

## Assessment of Na<sup>+</sup>/K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase, Ca<sup>2+</sup>-ATPase, and Total-ATPase Activities in Gills of Freshwater Mussels Exposed to Penconazole

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**Abstract:** Penconazole, a triazole fungicide, is widely used worldwide to increase agricultural production and to control fungus. Bivalves, especially mussels, are specific biomonitor organisms of pollution in aquatic ecosystems. This study deals with the possible toxic effects of exposure to penconazole on Na<sup>+</sup>/K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase, Ca<sup>2+</sup>-ATPase, and Total-ATPase activities in the gills of freshwater mussels (*Unio mancus*). In this context, freshwater mussels were exposed to different concentrations of penconazole (1, 10, 100, and 1000 µg AI L<sup>-1</sup>) in 96 hours static-renewal test system. The commercial form of penconazole (TOPAS® 100 EC, Syngenta, Germany) was used in toxicity tests. The actual penconazole concentrations in the exposure media were determined by LC-MS/MS analysis. According to the LC-MS/MS analysis results, the actual penconazole concentrations in the exposure media were determined to be approximately 25% lower than the nominal penconazole concentrations. After 96 hours of exposure, the highest concentration of penconazole caused significant inhibition of ATPase activities except Mg<sup>2+</sup>-ATPase activity (P < 0.05). The observed data indicated that acute penconazole exposure may be a potential risk for freshwater mussels and that ATPase enzymes are important and useful biochemical markers for aquatic toxicology studies.

**Keywords:** Penconazole toxicity, freshwater mussel, ATPases, LC-MS/MS.

### Penkonazole Maruz Bırakılan Tatlı Su Midyelerinin Solungaç Na<sup>+</sup>/K<sup>+</sup>-ATPaz, Mg<sup>2+</sup>-ATPaz, Ca<sup>2+</sup>-ATPaz ve Total-ATPaz Aktivitelerinin Değerlendirilmesi

**Öz:** Triazol bir fungusit olan penkonazol, tarımsal üretimi arttırmak ve fungusları kontrol etmek için dünya çapında yaygın olarak kullanılmaktadır. Çift kabuklu yumuşakçalar, özellikle midyeler, sucul ekosistemlerde kirliliğin spesifik biyomonitor organizmalarıdır. Bu çalışmada tatlı su midyelerinin (*Unio mancus*) solungaç Na<sup>+</sup>/K<sup>+</sup>-ATPaz, Mg<sup>2+</sup>-ATPaz, Ca<sup>2+</sup>-ATPaz ve Total-ATPaz aktiviteleri üzerine penkonazol maruziyetinin olası toksik etkileri değerlendirilmiştir. Bu amaçla, 96 saatlik statik yenileme test sisteminde tatlı su midyeleri farklı konsantrasyonlarda penkonazole (1, 10, 100 ve 1000 µg AI L<sup>-1</sup>) maruz bırakılmıştır. Toksikite testlerinde penkonazolün ticari formu (TOPAS® 100 EC, Syngenta, Almanya) kullanılmıştır. Maruziyet ortamındaki gerçek penkonazol konsantrasyonları LC-MS/MS analizi ile belirlenmiştir. LC-MS/MS analizi sonuçlarına göre, maruziyet ortamındaki gerçek penkonazol konsantrasyonlarının, nominal penkonazol konsantrasyonlarından yaklaşık %25 oranında daha düşük olduğu belirlenmiştir. 96 saatlik maruziyetten sonra, en yüksek penkonazol konsantrasyonu Mg<sup>2+</sup>-ATPaz hariç diğer ATPaz aktivitelerinin önemli düzeyde inhibisyonuna neden olmuştur (P < 0.05). Elde edilen veriler, akut penkonazol maruziyetinin tatlı su midyeleri için potansiyel bir risk olabileceğini ve ATPaz enzimlerinin akut toksikoloji çalışmaları için önemli ve faydalı biyokimyasal belirteçler olduğunu göstermiştir.

**Anahtar kelimeler:** Penkonazol toksisitesi, tatlı su midyesi, ATPazlar, LC-MS/MS.

### 1. Introduction

Pesticides significantly threaten both environment and human health due to their excessive use in agriculture (Chaabane et al., 2018). Penconazole, a triazole fungicide, is widely used worldwide to increase agricultural production and to control fungus (Lv et al., 2017; Aksakal & Ciltaş, 2018). Penconazole [1-(2,4-dichloro-b-propylphenethyl)-1H-1,2,4-tri-azole] acts by inhibiting the ergosterol biosynthesis of fungi (Zhang et al., 2019). This fungicide is normally applied by spraying directly to the plant (Chaabane et al., 2016). However, penconazole residues can reach to the soil, groundwater, and surface waters due to drifting, rain washing, and falling out. Therefore, it may adversely affect non-target organisms in both aquatic and terrestrial ecosystems (Husak, Mosiichuk, Storey, & Lushchak, 2017). In addition, high concentrations of triazole fungicides cause various toxic effects such as carcinogenicity, reproductive toxicity, and hepatotoxicity in mammals (Juberg, Mudra, Hazelton, & Parkinson, 2006; Peffer et al., 2007).

Bivalves which are commonly used as biomonitor organisms in ecotoxicological studies, play significant roles in organic matter recycling and control of phytoplankton levels (Iummato et al., 2018). Bivalves, especially mussels, have various characteristics such as a wide geographic distribution, sessile life, deep burrowing, accumulation of many types of pollutants, and a high tolerance for chemical pollution (Okay et al., 2016; Burgos-Aceves, & Faggio, 2017; Guidi et al., 2017; Savorelli et al., 2017; Sureda, Capó, Busquets-Cortés, & Tejada, 2018). They accumulate pollutants in the surrounding water due to their filter-feeding characteristics (Parisi et al., 2019). In the present study, the *Unio mancus* Lamarck, 1819 commonly found in the Atatürk Dam Lake was chosen as a biomonitor species.

Adenosine triphosphatases (ATPases) are potentially useful biochemical markers of pollution stress in aquatic organisms, as they are sensitive to the toxicity of organic pollutants such as pesticides and drugs (Pham, Miranda, Allinson, & Nugegoda, 2017). ATPases, which are

membrane-bound enzymes, have important roles in regulating membrane permeability and osmotic balance because they are responsible for ion transport (Kulac, Atli, & Canli, 2013). Alterations in ATPase levels are significant evidence for detecting the harmful effects of environmental pollutants. Furthermore, ATPase levels reflect disturbances in physiological processes that require these membrane-bound enzymes (Vijayavel, Gopalakrishnan, & Balasubramanian, 2007).

There are limited numbers of studies evaluating the effects of penconazole on aquatic organisms. In particular, there are no studies evaluating the effects of penconazole on ATPase activities in freshwater mussels. In this context, the main objective of this study is to evaluate ATPase activities in the gills of freshwater mussels exposed to commercial form of penconazole.

## 2. Material and Methods

### 2.1. Mussel collection

In March 2019, the freshwater mussel *U. mancus* samples were collected by a fisherman from Atatürk Dam Lake. The collected mussels were transferred to the laboratory within 1-2 hours in plastic containers filled with dam lake water. After the mussels were transferred to the laboratory, they were adapted to the laboratory for 14 days in a 50 L tank filled with bottled water. The mussels were fed ad libitum twice a week with a commercial concentrate phytoplankton (Roti-Rich™) during adaptation period. Both during the adaptation period and during the toxicity test, the tanks were continuously aerated and the mussels were kept at  $14 \pm 10$  hours of light / dark cycle and  $21 \pm 1$  °C.

### 2.2. Toxicity tests

After 14 days, mussels were divided into five test groups (one control group and four exposure groups) for toxicity testing. All test groups were set in three replicates and twelve mussels were used in each test group. Mussels were exposed to four nominal concentrations of penconazole (1, 10, 100, and 1000  $\mu\text{g AI L}^{-1}$ ) in the 96 h static renewal test system. The commercial form of penconazole (TOPAS® 100 EC, Syngenta, Germany) was used in toxicity tests. The active ingredient (AI) of this commercial form is labeled as 100 g  $\text{L}^{-1}$ . The legal limit value of penconazole in drinking and ground waters is 0.1  $\mu\text{g L}^{-1}$  (EU, 2006; EU, 2013; Nikolaou et al., 2017; IUPAC, 2019). In addition, the 96-hour  $\text{LC}_{50}$  value of penconazole for *Oncorhynchus mykiss* was determined as 1.13  $\text{mg L}^{-1}$  by the International Union of Pure and Applied Chemistry (IUPAC, 2019). The nominal penconazole concentrations used in toxicity tests were determined based on these values. The gills were dissected at the end of toxicity tests and stored at -80 °C until the biochemical assays.

The conductivity, dissolved oxygen, temperature, and pH of the test waters were measured using a multi parameter instrument (YSI Pro Plus Multi-Parameter) at the beginning of the toxicity tests (0. hour). The mean values of the dissolved oxygen, conductivity, temperature, and pH were determined as 7.11  $\text{mg L}^{-1}$ , 22.65  $\mu\text{S cm}^{-1}$ , 16.9 °C, and 7.55, respectively.

### 2.3. Penconazole analysis

The actual penconazole concentrations in the exposure media were determined using a liquid chromatography tandem mass spectrometry (LC-MS/MS, Shimadzu

Quadrupole 8040) at Adiyaman University Central Research Laboratory. Each test water sample was analyzed in triplicate. The conditions of LC-MS/MS were presented in Table 1. The calibration curve constructed from the standards (Dr. Ehrenstorfer GmbH with 99.8% purity) for the calculation of actual penconazole concentrations was in the range of 1-50  $\mu\text{g L}^{-1}$ . The limit of detection (LOD), the limit of quantification (LOQ), and the coefficient of determination ( $r^2$ ) for penconazole were 0.05  $\mu\text{g L}^{-1}$ , 0.16  $\mu\text{g L}^{-1}$ , and 0.998, respectively.

Table 1. LC-MS/MS conditions

Mobil phase A	1 mM ammonium formate in 100% water
Mobil phase B	100% methanol
Column	Inertsil ODS-4 (2.1 mm I.D. × 50 mm L., 3 $\mu\text{m}$ )
Column oven temperature	40 °C
Flow rate	0.4 $\text{mL min}^{-1}$
Interface voltage	4.5 kV
Nebulizing gas flow rate	3 $\text{L min}^{-1}$
Drying gas flow rate	15 $\text{L min}^{-1}$
DL temperature	250 °C
Heat Block temperature	450 °C

### 2.4. Determination of ATPase Activities

The tissues were weighed and homogenized in 1:4 (w/v) ratio of 0.1 M K-phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA, 1 mM DTT) using a Teflon-glass homogenizer (Heidolph RZR 2021). The homogenates were centrifuged at 16.000 × g for 20 min at 4°C (Hettich 460 R) and then, the supernatants were transferred to eppendorf tubes for measurements of ATPase activity and total protein concentration. All biochemical assays were performed using a microplate reader spectrophotometer (Thermo, Varioscans Flash 2000). Assays were run at 25°C in triplicate. The total protein concentrations of tissue samples were measured by Bradford (1976) method using bovine serum albumin as the standard.

The ATPase activities were determined according to a modified procedure of Atli and Canli (2011) for a microplate reader system. Incubation media (pH 7.7) containing 100 mM NaCl, 20 mM KCl, 4 mM  $\text{MgCl}_2$ , 40 mM Tris-HCl, and 1 mM ouabain was used to measure  $\text{Mg}^{2+}$ -ATPase and  $\text{Na}^+/\text{K}^+$ -ATPase activities. For  $\text{Ca}^{2+}$ -ATPase activity, incubation media (pH 7.7) containing 1 mM  $\text{CaCl}_2$ , 4 mM  $\text{MgCl}_2$ , 40 mM Tris-HCl, and 1 mM EGTA was used. To measure enzyme activities, 5  $\mu\text{L}$  of supernatant and 60  $\mu\text{L}$  of incubation medium were pipetted into the microplate wells and incubated at 37 °C for 5 minutes. After preincubation, the reaction was initiated by adding 10  $\mu\text{L}$  of 3 mM  $\text{Na}_2\text{ATP}$  and incubated for 30 min at 37 °C. After incubation, the reaction was stopped by adding 35  $\mu\text{L}$  of ice-cold distilled water ( $\text{dH}_2\text{O}$ ). In addition, the ouabain-free media, supernatant and  $\text{dH}_2\text{O}$  were added to microplate wells to be used as ATP blank. The ouabain-free media,  $\text{Na}_2\text{ATP}$  and  $\text{dH}_2\text{O}$  were added to the microplate wells to be used as sample blank. These blanks were used to correct non-enzymatic hydrolysis of ATP. The inorganic phosphate (Pi) released from 3 mM ATP added as a substrate to the media during incubation was measured spectrophotometrically according to the method proposed by Atkinson et al.

(Atkinson, Gatenby, & Lowe, 1973). At the end of the reaction, 190  $\mu\text{L}$  of the main reagent containing 5% polyoxyethylene 10 lauryl ether and 2% ammonium molybdate were pipetted into microplate wells. After 10 min incubation at room temperature, the absorbance was read at 390 nm.  $\text{KH}_2\text{PO}_4$  (100–1000  $\mu\text{M}$ ) was used as the Pi standard.

$\text{Na}^+/\text{K}^+$ -ATPase activity was calculated using the difference between total-ATPase activity (ouabain-containing media) and  $\text{Mg}^{2+}$ -ATPase activity (ouabain-free media). The  $\text{Ca}^{2+}$ -ATPase and the  $\text{Mg}^{2+}$ -ATPase activity was calculated using the difference between the enzyme activities measured in the presence and absence of  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , respectively. Enzyme activities were expressed as  $\mu\text{mol Pi/mg protein/hour}$ .

### 2.5. Data Analysis

All statistical tests performed with the software package SPSS 22 (USA). All data were presented as mean values  $\pm$  standard errors calculated on 12 samples. Using the Shapiro-Wilk and the Levene tests, all variables were analysed for normality and homogeneity, respectively. Kruskal-Wallis test was used to test the difference between the groups. If any significant differences were detected between the groups, Mann Whitney-U test was used to test the differences within the groups.  $P < 0.05$  was considered statistically significant for all analysis.

## 3. Results and Discussion

### 3.1. Actual concentrations of penconazole

The actual penconazole concentrations in the exposure media obtained from LC-MS/MS analysis were depicted in Table 2. The actual concentrations were determined to be approximately 25% lower than the nominal concentrations. These differences between the nominal and actual concentrations may be due to other compounds such as surfactants, solvents, and preservatives used in commercial formulations of pesticides. In addition, insufficient water dissolution of pesticides could cause a difference between nominal and actual concentrations (Korkmaz, Güngördü, & Ozmen, 2018). Furthermore, changes in pH, liquid formulations, and photolytic degradation of the active ingredient may also be related to these differences between nominal and actual concentrations (Vieira, Pérez, Acayaba, Raimundo, & dos Reis Martinez, 2018).

### 3.2. ATPase activities

As a result of the short-term exposure to penconazole, various fluctuations have been observed in the gill ATPase activities of freshwater mussels. The highest concentration of penconazole caused significant inhibition of ATPase activities except  $\text{Mg}^{2+}$ -ATPase activity ( $P < 0.05$ ). The lowest  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Mg}^{2+}$ -ATPase,  $\text{Ca}^{2+}$ -ATPase, and Total-ATPase activities were determined at 1000  $\mu\text{g AI L}^{-1}$  as  $15.15 \pm 1.26 \mu\text{mol Pi/mg protein/h}$ ,  $31.80 \pm 1.90 \mu\text{mol Pi/mg protein/h}$ ,  $41.95 \pm 3.85 \mu\text{mol Pi/mg protein/h}$ , and  $23.10 \pm 2.53 \mu\text{mol Pi/mg protein/h}$ , respectively (Figures 1–4). In general, these inhibitions in ATPase activities may be the result of entering the energy economy during the hypometabolic state in order to maintain the viability of freshwater mussels (Balasundaram, Ramalingam, & Selvarajan, 1995). However, these alterations in ATPase activities may be considered as early warning signals of damage caused by environmental pollutants in the

osmoregulatory and acid-base regulatory system in the gills (Atli & Canli, 2011).

Table 2. Actual penconazole concentrations measured by LC-MS/MS in the exposure media (n=3)

Nominal concentrations ( $\mu\text{g AI L}^{-1}$ )	Actual concentrations ( $\mu\text{g L}^{-1} \pm \text{SE}$ )		
Control (0)	0	$\pm$	0
1	0.74	$\pm$	0.005
10	7.18	$\pm$	0.124
100	75.89	$\pm$	0.299
1000	785.30	$\pm$	16.994

According to the data of the present study,  $\text{Na}^+/\text{K}^+$ -ATPase activity was inhibited by approximately 47% and 72% at 100 and 1000  $\mu\text{g AI L}^{-1}$  penconazole concentrations compared to the control group, respectively ( $P < 0.05$ ) (Figure 1).  $\text{Na}^+/\text{K}^+$ -ATPase is an ionic pump which is mainly involved in the maintenance of cell membrane potential and osmotic balance. It is well known that environmental chemicals usually affect the  $\text{Na}^+/\text{K}^+$ -ATPase enzyme (Begum, 2011). In bivalve molluscs,  $\text{Na}^+/\text{K}^+$ -ATPase has a significant role in regulating intracellular  $\text{Na}^+$  concentration in response to different environmental conditions (Parisi et al. 2019). Because membrane fluidity allows membrane-bound proteins such as  $\text{Na}^+/\text{K}^+$ -ATPase to function, changes in the lipid composition and structure of the membrane may cause a decrease in  $\text{Na}^+/\text{K}^+$ -ATPase activity (Pałecz, Komuński, & Gabryelak, 2005). In addition, Yang et al. have suggested that increases or decreases in  $\text{Na}^+/\text{K}^+$ -ATPase activity caused by xenobiotics may lead to adverse effects on organisms (Yang, Lu, Zhang, & He, 2002). These studies support the present findings.

Although penconazole exposure caused various fluctuations in  $\text{Mg}^{2+}$ -ATPase activity, these changes were not statistically significant ( $P > 0.05$ ) (Figure 2).  $\text{Mg}^{2+}$ -ATPase provides the homeostasis of  $\text{Mg}^{2+}$  which is critical in various toxicological processes (David, Sangeetha, Harish, Shrinivas, & Naik, 2014).  $\text{Mg}^{2+}$ -ATPase is the most important factor associated with energy metabolism and inhibition of this enzyme results in degradation of oxidative phosphorylation. However,  $\text{Mg}^{2+}$ -ATPase has lower sensitivity to environmental chemicals than other ATPases (Dogan, Atli, & Canli, 2015). The observed decrease in  $\text{Mg}^{2+}$ -ATPase activity in pesticide exposure may lead to reduction of ATP production due to the enzyme's role in oxidative phosphorylation (Parvez, Sayeed, & Raisuddin, 2006). In addition, Begum (2009) suggested that non-significant inductions in renal  $\text{Mg}^{2+}$ -ATPase activity in *Clarias batrachus* species exposed to cypermethrine may be due to increased ATP production to cope with pesticide stress.

Gill  $\text{Ca}^{2+}$ -ATPase activity of freshwater mussels exposed to penconazole was observed to be suppressed by approximately 42% at the highest penconazole concentration compared to the control group (Figure 3) ( $P < 0.05$ ).  $\text{Ca}^{2+}$ -ATPase, which is localized in sarcoplasmic reticulum tubules, is activated by  $\text{Ca}^{2+}$  ion which is necessary for the stabilization of cell membrane. This enzyme allows the removal of  $\text{Ca}^{2+}$  from the cytoplasm using energy released from ATP hydrolysis and thus, plays a role in the maintenance of low intracellular  $\text{Ca}^{2+}$  levels (Saxena, Zachariassen, & Jorgensen, 2000; Dogan et al., 2015).  $\text{Ca}^{2+}$ -ATPase is very important for the environmental pollution studies due to its importance in  $\text{Ca}^{2+}$  metabolism and its functional sulfhydryl groups

(Wong & Wong, 2000). Inhibition of  $\text{Ca}^{2+}$ -ATPase may be associated with increased intracellular cytosolic  $\text{Ca}^{2+}$  due to oxygen radicals or tissue damage as a result of pesticide toxicity (Balasundaram et al., 1995). Free oxygen radicals cause disruption of  $\text{Ca}^{2+}$  homeostasis resulting in oxidative cell damage (Vijayavel et al., 2007).

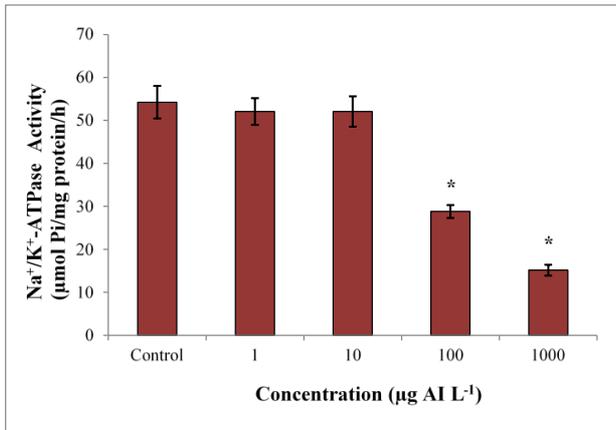


Figure 1. Alterations in  $\text{Na}^+/\text{K}^+$ -ATPase activity levels in gills of freshwater mussels after 96 h penconazole exposure ( $\text{Na}^+/\text{K}^+$ -ATPase activity expressed as  $\mu\text{mol Pi/mg protein/h}$ ,  $n=12$ ). \*:  $P < 0.05$  showed statistical importance compared with control group.

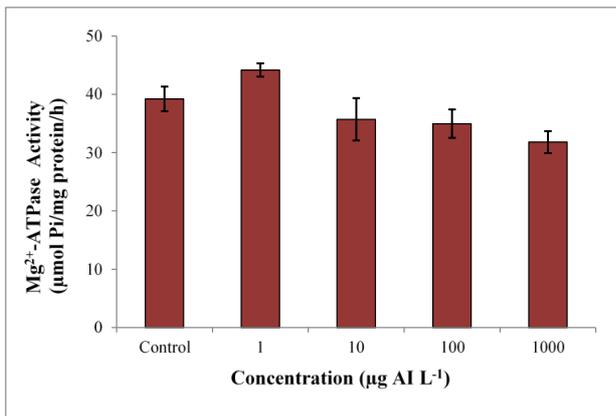


Figure 2. Alterations in  $\text{Mg}^{2+}$ -ATPase activity levels in gills of freshwater mussels after 96 h penconazole exposure ( $\text{Mg}^{2+}$ -ATPase activity expressed as  $\mu\text{mol Pi/mg protein/h}$ ,  $n=12$ ). \*:  $P < 0.05$  showed statistical importance compared with control group.

Data for total-ATPase activities in the gills analysed in the present study were displayed in Figure 4. According to the results, gill total-ATPase activities at  $100 \mu\text{g AI L}^{-1}$  and  $1000 \mu\text{g AI L}^{-1}$  penconazole concentrations were inhibited by approximately 45% and 55% compared to the control group, respectively ( $P < 0.05$ ). Pesticides affect membrane-bound enzymes such as total-ATPase as they disrupt the structural and functional integrity of the cell membrane. The decrease in ATPase activity may be due to an allosteric change resulting from partitioning in the enzyme complex caused by aquatic pollutants (Pham et al., 2017). Furthermore, the decrease in enzyme activity may be related to the high affinity of pollutants such as pesticides and metals to the -SH groups (Atli & Canli, 2011). Previous studies with aquatic organisms also reported that pesticides cause suppression of total-ATPase activity. Comoglio et al. reported that methyl parathion inhibited total-ATPase activity in white leg shrimps (Comoglio et al., 2005). Similarly, another study reported that thiobencarb inhibits total-ATPase activity in some tissues of *Anguilla anguilla* (Sancho et al., 2003).

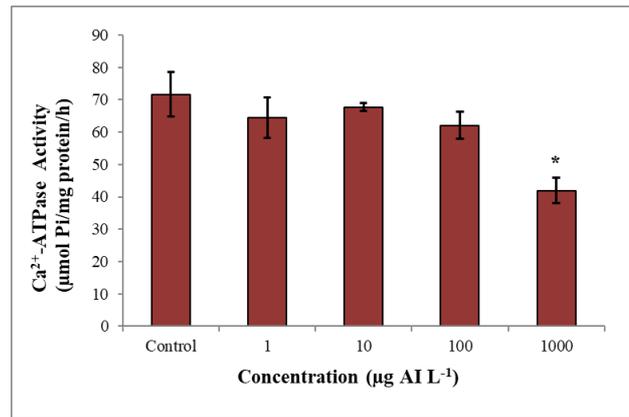


Figure 3. Alterations in  $\text{Ca}^{2+}$ -ATPase activity levels in gills of freshwater mussels after 96 h penconazole exposure ( $\text{Ca}^{2+}$ -ATPase activity expressed as  $\mu\text{mol Pi/mg protein/h}$ ,  $n=12$ ). \*:  $P < 0.05$  showed statistical importance compared with control group.

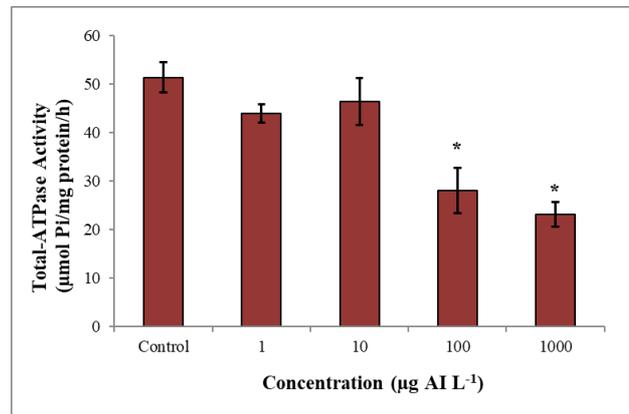


Figure 4. Alterations in total-ATPase activity levels in gills of freshwater mussels after 96 h penconazole exposure (Total-ATPase activity expressed as  $\mu\text{mol Pi/mg protein/h}$ ,  $n=12$ ). \*:  $P < 0.05$  showed statistical importance compared with control group.

#### 4. Conclusion

In this study, toxic effects of acute penconazole exposure on gill ATPase activities in freshwater mussels were evaluated and it was clearly observed that the highest penconazole concentration caused significant suppression of ATPase activities. In addition, this study is more realistic in terms of environmental effects of penconazole, since the effects of the commercial form of penconazole, which are widely used in agricultural activities, were evaluated. In addition, the data obtained from this study is considered to be a basis for further studies, since there are no studies in the literature on the effects of penconazole on freshwater mussel ATPase activities. However, more detailed laboratory studies are needed to better understand the effects of penconazole on ATPase activities.

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