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Behavioral and Biochemical Variations in *Unio tigridis* After Exposure to Lead Nitrate

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Abstract

This study was carried out with the freshwater mussel *Unio tigridis* exposed to lead nitrate. The samples of water and mussels were collected from Qandil water resources situated in Qandil village at 36° 37' 39.55" north latitude and 44° 10' 51.80" east longitude, and it is 322 meters above sea level. In January 2022, rivers and aquariums water were tested for temperature, pH, EC, TDS, DO, total alkalinity, total hardness, and calcium and magnesium ions. Studied organisms were exposed to variable concentrations of lead nitrate ranging from 0 to 900 ppm. The 96-h median lethal concentration (LC_{50}) was measured and a comparison was made between the behavioural responses of the control and treatment groups. Also, the concentrations of lead in the experimental media, shell, and soft tissue of mussels were measured. A sample of mussels collected from aquarium was tested for biochemical markers, including acetylcholinesterase (AChE), Glutathione S-Transferase (GST), Catalase (CAT) and malondialdehyde (MDA). After 96 hours, the LC_{50} was estimated to be 782.7 ppm. The findings on behaviours revealed that lead slows down species' activities. Lead concentratiom increased within the mussel body as the water lead level decreased. The value of AChE was inversely related to lead concentration. However, GST, CAT and MDA increased with lead exposure.

Keywords: *Unio tigridis*, LC50, Mussel, Lead nitrate, Biomarkers

التغيرات السلوكية والكيموحيوية في *Unio tigridis* بعد التعرض لنترات الرصاص

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الخلاصة

أجريت هذه الدراسة على ذوات الصدفتين المائيه *Unio tigridis* التي تعرضت لنترات الرصاص. جمعت عينات الماء و ذوات الصدفيتين من مصادر مياه قنديل في قرية قنديل عند خط عرض 36 ° 37 '39.55 شمالا وخط طول 44 ° 10' 51.80 بوصة شرقا، و بلغ ارتفاعه 322 مترًا فوق مستوى سطح البحر في يناير 2022. تم اختبار مياه النهر والأحواض لدرجة حرارة وإ الأس الهيدروجيني ، التوصيل الكهربائي ، إجمالي المواد الصلبة الذائبة ، الأكسجين المذاب ، القاعدية، العسرة ، أيون الكالسيوم ، أيون المغنيسيوم. عُرضت الكائنات الحية قيدالدراسة لتركيزات مختلفه من نترات الرصاص تراوحت بين صفر إلى 900 جزء في المليون ، وتم قياس متوسط التركيز المميت بعد 96 ساعة LC_{50} ، وتم إجراء مقارنة بين الاستجابات السلوكية لمجموعتي السيطرة والمعالجة. كما تم قياس تراكيز الرصاص في الوسط التجريبي والقشور والأنسجة لذوات الصدفيتين. تم

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اختبار عينة من ذوات الصدفتين التي تم جمعها من الاحواض للعلامات الكيموحيوية ، بما في ذلك AChE و GSTو CAT و MDA . بعد 96 ساعة ، قُدر LC_{50} بـ 782.7 جزء في المليون. كشفت النتائج المتعلقة بالسلوك أن الرصاص يبطئ أنشطة الأنواع. كما لوحظ زيادة تركيز الرصاص داخل جسمذوات الصدفتين مع انخفاض مستوى الرصاص في الماء. إضافة الى ذلكارتبطت قيمة AChE عكسياً بتركيز الرصاص ؛ ومع ذلك ، زادت مستويات GST ، CATو MDA مع التعرض للرصاص.

Introduction

Anthropogenic activities are major sources of contaminants in aquatic ecosystems, including metalworking, mineral extraction, and energy production and usage which contribute to the contamination of aquatic ecosystems with heavy metals. Alarming levels of heavy metals have been found in several bodies of water, especially ones close to industrial sites where effluents are frequently discharged [1, 2]. Metals and other pollutants can be substantial environmental stresses, impacting the biomarkers of mollusks. When exposed to heavy metals, the green mussels experience significant physiological changes [3]. In freshwater mussels, environmental pollutants can be readily taken in by the gills and circulating hemocytes and then get transferred to and accumulated in the different organs, including the digestive system, liver, and the male reproductive organs [4]. Mainly as a result of their sedentary lifestyle and diet, mussels are a great indicator when assessing water quality. *In vivo* modifications in biochemical parameters and the development of oxidative stress make biomarkers important ecotoxicological techniques to evaluate the impact of toxicants on aquatic taxa [5-7]. While the half-lethal concentration (LC_{50}), which is the end-point of lethality, is the most commonly used preliminary evaluation of toxic effects, chronic or sublethal toxicological studies take a more practical approach, exposing organisms to toxicant levels that are both low and realistic. Sublethal tests that combine behavioral and biochemical markers have been recommended as the most effective approach in ecotoxicity [8, 9].

Across the world widespread usage of lead, a hazardous element with no recognized biological function, has contaminated the environment. Contamination of water bodies with lead can result in aquatic taxa, such as mussels, producing activated oxygen, resulting in oxidative stress [10]. Catalase (CAT) is a primary enzyme in the cellular antioxidant defense system that detoxifies O_2 and H_2O_2. The hepatopancreas of mussels exposed to heavy metals showed a considerable reduction in CAT activity. Glutathione S-Transferase (GST) is a multifunctional stage II enzyme that is essential in reducing oxidative stress and facilitating the detoxification of xenobiotic compounds. Other enzymes and intermediate products like malondialdehyde (MDA) which is produced even during processes of lipid peroxidation and acetylcholinesterase (AChE), is a key enzyme participating in the synaptic transmission of nerve impulses, appear to be susceptible to pollution [11]. Biomarkers are physiologic, biochemical or molecular expressions of the functional measurements of exposure to environmental stressors. They may offer important details regarding the potential impact of harmful chemicals on the health of individuals [12]. The current study seeked to evaluate the toxicity of lead nitrate on *Unio tigridis* species collected from Qandil water resources using specific enzyme activities.

Materials and Methods

Mussel Collection and Acclimatization

Toxicity test was conducted using the freshwater mussel, *U. tigridis*. Mussels were manually collected in January 2022 from sediment along the Qandil water resources on the Greater Zab River in Erbil, Iraq, at a depth of 30 to 50 cm. The studied site is situated in Qandil village at 36° 37' 39.55" north latitude and 44° 10' 51.80" east longitude. It is 322 meters above sea level.

For acute toxicity tests, 180 individuals of one experimental group of freshwater mussels, *U. tigridis* with a shell length of 7.0 ± 0.5 cm, a width 4±0.5 cm, and weighing 26.5 g, were selected. The mussel species was identified using the common keys [13, 14]. The mussels were brought to the laboratory and acclimatized for 7 days in glass aquaria before commencing the toxicity tests. They were not fed anything during the acclimatization period to allow their digestive systems to empty. Oxygen was supplied continuously and the water was changed daily. Dead individuals were removed daily to avoid pollution.

Molecular Analysis

Following the morphological identification, DNA sequencing was performed on a sample using four replications. The GeneAll® ExgeneTM for Clinic Cell SV small kit was used to separate and purify the genomic DNA from the adductor muscle (Songpa-gu, Seoul, Korea). The amount of genomic DNA was extracted before PCR was determined by agar gel electrophoresis [15]. To ascertain the quantity and quality of DNA, the Nano Drop 1000 spectrophotometer was equipped to detect the optical density of a DNA sample as well as the concentration and purity of genomic DNA extraction. The primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3 and HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', which were ordered from Macrogen (Korea), effectively amplified the COI gene [16]. The PCR test was run in a 50-liter reaction cocktail that contained 3 liters of genome template, 1.0 liter of each primer and 10 pmol of each master mix (AMPLIQON, Denmark). Using PCR-grade water, the quantity was increased to 50 L. The COI gene's DNA was amplified for 5 minutes at 94°C in the thermal cycler to ensure that the DNA templates had been completely denaturized. Following that, the PCR was conducted as follows: 94°C for denaturation for 50 sec, 50°C for annealing for 45 sec, and 72°C for an extension for 50 sec. The final cycle included a 7-minute extension at 72°C after these components had been repeated forty times. The PCR products 21 were examined using 0.8% agarose gel electrophoresis in a 1 TAE buffer before being transmitted to Macrogen (South Korea) for sequencing [17]. The sequences that were obtained were analyzed and modified using the MEGA program.

Water Quality

Water temperature, pH, electrical conductivity EC, total dissolved solids TDS, dissolved oxygen, total alkalinity, total hardness, calcium, and magnesium ions of both river and aquaria water were measured [18] prior to the bivalves being placed in the test chambers. During the test period, the lead concentration in aquaria water was also measured. The water samples were kept at 4°C till their analysis in an atomic absorption spectrophotometer.

Toxicity Test

Before starting the experiment, a detection test was performed to determine the likely range of concentrations. Taxa of approximately equal size were used with 10 individuals assigned to the control and each Pb $(NO_3)_2$ treatment concentration (100, 300, 500, 700, and 900 ppm). Food was not provided throughout the experiment. Dead individuals, if any, were collected at 24-hour intervals [19]. The half-lethal concentrations (LC_{50}) were determined by probit analysis using SPSS 25 statistical software. The end point was indicated by immobility, excessive milky white mucus discharge, open valves, prolonged foot protrusion outside the shell, floating mussels and a lack of reaction to mechanical stimuli. The distinction between behavioral alterations was observed before the toxification and during exposure, taking protective behavior, foot movement, mucus secretion and tentacular movement into consideration.

Lead Concentration

Lead concentration was measured in the exposure medium during the test period. First, acid digestion was performed by nitric acid, and then kept at 4°C till being analyzed in an atomic absorption spectrophotometer (AAS Perkins Elmer USA 1100D) [20]. The concentration of the lead standard solution from Merck, Germany, was 1000. In addition to the water samples, the concentrations of lead were measured in the soft body and shell of mussels. Mussel samples were washed with distilled water and immersed in boiling water for a few minutes. The soft body of the mussels was taken out of the shell, cleaned with deionized water and dried at 70°C for 48 h. The dried samples in powder form were stored in a desiccator for metal analysis. The mussel shells were cleaned with deionized water and dried at 70°C for 48 hours. The sample was stored in a desiccator for subsequent analysis [21]. About 0.5 g of dry animal tissues and shelsl were placed into digestion containers and treated with HNO_3. The samples were then digested with HNO_3 and H_2O_2. Just after digestion, the acid was removed from the samples, before diluting them with 10 mL with 1% HNO3. An atomic absorption spectrophotometer was used to measure Pb concentrations. Pb levels in the animal body were expressed in micrograms per gram of body weight (w/w) [22].

Biochemical Markers

The mussel was dissected and saline solution was used to wash the gills. The gills were homogenized in the extraction solution using a hand-held glass homogenizer. At 4°C, the homogenates were centrifuged for 10 minutes at 8000 rpm. The supernatants were removed and kept at -80°C prior to analysis [23]. Concentrations were determined using commercial assay kits for acetylcholinesterase AChE, catalase CATactivity, glutathione S transfer GST and malondialdehyde MDA.

Statistics Analysis

Every determination was made in triplicate. The SPSS program Version 25 was used to analyze the data. ANOVA one-way method was adopted for dealing with data on water quality, lead concentration, and biochemistry. Duncan's post hoc test was used to look for significant differences between treatments. Additionally, a correlation was made between heavy metal concentrations in the water, shell and soft body of the mussels. The limit for statistical significance is a p-value of 0.05.

Results and Discussion

Metals play critical roles in a variety of mechanisms in living organisms. Although some metals such as cadmium, lead, copper and iron can be toxic to living things when present in large concentrations. It may be useful to monitor these factors and evaluate biochemical indicators in biota treated with these chemicals to learn more about the ecosystem's health [24].

The identification of the mussel molecularly by using comprehensive or specific initial gene magnification was consistent with phenotypic evaluation. CDS nucleotide sequencing provided data for the molecular sample as well as isolation, diagnostics, and minute properties. The DNA sequence of the mussel included nucleotide sequencing from *U. tigridis* gene bank accession numbers (ON872361, ON872362, ON872363, and ON872364).

The physicochemical parameters of the test water mediums were: temperature, 19 °C; pH, 7.79; EC, 654.33 µS/cm; TDS, 328 mg/l; DO, 7.5 mg/l; alkalinity, 287 mg $CaCO_3$/l; total hardness, 380 mg $CaCO_3$/l; calcium and magnesium ions, 100 and 31.2 mg/l respectively. However, the aquaria water physicochemical characteristics included the following:

temperature 20 °C; pH 7.74–8.02; EC 392.17–400.56 µS/cm; TDS 256.36-262.75 mg/l; DO 7.16-7.25 mg/l; alkalinity 95.28–112.39 mg $CaCO_3$/l; total hardness 217.99–218.50 mg $CaCO_3$/l; calcium and magnesium ions 62.69–67.86 and 14.41–17.01 mg/l respectively.

Metal-induced stress was found to be high at various concentrations upon exposing the mussels to the acute toxicity of lead nitrate. Consequently, a comparison was made between the behavioral responses of the exposed groups and the control group. These alterations were noticed after exposure for 24, 48, 72 and 96 hours (Table 1). Changes in animal behavior could also be used to detect ecotoxicity. Similar outcomes were noted by Yasmeen and Pathanf [25]. Over and above that, the metal-induced mortality was high enough to calculate an LC_{50} for *U. tigridis*; the 96 hr. LC_{50} was 782.70 ppm. According to Sokolova and Lannig [26], the element and species will have a significant impact on how many metals mussels take up.

Table 1 : The control group's usual behavior and changes in behavior in freshwater mussels *Unio tigridis* resistance to lead nitrate intoxication various exposure times.

Response	Behavior				
	Control	**24 h**	**48 h**	**72 h**	**96 h**
Protective	rapid response	tolerate toxicity using operculum	valves closed tightly	Valves closed tightly	Valves closed tightly
Activities of the foot and its secretion	was quick and firmly attached to the surface	extended initially	was slowed down	retracted	Not observed
Reaction to environmental stimulation	when immersed in water, close the shell valves	rapid	reduced	poor	no
Gill mucus secretion	not observed	initiated	increased	quantitatively increased	thick white mucus observed

Water samples from the experimental media were collected at 24, 48, 72 and 96 h after the test for quantifying the concentration of lead. Figure 1A illustrates the variation in lead concentrations during hours 24, 48, 72 and 96 of the tests in all aquaria with different concentrations of lead nitrate. Lead concentration during the study ranged from 0.015 ppm in the control aquarium after 96 h of the test period to 77.966 ppm in the aquarium with 900 ppm after 24 h of the test. There was no significant difference ($p > 0.05$) in lead levels in aquaria with 0 and 100 ppm lead nitrate concentrations during the testes period. However, lead concentrations varied significantly ($p \leq 0.05$) in 24 h and all other studied periods in all other aquaria. This was due to the bioaccumulation of heavy metals by mussels. Bivalve are important ingredients of the aquaculture biogeochemical cycle and, as filter feeders, are extremely sensitive to pollutants [27]. Moreover, the lead concentrations were measured in both the shell and tissues of the mussels. As is obvious from Figures 1B and 1C that the efficiency of the shell to collect heavy metals was greater than that of a soft body [21]. The lead concentrations in the shell ranged from the minimum value of 35.3 µg/l in the control aquarium after 24 h of the test period to the maximum value of 425.833 µg/l in the aquarium, with 900 ppm lead nitrate concentration after 96 h of the test period. The lead concentrations estimated in the shells of mussels collected from aquaria increased significantly ($p \leq 0.05$) during the studied periods, except in the control aquarium where the lead level remained steady. While, the lead concentrations measured in the tissues of the collected mussels ranged from the minimum value of 9.36 µg/l. Also, in control aquarium after 24 h of the test period to the maximum value of 793.4 µg/l in aquarium with 900 ppm lead nitrate concentration after 96 h of the test period. The mussel shells had the highest concentration of Pb. This result is comparable to Yap et al.

[28] and could be attributed to the chemical composition of the shells. Due to surface adsorption, shells accumulated higher concentrations of metals at first [29]. Lead and calcium share some similarities in their metabolic pathways, but they also have important differences. Calcium plays a crucial role in the formation and maintenance of bivalves' shells. Bivalves are able to absorb calcium ions from their environment and incorporate them into their shells. Lead, on the other hand, is a toxic heavy metal that is not essential for biological processes. Both lead and calcium can be transferred to the shell through passive adsorption, ingestion, or gill respiration, even though they have different metabolic pathways and effects on the organism [30].

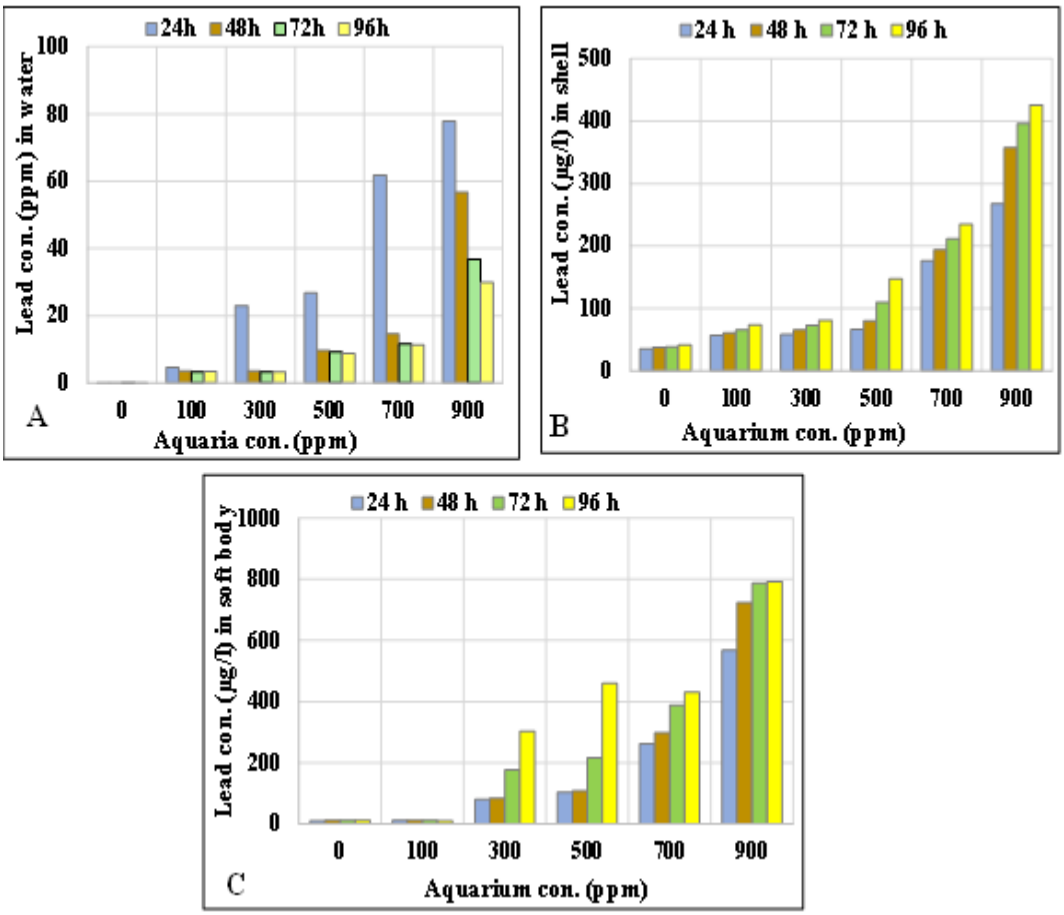

Figure 1: Concentration of lead in the A-experimental media, B-shell and C-soft body of the mussels during test periods.

The biochemical responses of each treatment group are shown in Figure 2. The influence of treatment and time on biochemical biomarkers such as AChE, GST, CAT, and MDA was found to have a clear interaction ($P \leq 0.05$). The enzyme AChE had a significant impact on the hydrolysis of the neurotransmitter acetylcholine and happened to be a good biomarker for detecting heavy metal toxicity in the ecosystem [31]. Pesticides, as well as some heavy metals, have been shown to strongly inhibit AChE activity [11]. The level of AChE in *U. tigridis* treated with lead nitrate was significantly lower than in the control group (Figure 2A). The results revealed that lead reduces AChE activity in a dose-dependent manner. The lowest level of AChE was recorded in aquaria with 900 ppm of lead nitrate after 96 h of the test period. The inhibition ofAChE activity and metal concentrations have an inverse relationship. However, it is well known that metals, as well as other environmental pollutants, inhibit AChE [32]. Yololu et al. discovered similar results [24]. GST is an enzyme group that aids in the detoxification of

metabolites produced during oxidative stress. Glutathione is regarded as a key agent because it protects cell membranes from lipid peroxidation by prohibiting oxygen radicals from passing through the cell membrane. The rise in GST levels in mussels is a sign of exposure to chemical pollution [33]. During the test period, the value of the GST increased slowly with increasing exposure concentration and the test period (Figure 2B). Catalase is essential for the conversion of H_2O_2 to H_2O and O [34]. CAT concentrations in mussel gill tissues increased significantly at all concentrations over the course of the study. After a 48-hour test period, the maximum concentration of CAT was recorded in aquaria with a lead nitrate concentration of 500 ppm. Cadmium significantly increased catalase activity in the mussel *Pernacanaliculus* [35]. Similar findings have been noted in manganese-exposed *Carassius auratus* serum, with significant rises in CAT and glutathione s-transferase [36]. Treating *U. tigridis* with lead nitrate resulted in a significant ($p \leq 0.05$) increase in MDA in comparison to the control group (Figure 2 D). The maximum value of MDA was observed after 96 h of testing in an aquarium with a 900ppm lead nitrate concentration. The antioxidant defense system, whether enzymatic or non-enzymatic, is a biochemical marker that can be used to assess the impact of contaminants on bivalves [37]. These findings supported prior findings indicating that lead-induced lipid peroxidation causes the formation of aldehydic byproducts such as MDA *in vivo* [38].

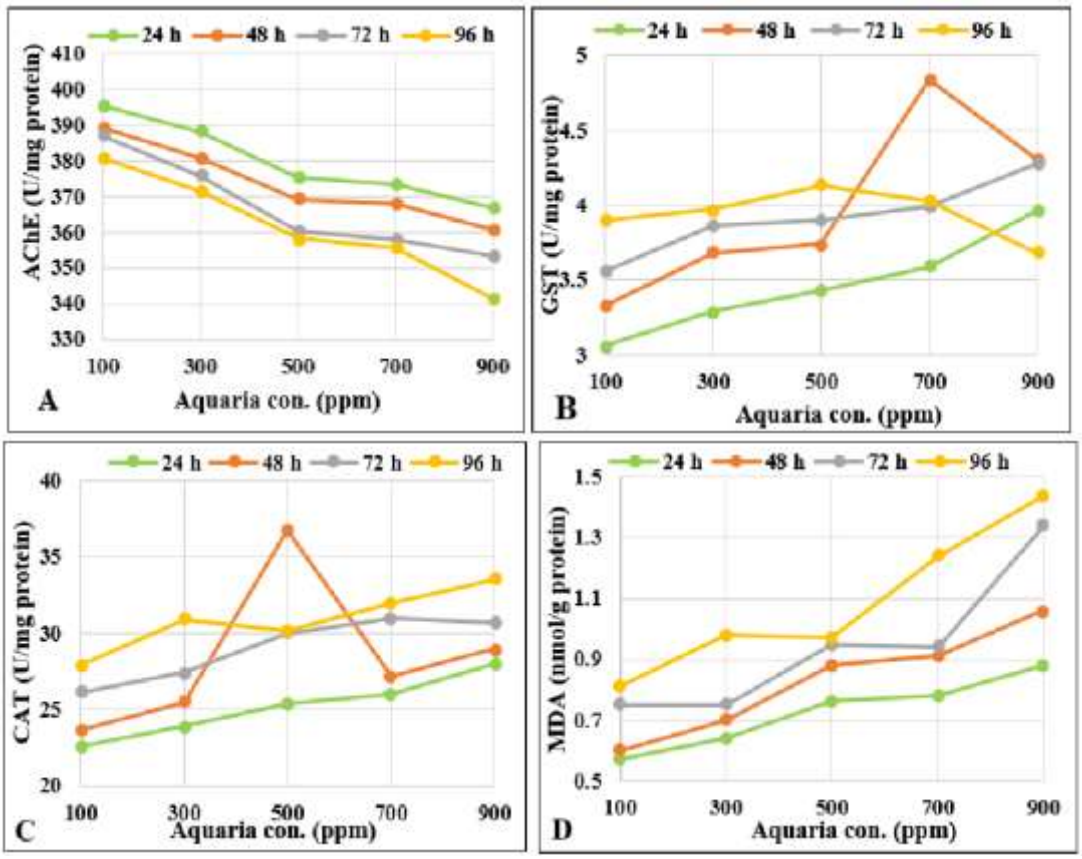

Figure 2: Change in acetylcholinesterase (AChE) (A), glutathione s-transferase (GST) (B), catalase (CAT)(C) and malondialdehyde (MDA) (D) levels of *Unio tigridis* exposed to 100, 300, 500, 700 and 900 ppm lead nitrate along the 24, 48, 72 and 96 h.

Conclusion

This study was carried out with the freshwater mussel *U. tigridis* exposed to lead nitrate. The study on the taking of lead by mussels in experimental media revealed that the metal level in aquaria water decreased as the level increased within the test organismshell and soft tissues, thus leading to behavioral alterations. Changes in biomarker levels were affected by pollutant

concentrations and exposure times. According to our *in vivo* findings, exposure to lead concentrations induced several biochemical responses in antioxidant enzymes, resulting in lipid peroxidation. Enzyme activity was inhibited during the exposure period which may have had some relationship with oxidative stress. The value of AChE was found to be inversely related to lead concentration. However, the values of all other biomarkers, including GST, CAT, and MDA, increased as lead exposure concentration increased.

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