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Does Fipronil Affect on Aquatic Organisms? Physiological, Biochemical, and Histopathological Alterations of Non-Target Freshwater Mussel Species

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Abstract: Fipronil is widely used against insects in agriculture and ectoparasites in domestic areas and veterinary medicine. However, fipronil may influence non-target species as a result of the contamination of aquatic ecosystems. The present study aimed to investigate the acute and sublethal effects of fipronil in freshwater mussels (*Unio delicatus*), a non-target species, with physiological, antioxidant action mechanisms and histopathological observations. The 96-h LC_{50} value of fipronil was found to be 2.64 (1.45–4.56) mg/L. Sublethal concentrations were applied at $^{1}/_{10}$ and $^{1}/_{5}$ of 96-h LC_{50} as 0.264 mg/L and 0.528 mg/L for 48-h and 7-d. Haemolymph samples, digestive gland and gill tissues of mussels were taken after exposure times. While the Total Haemocyte Counts decreased in 48-h of exposure, it was only high at 0.264 mg/L fipronil-exposed for 7-d ($p < 0.05$). While glutathione values in digestive glands and gills were higher in the fipronil applied groups ($p < 0.05$), the AOPP values were only higher in the digestive glands at 7-d of exposure ($p < 0.05$). Moreover, fipronil caused histopathological alterations on gills and digestive glands. These things considered, the principal component analysis revealed that the most pronounced changes in the antioxidant action mechanisms were caused by the fipronil exposure. These results show that sublethal concentrations of fipronil are toxic to freshwater mussels.

Keywords: mussels; LC_{50}; AOPP; glutathione; total hemocyte counts; histology

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1. Introduction

Fipronil (C12H4Cl2F6N4OS, 5-amino 1-[2,6-dichloro-4-(trifluoromethyl)phenyl] 4-(trifluoromethyl sulfinyl) pyrazole-3-carbonitrile; CAS No: 120068-37-3) is used as a systemic herbicide in some monocotyledonous plants, and is frequently used in veterinary medicine as an acaricide and insecticide, including in pet animals against external parasites such as insects, lice, fleas, and ticks [1–3].

Fipronil is a highly active molecule and a potent disruptor of the insect central nervous system, interfering with the passage of chloride ions through the chloride channel regulated by γ-aminobutyric acid (GABA). This reaction results in uncontrolled central nervous system activity and the subsequent death of the insect. Although the GABA channel is important in nerve conduction in both vertebrates and invertebrates and fipronil binds to GABA receptors, the binding is less tight in terms of selectivity than in invertebrates. However, some of the toxicity of fipronil observed in mammals seems to include interference with the normal functioning of the GABA receptor [1].

According to Section 18 of the "Ecological Risk Assessment for Fipronil Use to Control Cabbage—Maggot in Turnip and Rutabaga" report by the U.S. Environmental Protection Agency, fipronil causes a highly toxic effect on birds, mammals, freshwater fish, freshwater invertebrates, algae, and vascular plants [4]. Due to its persistence in soils, fipronil accumulates in soils and sediments [5]. Via anthropogenic activities and runoff to agricultural areas, fipronil has contaminated surface and groundwater ecosystems [6]. For these reasons,

fipronil has been detected in various parts of the aquatic ecosystem. For example, fipronil ranging from 0.117 µL to 8.41 µL was detected in the water samples taken from the aquatic systems of Los Angeles. In addition, in the sediment samples from the same ecosystems, fipronil sulphide, fipronil desulfinyl, and fipronil sulfone were found to range from 0.636 to 24.8 µg/kg, 0.55 to 7.01 µg/kg, and non-detected to 10.5 µg/kg, respectively [4].

Reactive Oxygen Species (ROS) are produced in cells in different amounts. Low concentrations of ROS express physiological characteristics, including immune response, while high concentrations of ROS proceed from biomolecules of cells, such as protein and lipids, and alter their functions [7,8]. Due to the adverse effects of these biomolecules on cells and tissues, it becomes necessary to remove ROS from the cells, arrange homeostasis inside the cells, and prevent pathological development in the tissues [7,9–11]. In this way, the antioxidant enzymatic and non-enzymatic systems in the cells keep ROS at normal concentrations [8]. While glutathione is one of these antioxidant non-enzymatic systems for maintaining cellular homeostasis [12], Advanced Oxidative Protein Products (AOPP) are a novel oxidative stress marker of non-enzymatic systems [13].

In order to determine the effects of pollutants on organisms, it is advantageous to strategically use antioxidants to understand the causes of oxidative stress and control [14]. Even though the traditional model organisms, including *Danio rerio*, *Caenorhabditis elegans*, and *Daphnia magna*, are still largely used to investigate the adverse effects of pollutants on organisms [15–18], the use of alternative organism models has also become popular [19]. In terms of organism complexity, aquatic invertebrates occupy an intermediate position as concerns conventional animal models of toxicology [20]. Aquatic invertebrates have the capacity to absorb and store pollutants, including insecticides, in a variety of ways, including through the digestive tract and gills by ingesting food and water. This makes it very suitable for ecotoxicology [7,19–21]. Unlike gills, which have tasks such as respiration, osmoregulation, and removal of nitrogenous waste, ROS produced in high concentrations by the digestive system reflects the function of biotransformation mechanisms of the aquatic invertebrates [7,22,23]. Similar to the screening of oxidative stress parameters, the histopathology of aquatic invertebrates has also been investigated in aquatic toxicology studies [24,25]. Moreover, Total Haemocyte Counts are a biomarker which indicates the health and the stress of aquatic invertebrates dependent on environmental conditions, including pollutants [25].

Among aquatic invertebrates, mussel species are used extensively in aquatic toxicology studies [26–30]. Due to filter-feeding habits, mussels are important in aquatic ecosystems [26]. Therefore, they constitute one of the indicator groups used in aquatic biological monitoring studies and laboratory studies [26–30]. Among freshwater mussel species, the Unionid family is distributed worldwide with more than 620 species [31]. One of these species found in Turkey is *Unio delicatus* (Lea, 1863) [32].

To our knowledge, thus far no studies have assessed the acute and sublethal effects of fipronil on freshwater mussels. In this regard, the current study aimed at enhancing knowledge of the toxic effects of fipronil on the freshwater mussel *U. delicatus*. After evaluating the acute toxic values of fipronil, the effects of fipronil were investigated using different biomarkers in mussels exposed to sublethal levels. These biomarkers included physiological/immunological (Total Haemocyte Counts (THCs)), biochemical (Total Antioxidant Status (TAS), Total Oxidant Status (TOS), glutathione, and AOPP), and histopathological parameters.

2. Materials and Methods

2.1. Test Organisms

The freshwater mussels *Unio delicatus* (Lea, 1863) were obtained from fishermen from Antakya (Turkey) and kept in aerated water until they were brought to Gazi University (Ankara, Turkey). To adapt to the laboratory conditions and depuration, the mussels were placed in dechlorinated municipal water with constant aeration and light, including a dark photoperiod (16:8) for 15 d before the bioassays. During this time, the mussels were fed

Chlorella sp. The mean weight, length, height, and thickness of the test organisms used for the acute and sublethal bioassays were 35.45 ± 6.93 g, 5.12 ± 0.42 cm, 2.32 ± 0.28 cm, and 1.36 ± 0.24 cm, respectively. Twenty-four hours before the bioassays, the feeding was stopped. The experiments were conducted with a modification of the OECD [33] and APHA [34] bioassay procedures.

2.2. Chemicals

Technical grade (98%) fipronil (Shenzen Agro Hunter Co., Shenzhen, China) was used and a stock solution of fipronil was prepared using an analytical grade of dimethyl sulfoxide (DMSO) at 10 mg/L before the 24-h experiment and kept at +4 °C.

2.3. Acute Experiments

To detect the mean Lethal Concentration (LC_{50}), the freshwater mussels were exposed to different concentrations of the fipronil for 96-h after range finding tests. There were also control groups: a control and solvent control group. All the acute experiments were conducted in triplicate (10 organisms in each concentration/aquarium).

2.4. Sublethal Experiments

After evaluating the 96 h of LC50, the freshwater mussels were exposed during 48 h and 7 d to 1/10 and 1/5 of 96 h LC50 values of fipronil. The sublethal concentrations were selected according to Sprague [35]. There were also two control groups: a control (water and mussels) and solvent control (DMSO, water and mussels). All the sublethal experiments were conducted in triplicate (10 organisms in each concentration/aquarium). After the exposure times, the samplings of the haemolymph and the tissues were carried out. First, the haemolymph samples were collected from the adductor muscle by a 2.5 mL syringe. Second, the gill and digestive gland tissues of the mussels were removed by dissection. Third, half of the gill and digestive gland tissues were immediately kept in an aluminum folio and put into liquid nitrogen for biochemical analysis. Fourth, the other half of the gills and digestive gland tissues, and the rest of the body tissues were put into tissue cassettes and fixed into a Davidson solution (330 mL 95% ethyl alcohol: 220 mL formalin: 115 mL glacial acetic acid: 335 mL dH_2O).

2.5. Total Haemocyte Counts

After taking the haemolymph samples, 1 mL of haemolymph was fixed with 4% formalin (1:1) in the ependorf tubes. The haemocytes in the haemolymph samples were counted under the light microscope using a Thoma chamber. To detect the Total Haemocyte Counts (THCs) values were calculated according to Yavuzcan and Benli [36]. The results were expressed as cells/mL.

2.6. Biochemical Analysis

The haemolymph values of the Total Antioxidant Status (TAS) and Total Oxidative Stress (TOS) were measured by using the commercially available ELISA kits (REL assay, Baran Medical, Gaziantep, Turkey, product numbers RL0017 and RL0024, respectively). The TAS and TOS values were expressed as mmol/L Trolox Equiv./L and μmol H_2O_2 Equiv./L, respectively.

The glutathione activities of the digestive gland and gill tissues of the mussels were determined by assaying the as described by Ellman [37]. After "the 100 mg of tissue samples were homogenized using 900 μL of metaphosphoric acid (0.5 M, pH: 8), the samples were centrifugated at 3500 rpm +4 °C for 10 min and collected the supernatant". The Ellman's reagent was added to the 200 μL of the supernatant and the glutathione activity was measured spectrophotometrically at wavelengths of 410 (A1) and 420 (A2) nm against a blank. Then, the protein concentrations of supernatants were determined using a Bradford [38] assay. The (A1-A2)/protein equation was used for the measurement of glutathione activity. The glutathione values were expressed as μM/mg protein.

The level of the digestive gland and gill tissues' advanced oxidative protein products was determined as described by Witko-Sarsat et al. [39]. The "100 mg of tissue samples were homogenized using 400 µL of Tris-HCl buffer (20 mM, pH: 7.4), centrifugated at 5000× *g* +4 °C for 10 min and collected the supernatants. After the 100 µL of supernatant samples were diluted with phosphate buffer solution (1 M, pH: 7.4) (1:5), 10 µL potassium iodide (1.16 M) and 20 µL acetic acid were added and read of absorbance at 340 nm". Like in the glutathione assay, the protein concentrations of the supernatants were determined using a Bradford [38] assay. The AOPP values were expressed as µM/mg protein.

2.7. Histopathological Analysis

After fixing the gill, digestive gland, and mantle tissues in a Davidson solution for 24-h, they were put into ethyl alcohol series to dehydrate before the processes including of paraffin embedding, cutting the paraffin blocks at 5 µm thickness, and staining with Haematoxylin and Eosin (H&E) as described by Luna [40]. The slides were observed under a light microscope (Zeiss Primostar, Oberkochen, Germany). The histopathological alterations were scored using the method of Benli et al. [41].

2.8. Statistical Analysis

The LC50 of fipronil at 24-h, 48-h, 72-h, and 96-h were evaluated by using an EPA computer program according to Finney's probit analysis method, (U.S). [42]. The data in the graphs were expressed as mean ± SEM. The GraphPad Prism program (GraphPad Prism, version 5, Boston, MA, USA) was used for the statistical analysis. The Principal Compound Analysis (PCA) was determined using the glutathione and AOPP results of the tissues by calculating Microsoft Excel.

3. Results and Discussion

Insecticide pollution in aquatic ecosystems and the toxicity of these pollutants in bivalves are highly worrying. Studies have shown that insecticides are filtered by filter-feeding organisms. By compromising the integrity of the bivalve's immune system, mussels activate their antioxidant mechanisms or cause changes in their tissue structures [23,43–47]. Therefore, this study aimed to evaluate physiological, antioxidant, oxidant, and histopathological alterations after sublethal exposure to fipronil in the freshwater mussels *U. delicatus*.

3.1. Acute Toxic Effects of Fipronil on the Freshwater Mussels

During the acute toxic tests, there were no dead organisms in the control groups. In the fipronil applied groups, the dead mussels opened the valves. As a result of the experiments, the 96-h LC_{50} value was estimated as 2.64 (1.45–4.56) mg/L (Table 1).

Table 1. The LC_{50} values of fipronil and 95% confidence interval.

Exposure Times	LC_{50} Values	95% Confidence Interval
24-h	18.71 mg/L	13.20–25.75
48-h	8.66 mg/L	5.02–13.81
72-h	5.87 mg/L	3.28–9.88
96-h	2.64 mg/L	1.45–4.56

Although there are few studies examining the acute toxic values of fipronil on aquatic organisms, there are studies on some aquatic invertebrates and aquatic vertebrates. In a study by Connelly [48] the acute toxic values of fipronil in different aquatic organisms were investigated. Accordingly, after 96-h of exposure, the acute toxic value (LC_{50}) of fipronil was found to be 0.248 ppm for rainbow trout, 0.085 ppm for copper nose, 0.13 ppm for *Cyprinodon variegatus*, and 0.000140 ppm for opposum shrimp, while for 48-h of exposure to the LC_{50} value of fipronil was found to be 0.19 ppm for *Daphnia* sp. The LC_{50} values of the freshwater invertebrate species *Procambarus clarkii* and *P. zonangulus*, after 96-h of exposure to fipronil, were determined as 14.3 and 19.5 µg/L, respectively [49]. In the study

of investigating the LC_{50} values of fipronil for *Anodonta* sp., the values were found to be 1.21 mg/L for rac-fipronil, 3.27 mg/L for R-fipronil, 0.63 mg/L for S-fipronil, 1.19 mg/L for fipronil desulfinil, 0.32 mg/L for fipronil sulfide, and 0.24 mg/L for fipronil sulfone [50]. The LC_{50} values of fipronil at 24, 48, 72, and 96-h were found in fipronil exposure studies with mussel species belonging to different life cycles. Fipronil applied to *Villosa constricta*, *Elliptio complanate*, *Lampsilis fasciola*, and *Lampsilis siliquoidea* in the life cycle of glochidia had an LC_{50} value higher than 2 mg/L for 24-h. Similar results were found in the 48-h application, except for *E. complanate*. In addition, the same results were obtained in the juvenile species of *L. fasciola*, and *L. siliquoidea* [51]. These values obtained in the literature compared with the current study's results are consistent in that the acute toxic values of fipronil vary from μg/L to mg/L.

3.2. Total Haemocyte Counts

The Total Haemocyte Counts (THCs) were performed in the haemolymph samples from the freshwater mussels following exposure to fipronil. The results are shown in Figure 1. Accordingly, a lower amount of the THCs was detected in the experimental groups when compared to the control in the 48-h exposure time. On the other hand, after 7-d of exposure, it was observed that the THCs increased in the low concentration fipronil-exposed group compared to the control groups and decreased in the high concentration fipronil-exposed group when compared to the control groups. Findings obtained from the THCs showed a significant difference between the experimental groups compared to the control groups ($p < 0.05$).

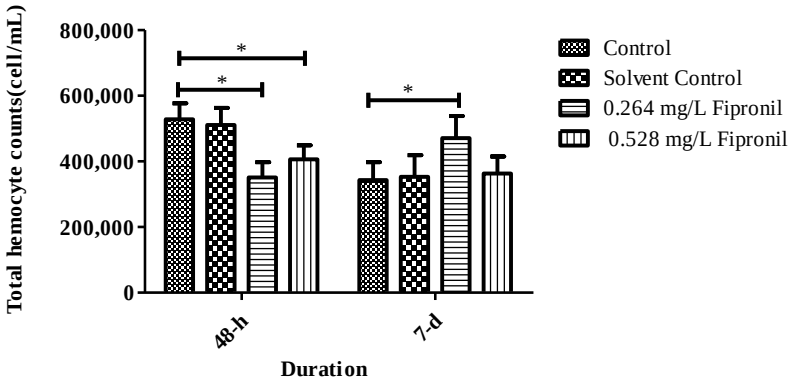


Figure 1. The Total Haemocyte Counts (THCs) (mean ± SEM) of freshwater mussels (* indicates $p < 0.05$).

The immune systems of bivalves contain cellular/non-cellular defense responses and circulating haemocytes respond to different types of pollutants in the aquatic ecosystems. Due to an early internal defense by circulating haemocytes against various environmental stressors, any decrease/increase in THCs can impair the defensive response of bivalves. Therefore, the values of THCs are an important immunological parameter [52]. In this regarding this, the alterations of THCs in bivalves in response to various types of pollutants exist in the literature. In particular, significant decreases in THCs have been reported for the black clam, *Villorita cyprinoides* var. *cochinensis*, exposed to copper [53]. In a study in which in field collected the Mediterranean mussels, *Mytilus galloprovincialis* was collected, the THCs varied dependent on the collecting sites due to pollution [54]. In a study performed on *Lamellidens marginalis*, where chlorpyrifos 20 EC was applied in different concentrations for 35-d, THCs gradually decreased as the dosage increased [55]. In contrast, some studies have reported significant increases in THCs for Sydney rock oysters exposed to imidacloprid [56], and for *U. delicatus* exposed to cyfluthrin [57]. This study found that for 48-h of exposure time, the THCs of fipronil applied groups were decreased whereas for 7-d of exposure time, the THCs were only increased in low-concentration fipronil applied group. This may be an indication that the immune system of mussels is activated immediately in short-term

exposure. Obtaining high total haemocyte counts at low doses in longer exposures may also be due to the chemical structure of the substance.

3.3. Biochemical Parameters

The results of the oxidative stress biomarkers, TAS and TOS, in haemolymph tissues are shown in Figure 2a,b. In accordance with the current results, the TOS values of haemolymph were higher than the TAS values. However, TAS values in 7-d fipronil exposure groups were significantly higher in than the control group ($p < 0.05$). Moreover, the mean TAS/TOS ratio was 57.8 and 99.8 mmol/L in the 48-h and 7-d control groups, respectively. While the mean TAS/TOS ratio was 56.9 and 34.2 mmol/L in the 0.264 mg/L fipronil applied groups for 48-h and 7-d, the mean TAS/TOS ratio was 37.3 and 15.4 in 0.528 mg/L fipronil applied groups for 48-h and 7-d, respectively.

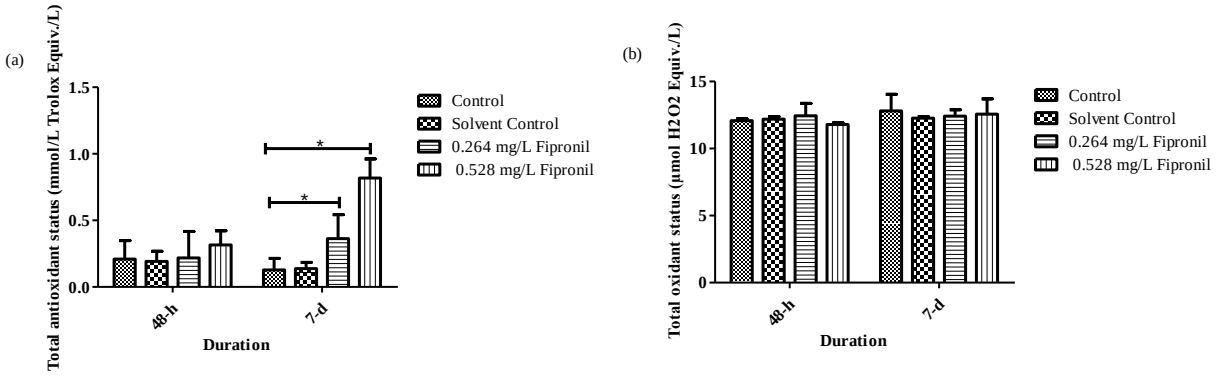


Figure 2. (**a**) Total Antioxidant Status and (**b**) Total Oxidant Status of haemolymph tissues of freshwater mussels. (mean ± SEM) * indicates $p < 0.05$.

Cells have the ability to balance the harmful effects of ROS with their antioxidant defense system consisting of free radical scavengers under normal physiological conditions. If these free radicals produce more than normal, they exceed the antioxidant defense system of the cell and result in oxidative stress [57,58]. Due to the failure of the antioxidant mechanisms in the cell, the biomolecules containing proteins and lipids are destroyed and cause negative effects on the cell [59]. Insecticides are known for the alteration of ROS and cause oxidative stress in mussels [24,60,61]. Glutathione activities and advanced oxidative protein products are used to monitor the effects of pollutants [12,13]. In addition, TAS and TOS are important indicators for evaluating the antioxidant and oxidative stress status of the organisms [62].

While no significant change was observed in TOS in this study, an increase in TAS was observed after 7-d of exposure to fipronil. Results similar to those of the present study were shown in other studies with different aquatic invertebrates. It was reported that an increase in TAS was observed in the whiteleg shrimp *Litopenaeus vannamei* when acutely exposed to ammonia [62]. Another study showed that exposure to PFOS, one of the persistent organic pollutants, increased the TAS and TOS of the hemolymph tissues of narrow-clawed crayfish [63].

In this study, glutathione parameters were measured in gill and digestive gland tissue samples taken from freshwater mussels following exposure to fipronil. The results obtained are shown in Figure 3a for the digestive gland and Figure 3b for the gill tissues. According to the results of the digestive gland samples, even though it was observed that the glutathione levels significantly increased in 48-h of fipronil exposure compared to the control groups, the 117.8% increase was observed in the 0.528 mg/L fipronil applied group ($p < 0.05$). There were also increases in the 7-d fipronil exposures compared to the 7-d control groups. Significant increases of 59.8% in the 0.264 mg/L and 82.4% in the 0.528 mg/L fipronil-applied groups for 7-d were observed compared to the 7-d control groups ($p < 0.05$). According to the results of the gill samples, the glutathione levels after

48-h decreased as a 24.7% ratio in the 0.264 mg/L and increased as a 55.7% ratio in the 0.528 mg/L fipronil-applied groups compared to the control group ($p < 0.05$). Similar to the digestive gland tissues, there were also increases in the 7-d fipronil-applied groups, but no significant changes were observed.

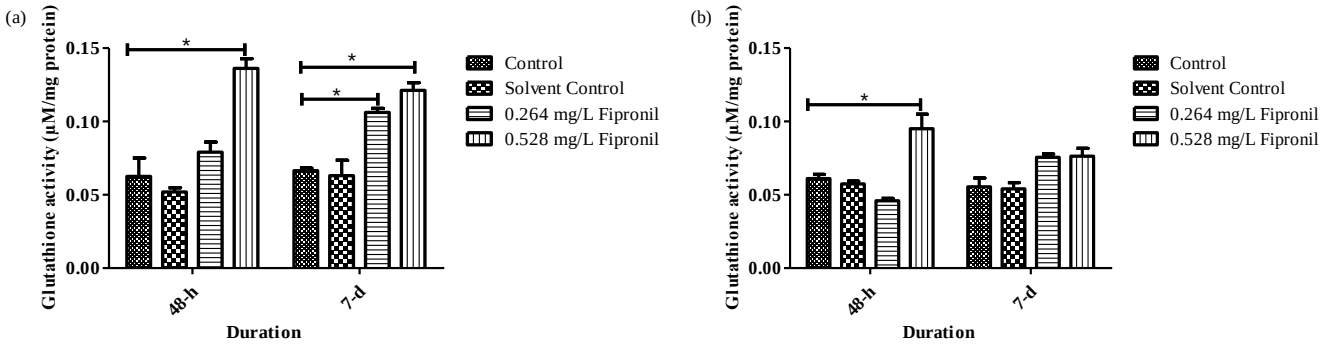


Figure 3. The glutathione activities (mean ± SEM) of (**a**) the digestive gland and (**b**) the gill tissues of freshwater mussels. * indicates $p < 0.05$.

The present study showed there was an increase in the digestive gland glutathione values in both exposure times to fipronil. When comparing fipronil concentrations over time, an increase was found at the 0.264 mg/L (low concentration) dose, while a decrease was found at the 0.528 mg/L (high concentration) dose. A similar situation occured for the glutathione findings in the gill tissue. Depending on the exposure time, an increase occurred in the fipronil experimental groups compared to the control groups. In the comparison of the fipronil experimental groups over time, an increase in the dose of 0.264 mg/L and a decrease in the dose of 0.528 mg/L were detected. This situation supports the conclusion that the effects of endocrine-disrupting substances may be higher at low concentrations [64]. In studies conducted with the marine mussel *Mytilus galloprovincialis*, some studies observed that different chemical substances cause changes in the mussel's glutathione mechanism, including glutathione-S-transferase and glutathione reductase [65–67]. In addition, in a study investigating the effect of nanoparticles on *Unio tigridis*, another species belonging to the *Unio* genus, it was reported that glutathione peroxidase and glutathione-S-transferase were significantly increased in the gill and digestive glands in the nanoparticle-applied groups [68].

In this study, Advanced Oxidation Protein Products (AOPP) parameters were measured in digestive gland and gill samples taken from freshwater mussels following exposure to fipronil (Figure 4). In freshwater mussels exposed to 0.264 mg/L and 0.528 mg/L of fipronil for 48-h and 7-d, the AOPP levels obtained from the digestive gland tissues were higher than the control groups ($p < 0.05$). According to digestive gland tissue results (Figure 4a), the same increase of 47% for the fipronil-applied groups compared to the control group at 48-h was observed ($p < 0.05$). In the 7-d exposure to fipronil, a 74% increase was observed in the 0.264 mg/L fipronil group compared to the control group ($p < 0.05$), while a 34% increase was observed in the 0.528 mg/L fipronil group ($p < 0.05$). The AOPP levels obtained from the gill tissues of the mussels exposed to fipronil concentrations for 48-h increased by 12% compared to the control group (Figure 4b). On the other hand, in the 7-d exposure to fipronil, the significant decrease of AOPP levels in the 0.264 mg/L and 0.528 mg/L fipronil-applied groups compared to the control group were 20.7% and 54.6%, respectively ($p < 0.05$).

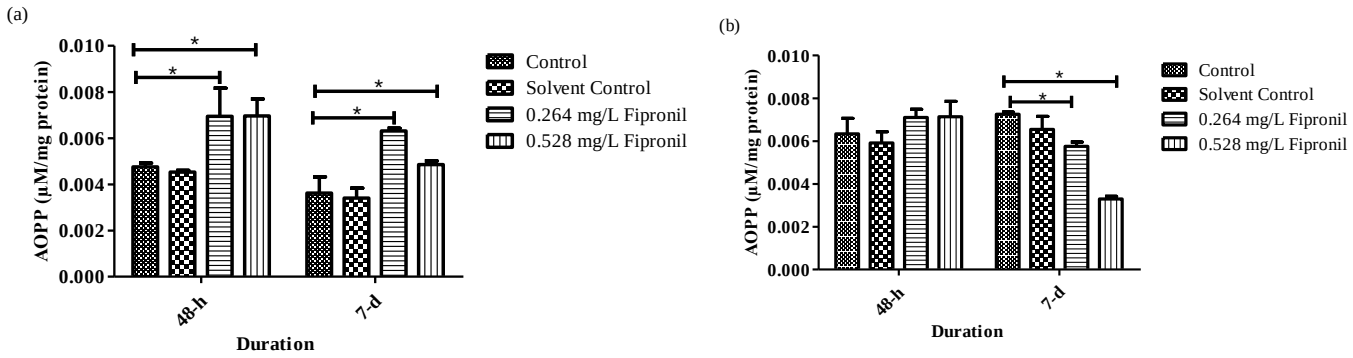


Figure 4. The AOPP levels (mean ± SEM) of (**a**) the digestive gland and (**b**) the gill tissues of freshwater mussels. * indicates $p < 0.05$.

The AOPP results of the study showed that higher AOPP values were obtained in the digestive gland and gill tissues exposed to fipronil compared to the control groups. Digestive gland and gill AOPP values increase after 48-h of exposure to fipronil. However, in 7-d exposure to fipronil, digestive gland AOPP values show an increase, while gill AOPP values show a decrease. This indicates that a detoxification event still occurs in long-term exposure in digestive gland tissues, where the detoxification mechanism and, therefore, CYP activities are higher than in other tissues [69]. In a study with another insecticide, chlorpyrifos, AOPP values were investigated in in vitro gill and digestive gland models of *U. delicatus*. In the gill cell culture, in the insecticide-applied groups, AOPP values decreased compared to the control group, while an increase was observed in only one group in the digestive gland cell culture compared to the control group [70].

3.4. PCA Results

The PCA results performed to understand the relationship between the glutathione and AOPP findings are shown in Figure 5. In both parameters, there is a negative relationship between the gill tissue and digestive gland tissue.

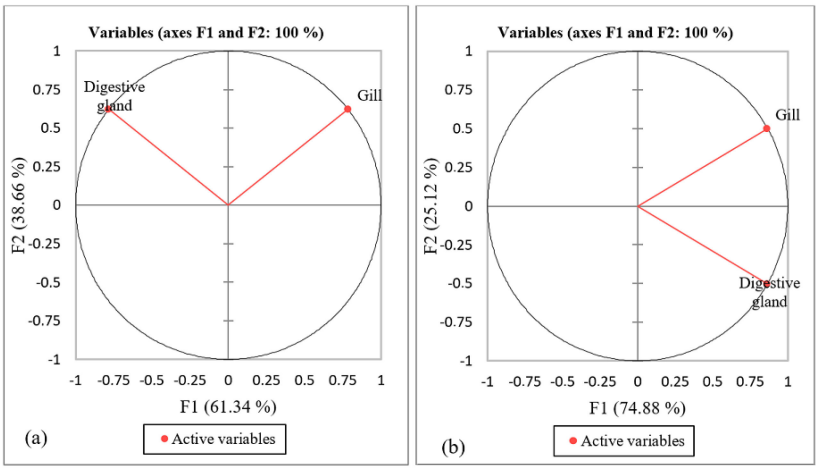


Figure 5. The PCA analysis results of the digestive gland and gill tissues of (**a**) glutathione and (**b**) AOPP.

Similar results were reported for other aquatic animals such as mussels [71,72] and crayfish [73] exposed to xenobiotics. The effects of different pollutants on tissues require further investigation of their interactions on organisms.

3.5. Histology Results

Mussel tissues of the mantle did not show any significant alterations after exposure to sublethal fipronil for 48-h and 7-d. Control groups also did not reveal any histopathological

changes in the tissues. Several histopathological changes were determined in the gill and the digestive gland tissues. The histopathological results of sublethal fipronil-exposed mussels are depicted in Table 2. It was noted that the control gill tissues had well-preserved lamellae made up of a single layer of epithelial cells and a tight haemolymphatic sinus (Figure 6a). Exposure to two sublethal concentrations of fipronil caused lamellar deformations (Figure 6b), haemocytic infiltrations (Figure 6c), and epithelial hyperplasia of lamella (Figure 6d) in the gill tissues according to concentration and duration. The alterations in the digestive gland tissue were more severe than in the gill tissues. The control groups' digestive gland tissues were normal in appearance with tubules (Figure 7a). The fipronil-exposed mussels' digestive gland tissues exhibited severe degenerations (Figure 7b,d) and necrosis of the digestive tubules (Figure 7c).

Table 2. Histopathological alterations of the freshwater mussel (*Unio delicatus*) exposure to sublethal fipronil concentrations.

Experiment Groups	Control		Control (DMSO)		0.264 mg/L Fipronil		0.528 mg/L Fipronil	
Tissues/Duration	**48-h**	**7-d**	**48-h**	**7-d**	**48-h**	**7-d**	**48-h**	**7-d**
Digestive Gland								
Degenerations of the tubules	-	-	-	-	+	++	+++	+++
Tubule necrosis	-	-	-	-	+	+	++	+++
Gill								
Deformations of the lamella	-	-	-	-	+	+	+	++
Haemocytic infiltration	-	-	-	-	-	+	+	+
Epithelial hyperplasia of lamella	-	-	-	-	-	+	++	++

The histopathological alterations were scored as "(-) none (no histopathological alterations), which represents normal histological structure; (+) histopathology in >20% of fields (mild); (++) histopathology in 20–60% of fields (moderate), and (+++) histopathology in <60% of fields (severe)".

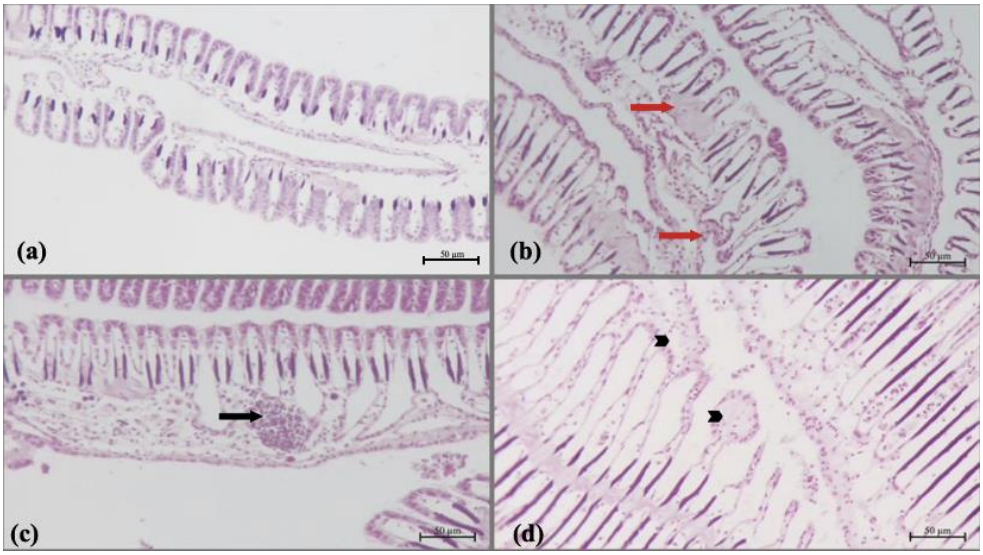


Figure 6. Gill tissue of freshwater mussel, the *Unio delicatus* (**a**) control, (**b**) lamellar deformation of the gills (red arrows) after exposure to 0.528 mg/L fipronil for 48-h, (**c**) haemocytic infiltration (black arrows) after exposure to 0.264 mg/L fipronil for 7-d, (**d**) epithelial hyperplasia of the lamella after exposure to 0.524 mg/L fipronil for 7-d (black arrow heads) (H&E).

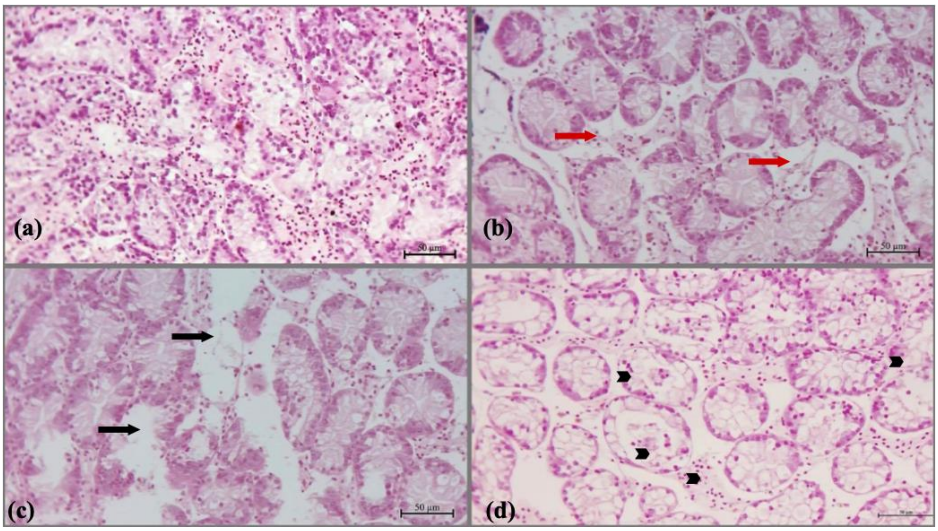


Figure 7. Digestive gland tissue of the freshwater mussel, *Unio delicatus* (**a**) control, (**b**) deformation of the tubules (red arrows) after exposure to 0.264 mg/L fipronil for 48-h, (**c**) necrosis of the digestive tubules (black arrows) after exposure to 0.528 mg/L fipronil for 7-d (**d**) deformation of the tubules after exposure to 0.524 mg/L fipronil for 7-d (black arrow heads) (H&E).

As filter-feeding organisms in the food web, mussels can easily accumulate waterborne toxicants from water bodies and reflect changes in water quality. Gills and the digestive gland tissues are the organs most affected by xenobiotics in the mussels [28,74]. Exposure to fipronil after 48 h and 7 d resulted in alterations in the gill tissues such as degeneration, haemolymph infiltration, and epithelial hyperplasia in the lamella, and tubule degenerations and necrosis in the digestive gland tissues in the present study. No study was found related to fipronil toxicity and its histological effects on mussels in the literature survey, but some studies on fish species were reported. Ardeshir et al. [75] determined aneurisms, extensive fusion, and necrosis in the gill tissues of white fish (*Rutilus frisii*) and El-Murr et al. [76] noticed focal necrosis, intense lymphocytes, epithelial proliferations, fusion, hyperplasia of epithelium, congestion, and haemorrhages in tilapia (*Oreochromis niloticus*) after exposure to 0.0042 and 0.002 mg/L fipronil for 10 weeks. Ghaffar et al. [77] studied common carp exposed to 0.02–0.10 mg/L fipronil for 12-d and observed disruption of cartilaginous core, degeneration/disruption of primary lamellae, fusion of secondary lamellae, necrosis, and aneurysm. This result confirms that fipronil has negative effects on gill histology in aquatic organisms. Digestive gland tissue is used to assess the overall health of mussels and the effects of xenobiotics [68]. Toxic exposure to xenobiotics has previously been linked to digestive tubule degeneration [69]. Exposure to fipronil resulted inaccumulated lipofuscin aggregates and caused mild degeneration of digestive tubules. Hepatotoxicity of fipronil was also reported by other aquatic and model mammalian organisms [75–81]. The histological alterations in the present study were also non-specific histological findings of mussels exposed to different toxicants [28,82].

4. Conclusions

To conclude, fipronil, which is widely used in agricultural, domestic, and veterinary medicine, has various effects on non-target species. In this study, the acute toxic value of fipronil for the freshwater mussel *Unio delicatus* species was found and contributed to the literature. The Total Haemocyte Counts were examined. In addition, increases/decreases were observed in non-enzymatic (glutathione) and the AOPP parameters within the scope of the organism's antioxidant defense mechanism. Histopathological evaluations supported the adverse effects of fipronil in the tissue levels.

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