hemocyte counts in *U. delicatus* was found to be non-linear exposed to cyphenothrin concentrations.

The changes in glutathione level, which is one of the oxidative stress parameters, were investigated in the gill and digestive gland tissues of freshwater mussels exposed to cyphenothrin (Fig. 2). It was observed that significantly higher values were obtained in the gill tissues in the groups at the dose of 10 µg/L at 24-h and 48-h exposure compared to the control group ($p < 0.001$). Glutathione levels at the dose of 10 µg/L increased 1.9 and 1.5 times in the gill tissue compared to the control group during 24 h and 48 h of exposure. Unlike gills, glutathione level at a dose of 10 µg/L decreased 1.6 times but increased 1.4 times in the digestive gland tissue compared to the control group in 24 h and 48 h of exposure, respectively ($p < 0.001$).

Similar results have been obtained in in vitro studies of freshwater mussels as well as in vivo experiments (Fig. 3). Glutathione levels in the gill cell culture were increased by 1.34 times at 24-h exposure and 1.61 times at 48 h of exposure in the 10 µg/L dose group compared to the control group ($p < 0.001$). Like gill cell cultures, in the digestive gland cell culture, a 0.79-fold increase in 24-h exposure and 1.52-fold increase in 48 h were observed compared to the control group cells ($p < 0.001$).

4 Discussion

Mussels, important species diversity among aquatic organisms, absorb aquatic pollutants through filtering the water and lead to bioaccumulation in the food chain. Due to these properties, they help to reduce the pollutant load in those systems by transferring them

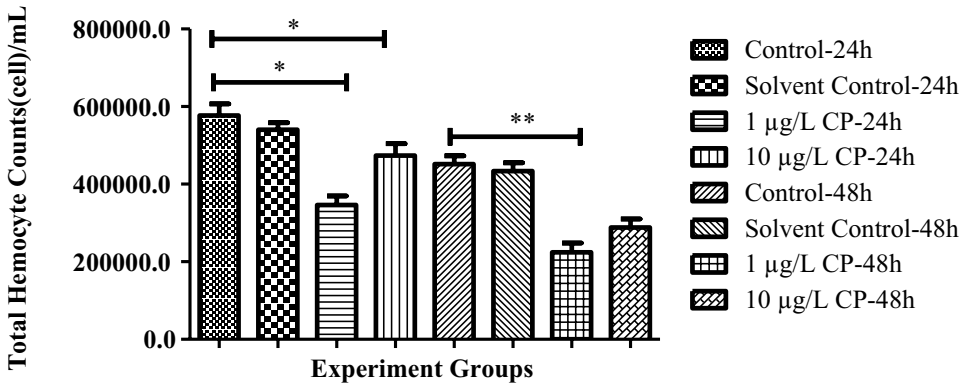

Fig. 1 Total hemocyte counts (* means $p < 0.05$; ** means $p < 0.005$)

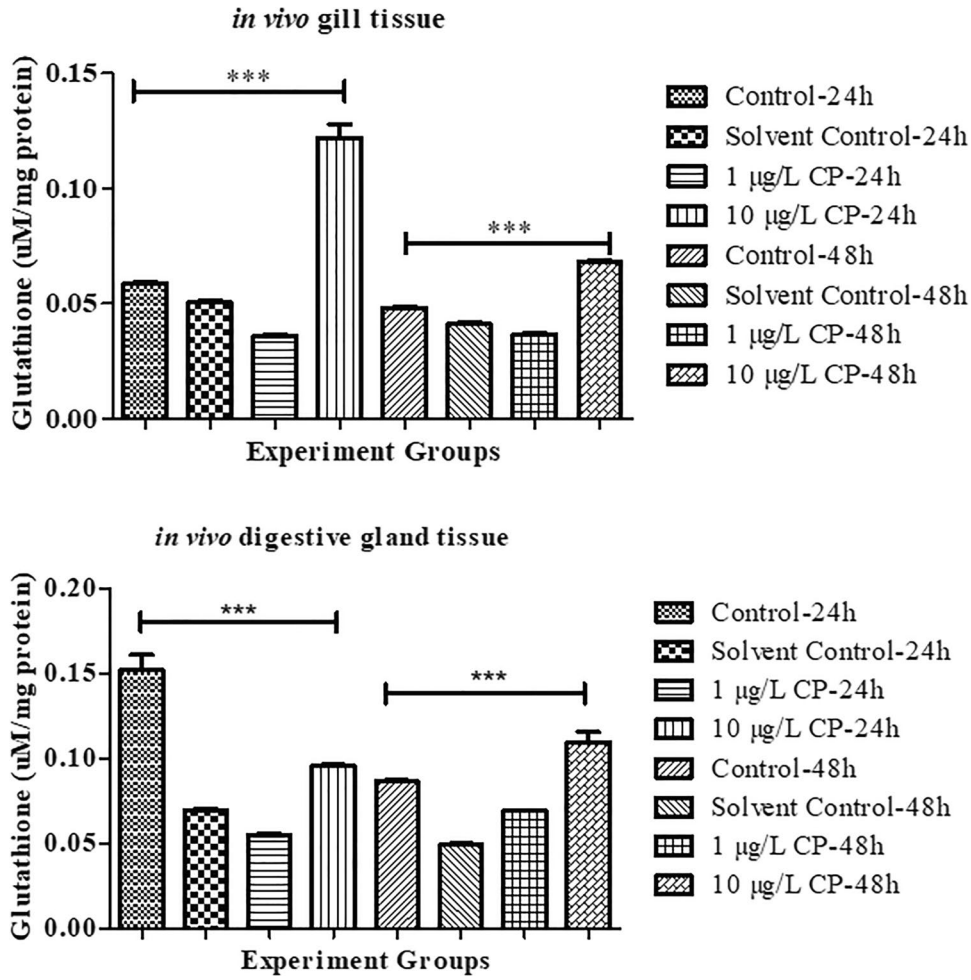

Fig. 2 The glutathione values of in vivo experiments of freshwater mussels (*** means $p < 0.001$)

to aquatic systems where the pollutant load is known to increase. Thus, they are used to reflect a load of pollutants, especially in field studies, as well as to investigate the effects of pollutants at different biological levels in laboratory studies (Doyotte et al., 1997; Stara et al., 2020). Therefore, they are considered model organisms to biomonitoring the aquatic system health status to provide accurate biological endpoints such as physiological, cellular, and biochemical (Faggio et al., 2018).

Due to the increase in pollution in aquatic ecosystems, the immune system of mussels weakens and the potential for disease increases. Against this phenomenon, the immune system, which consists of cellular and humoral components, comes into the situation. The hemocyte cells in the hemolymph tissue, which contains the immune system elements, reveal the physiological and immunological status of the mussels (de la Ballina et al., 2022). There are studies in which the total hemocyte counts decrease or increase in marine and freshwater mussels exposed to different pollutants (Arslan, 2022; Gürkan, 2022; Li et al., 2022). The THC results of this study showed a rapid decrease in the lowest concentration of cyphenothrin in both exposure times. These results indicate that hemocyte circulation decreased in the hemolymph tissues of organisms exposed to chemical (Gürkan, 2022). Moreover, the THCs at low dose of cyphenothrin are less than that at high dose can be explained by the slow response of the organism's metabolism. Ray et al. (2013) obtained similar results to the current study

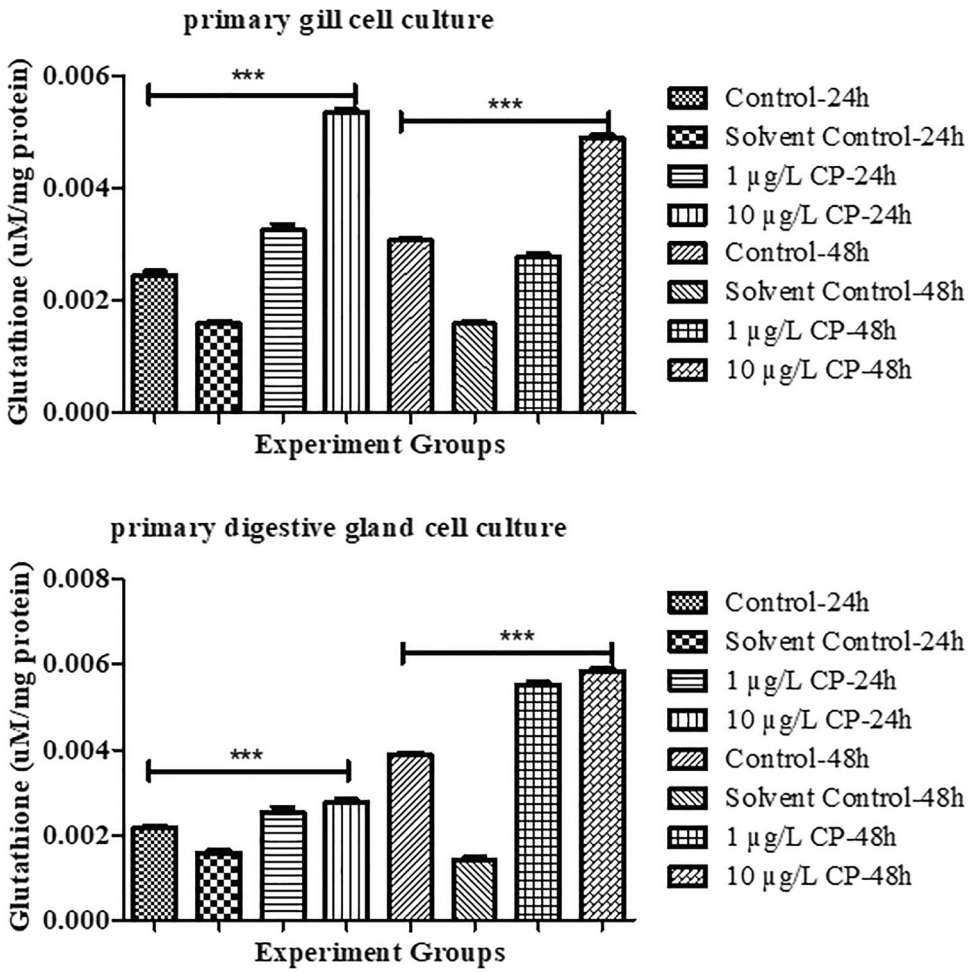

Fig. 3 The glutathione values of in vitro experiments of freshwater mussels (*** means $p < 0.001$)

in aquatic invertebrates exposed to cypermethrin and fenvalerate.

Glutathione, one of the compounds with a thiol group in the cell, is metabolically active in the organism against exposure to endogenous and exogenous chemicals and is one of the cell defense mechanisms (Canesi et al., 1999). Glutathione, which is among the antioxidant mechanisms, is used as a biomarker for contaminant-mediated in many aquatic organisms (Almeida et al., 2005; Regoli, 1998). In the current study, both models of freshwater mussels marked alterations in the glutathione levels of digestive glands and gills.

One of the tissues used in the screening of health indicators of mussels is the digestive gland tissue. The digestive gland is involved in processes such as secretion, enzyme production, and digestion. In addition, lysosomes in cells take part in the detoxification process of toxic substances (Faggio et al., 2018). Due to the importance of xenobiotics in the detoxification process in the organism, digestive gland tissues have been studied as in vivo or in vitro models in many studies (Wilhelm Filho et al., 2001; Canesi et al., 2007; Yurdakök-Dikmen et al., 2018; Gürkan, 2022). In this study, the effect of the insecticide on the glutathione parameter was investigated in the digestive gland tissue both in vitro and in vivo. In both models, the glutathione levels were higher in the high-dose group than in the control groups during the exposure times. Obtaining similar results in both models proves that digestive gland primary cell cultures can be used as an alternative

to in vivo experiments. In addition, these results support the use of digestive gland cell cultures made with marine and freshwater organisms as a usable model for investigating oxidative stress parameters caused by pollutants (Birmelin et al., 1999; Parolini et al., 2011; Balbi et al., 2017).

Gill tissue is responsible for the respiration of the mussels (Wu et al., 2022) and is the first organ contact the xenobiotics in the aquatic environment (Günal et al., 2021; Nimet et al., 2020). In this study, in which the oxidative parameter of the gills was evaluated in vitro and in vivo systems, it was observed that the glutathione level increased at high doses compared to the control groups, while a decrease occurred at low doses. Like digestive gland cell cultures, primary gill cell cultures are also useful tool for the evaluating the toxic effects of pollutants (Arslan et al., 2021; Parolini et al., 2011; Yurdakök-Dikmen et al., 2018).

In conclusion, cyphenothrin is toxic freshwater mussels. Among physiological parameters, the lowest THCs values were found in the lower concentrations of cyphenothrin in both exposure times, but the higher concentrations were also decreased compared to control groups. This is due to the difference in metabolism in the recovery process of the organism against the dose of insecticide. On the other hand, the glutathione levels of the digestive gland and gill tissues were increased in the higher concentration of cyphenothrin in both exposure times. Similar results were obtained in both in vitro and in vivo tissue comparisons. Therefore, the current study demonstrated that *Unio delicatus* primary gill and digestive gland cell cultures should be absolutely considered in screening ecotoxicological evaluations to assess the potential hazard of aquatic pollutants. Hence, the 3R rule can be adopted by turning to in vitro systems when carrying out the studies on aquatic pollutants.

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Author Contribution The author designed the study, performed the experiments, evaluated the results, and wrote the manuscript.

Data Availability The research has no associate data.

Declarations

Conflict of Interest The author declares no competing interests.

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