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ORIGINAL PAPER

Metal bioaccumulation, oxidative stress, and biochemical alterations in the freshwater snail (*Galba truncatula*) exposed to municipal sewage

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ABSTRACT

Urban wastewater contains various detergents, pesticides, pharmaceutical medications, cosmetics, hygiene products, and heavy metals. Discharge of municipal sewage into surface water affects the health of aquatic organisms through altering biochemical markers and accumulating heavy metals in various tissues. The present study aimed to evaluate the toxic effects of municipal sewage on the biochemical markers and bioaccumulation of cadmium and lead in freshwater snail (*Galba truncatula*) during 14 days. Concentrations of toxic metals in the snails were determined using ICP-OES-PerkinElmer, and biochemical parameters were measured via UV-Vis spectroscopy. The results indicated significantly lower levels of glycogen and total antioxidant in the cells, as well as the significantly lower activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, acetylcholine esterase, catalase, and glucose-6-phosphate dehydrogenase in the *Galba truncatula* exposed to sewage compared to the control. However, the activities of lactate dehydrogenase ,glutathione peroxidase, and malondialdehyde were significantly higher in the snails exposed to sewage compared to the control. Moreover, bioaccumulation of cadmium and lead was observed to increase in the experimental groups exposed to sewage. Alterations in biochemical parameters in the *G. truncatula* exposed to sewage could be due to the toxic effects of various environmental pollutants in municipal wastewater. According to the findings, oxidative damage to the vital tissues of *G. truncatula* was associated with the bioaccumulation of cadmium and lead, depletion of total antioxidant levels, changes in biochemical parameters, and lipid peroxidation in soft tissues.

Keywords: Municipal sewage, Biochemical markers, Freshwater snails, Metal bioaccumulation

Introduction

Recently, the global production of synthetic chemicals has risen to up to 400 million tons per year.[1] There are growing concerns regarding the adverse effects of emerging pollutants on aquatic ecosystems, while data is scarce on their potential environmental hazards. Some of these hazardous chemical compounds include pharmaceuticals, cosmetic and personal care products, heavy metals, and pesticides.[1,2]

To date, numerous *in-vitro* studies have investigated the toxic effects of environmental pollutants on aquatic ecosystems.[3.4] Nevertheless, aquatic organisms are constantly exposed to various contaminants.[5] Few studies have been focused on the influence of a combination of pollutants on aquatic organisms. According to the laboratory and field studies in this regard, emerging environmental pollutants could adversely affect numerous aquatic animals.

Municipal sewage is a significant contributing factor to the pollution of aquatic ecosystems. Even the most advanced wastewater treatment centers are not able to thoroughly remove pollutants from wastewater; consequently, these compounds enter the environment. Developing countries are faced

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with this issue more critically, especially the cities where there are no sewage treatment plants. For instance, in many southern cities in Iran (e.g., Behbahan, Khuzestan), domestic sewage and surface runoff outfall into water canals without any treatment and enter the Maroon River due to the absence of municipal sewage treatment centers and public ignorance.

Considerable amounts of sewage are discharged into the Maroon River through local channels on a daily basis.[5] If the concentrations of the pollutants are presumably low in the sewage outfall, exposure to these compounds could be a threat to the environment, leading to their bioaccumulation in aquatic organisms.[1]

The Maroon River is one of the main habitats of native aquatic species owing to the climate of Khuzestan province, as well as the physical and chemical properties of the river and its hydrological regimen (Table 1). Furthermore, the Maroon River is an important source of drinking water and agriculture for the residents in the region. On the other hand, the wastewater of Behbahan city and the sewage produced by at least 15 industrial units, agricultural farms, and the surrounding villages is eventually discharged into this river.[6] As a result, the pollution of this river could threaten aquatic organisms.

There are varied species of aquatic organisms in the Maroon River, including gastropods. Based on their way of life and nutrition, gastropods are considered to be the bio-indicators used to evaluate the pollution level of aquatic ecosystems.[7] This is due to the changes observed in the physiology, growth, and behavior of *Bellamya aeruginosa*,[8] oxidative stress, tissue damage, and alterations in the biochemical parameters in *Pila globosa*,[9] *Meretrix meretrix*,[10] *Biomphalaria alexandrina*,[11] *Lymnaea luteola*,[12] *Helix aspersa*,[13] and *Theba pisana,*[14] as well as the genetic damages in *Bellamya aeruginosa,*[8] which were exposed to various environmental pollutants.

Undoubtedly, these aquatic organisms play a pivotal role in the food chain of aquatic ecosystems. Moreover, freshwater snails could act as an intermediary host in the prevalence of parasitic infections. Aquatic mollusks are benthic organisms that are exposed to various chemical pollutants in water and riverbed sediments. Therefore, freshwater snails are proper bio-indicators to evaluate the levels of environmental pollutants in aquatic ecosystems. In addition, several mollusks are highly adaptable to living in contaminated environments. With this background in mind, assessing the physiological response of these organisms and bioavailability of environmental pollutants in their body could yield valuable data regarding the ecological conditions of aquatic ecosystems.[15]

Table 1. Physicochemical properties of maroon river water

Parameters	Maroon River Water
pH	8.1±0.2
COD (mg/l)	51±8
BOD (mg/l)	24±7
TSS (mg/l)	43±10
TDS (mg/l)	983±269
Water turbidity	12.5±4.5
EC (dS/m)	1.23±0.35
Ca (mg/l)	1045±25
Mg (mg/l)	36.6±0.42
N-NO_3 (mg/l)	2.62±0.15
P-PO_4 (mg/l)	0.28±0.02
Cd (mg/l)	0.07±0.02
Pb (mg/l)	0.09±0.03
Temperature (ºC)	20.5 ± 2.5
Salinity (g/l)	1.98±0.3
Dissolve Oxygen (mg/l)	7.4±1.7

Galba sp. (*Lymnaea* sp.) is a freshwater snail, which is found abundantly in freshwater ecosystems, including the Maroon River. These snails are also found in the vegetation grown by rivers and dams. *Galba truncatula* is a sedentary species that inhabits freshwater ecosystems. It could readily be collected, identified, and preserved *in-vitro*.

The present study aimed to investigate the toxic effects of the sewage collected from the water canals in Behbahan with various concentrations on the biomarkers of *G. truncatula*.

Materials and Methods

Tested Organisms

Sexually mature male and female *G. truncatula* (mean wet weight: 60±15 mg; mean shell length: 22±3 mm) were collected manually at the depth of 30-40 centimeters from a vegetated bank of the Maroon River with a less polluted water source. Afterwards, the snails were transferred to the laboratory of Khatam Alanbia University of Technology in Behbahan, Iran. The samples were washed to remove the mud and algae sticking to the shells.

The snails were preserved in aerated glass aquaria (8 L) filled with filtered, dechlorinated tap water with the mean pH of 7.4±0.2 and mean temperature of 22±2 ºC within a cycle of 14 hours of light and 10 hours of darkness as the artificial photoperiod regimen. Water exchange was carried out every day, and the dead snails were removed as soon as possible. The snails were fed with lettuce leaves once a day for 14 days.

The study was conducted during February-March at the beginning of the reproductive season of the snails. All the experiments were reviewed and approved by the Ethics Committee on Animal Use of the Faculty of Environment and Natural Resources at Khatam Alanbia University of Technology in Behbahan, Iran (code: 10.6.3521; 12 June 2017).

Wastewater Samples

Sewage effluents were collected from six stations of the sewage canal in Behbahan in February. Seven days before sampling, there was no rain in the sampling area, and the temperature was 17-26 ºC. Duplicates of each sample were collected in brown glass bottles with Teflon stoppers. In order to disinfect and remove pathogens from the wastewater, the samples were filtered and sterilized using an ultraviolet lamp for 30 minutes before use. In addition, the physicochemical properties of the municipal sewage samples were analyzed using standard methods.

Sub-lethal Toxicity Tests

Semi-static, sub-lethal toxicity tests were carried out in glass aquariums, which contained eight liters of dechlorinated tap water and were constantly aerated. The snails were divided into four treatment groups, each of which was triplicate. Each aquarium contained 30 freshwater snails, which were exposed to 0.1%, 0.2%, and 0.4% of the municipal wastewater for 14 days. The control groups were only preserved in dechlorinated water.

The aquaria were cleaned by siphoning, and 50% of the water was exchanged daily in order to reduce metabolic wastes. Afterwards, the municipal wastewater was added again so as to maintain its concentrations at the nominal level. Mortality was verified daily, and the dead snails were removed immediately.

After 14 days of exposure, the snails were collected and washed, and the shells were broken to separate the soft tissue. In each experimental group, the soft tissues were collected, pooled, and placed in micro-tubes. For the enzymatic and biochemical examinations, the samples were homogenized on ice in cold buffer (100 mM potassium phosphate, pH: 7.4, 2 mM of EDTA). Tissue homogenates were centrifuged at 15,000×g at the temperature of 4 ºC for 15 minutes. Following that, the supernatant was removed and frozen at the temperature of -25 ºC for further analysis.

Measurement of Biochemical Parameters

Concentration of malondialdehyde (MDA), which is a marker of lipid peroxidation, was estimated at 532 nanometers based on the method proposed by Placer *et al.*[16] In addition, the total antioxidant capacity of the cells was measured based on the ferric reducing ability of plasma (FRAP) at 593 nanometers.[17] The glycogen content was also measured 18 after glycogen hydrolysis to glucose using hydrochloric acid (HCl) and Sodium hydroxide (NaOH). Catalase activity was assessed following the reduction of the absorbance at 405 nanometers due to the use of hydrogen peroxide (H_2O_2) in the presence of ammonium molybdate.[19]

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were assayed based on the method proposed by Moss and Henderson using commercial biochemical kits (Pars Azmoun Co., Iran).[20] Acetylcholinesterase

(AChE) activity was determined at 405 nanometers using commercial biochemical reagents (Pars Azmoun Co., Iran).[21] In addition, the activity of glucose-6-phosphate dehydrogenase (G6PDH) was evaluated at 340 nanometers using $NADP^+$ as the substrate, and glutathione peroxidase (GPx) activity was assessed using diagnostic biochemical kits (Ransel and Randox Co., UK).[22]

In brief, GPx was analyzed using cumene hydroperoxide as the substrate at 340 nanometers. The enzyme activities were expressed per gram of protein in the entire measured tissues using commercial biochemical kits (Pars Azmoun Co., Iran).[23] All the biochemical parameters were measured using a UV-Vis spectrophotometer (model: Biochrom Libra S22).

Measurement of the Bioaccumulation of Cadmium and Lead

For sample digestion, a representative homogeneous tissue of the snail (wet weight: 1 g) was digested with the repeated addition of nitric acid (HNO_3; 5 ml) and 30% H_2O_2 (5 ml). After 60 minutes, five milliliters of HCl was added to the initial digestion, and the sample was refluxed. The digested samples were filtered, and the filter paper and residues were initially rinsed with HCl, followed by hot distilled water.

At the next stage, the digested samples were diluted to the final volume of 20 milliliters. The concentrations of cadmium and lead were also measured using ICP-OES-PerkinElmer (Optima 7300-DV). After drawing a diagram for the calibration of cadmium and lead, the concentrations of the metals in the prepared solutions were determined. Following that, the samples were filtered using a Whatman filter (0.22 µm), and the concentrations of cadmium and lead were identified in each sample using an ICP instrument. Cadmium and lead levels were measured in triplicate.

Statistical Analysis

Data analysis was performed in SPSS version 19 (IBM) using one-way analysis of variance (ANOVA), and the normality of the data was evaluated using the Shapiro-Wilk test. Comparison of the significant means was performed using Tukey's test at the significance level of $P<0.01$. Data were presented as mean and standard deviation (SD) in each treatment group. In addition, comparison of the significant differences between the obtained values with the control group was marked by alphabetical symbols ($P<0.01$).

Results and Discussion

The physicochemical properties of the urban sewage effluent samples and Maroon River are presented in Tables 1 and 2. The mean turbidity of the untreated sewage effluent was 126±10 mg/L, while electrical conductivity (EC) was estimated at 126±10 dS/m. The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) of the urban sewage were calculated to be 5205±85 and 639.5±47 mg/L, respectively. The mean levels of $N\text{-}NO_3$, $P\text{-}PO_4$, and sulfide in the municipal wastewater were estimated at 30.22±4.75, 21.8±4.3, and 186±7 mg/L, respectively.

Table 2. Physicochemical properties of municipal sewage effluent

Parameters	Raw sewage
pH	7.97±0.40
COD (mg/L)	5205±85
BOD (mg/L)	639.5±47
TSS (mg/L)	1252±25
TDS (mg/L)	4920±400
Water turbidity	126±10
EC (dS/m)	3.65±0.5
Ca (mg/L)	1658±202
Mg (mg/L)	129.85±22.5
$N\text{-}NO_3$ (mg/L)	30.22±4.75
$P\text{-}PO_4$ (mg/L)	21.8±4.3
Sulfide (mg/L)	186±7
Cd (mg/L)	15.5±5.5
Pb (mg/L)	19.7±8.2
Temperature (ºC)	22.5 ± 2.0
Salinity (g/l)	5.11±1.3
Dissolve Oxygen (mg/L)	4.7±0.8

In the present study, only a few snails died during the experiments, and the most significant mortality rate was observed in the snails exposed to the highest concentration of sewage. However, the mortality rate in the treatment groups was less than 10%.

With regard to the quality of urban sewage, its discharge into the Maroon River adversely affected the water quality. Subsequently, the water quality had a negative impact on the life of aquatic organisms, threatening their survival with chemical compounds. To evaluate the level of water pollution, the snails could be used as an appropriate bio-indicator since they are frequently found in freshwater ecosystems.

The biochemical parameters of the mollusks that are exposed to environmental pollutants are among the key biomarkers that could be applied in the monitoring of environmental pollutants.[13, 14, 24] In the present study, we investigated the changes in the *G. truncatula* samples that were exposed to various concentrations of municipal sewage. As is depicted in Figure 1, exposure to sewage significantly decreased the AChE, AST, ALT, and ALP activities in the soft tissues of the snails. Therefore, it could be concluded that exposure to sewage could significantly increase the activity of LDH in *G. truncatula* (Figure 1). Furthermore, out findings indicated that the activity of AChE was significantly lower in the snails exposed to sewage compared to the controls. The reduced activity of AChE could be due to the presence of AChE inhibitors in sewage, such as heavy metals, organophosphate and carbamate pesticides, and other chemicals.[25] These compounds could interact with cysteine on the active site of AChE and disturb the enzyme activity.[5] The reduced activity of AChE has also been reported in *Physa acuta.*[26] The results of the present study in this regard were consistent with the findings of Khalil.[27]

According to the current research, the exposure of the snails to sewage significantly reduced AST and ALT. Many cells regularly use glutamate to synthesize glutathione. These cells need α-ketoglutarate and glutamine in order to maintain the intracellular glutamate. AST and ALT play a key role in the biosynthesis of glutamate as a constituent amino acid of glutathione.[28] Therefore, the reduced activity of AST and ALT in the snails exposed to sewage may diminish the synthesis of cellular glutathione. Furthermore, ALT and AST are vital enzymes in the metabolism of amino acids, while they are also essential to the transfer of amino acids in the pathways involved in the production of energy in the tricarboxylic acid (TCA) cycle.[29] Therefore, the significant reduction of AST and ALT activity may indicate the disrupted process of energy supply through amino acid metabolism. Under normal circumstances, gastropods often supply their energy through aerobic metabolism. However, when snails are exposed to physiological stress, they may act through fermentation or anaerobic routes to provide energy.[30]

In the present study, a significant increase was observed in the activity of LDH in the *G. truncatula* exposed to sewage, which could be a physiological response to the stressful environmental. Increased LDH activity in the soft tissues of snails could be attributed to hypoxia and increased anaerobic glycolysis. This enzyme is involved in the metabolism of carbohydrates in cells and plays a key role in maintaining the balance between the catabolism and anabolism of carbohydrates in mollusks.[29] In a stressful environment, LDH converts pyruvate into lactate, which in turn leads to the enhanced concentration of carboxylic acid (lactic acid) in tissues and hemolymph. Therefore, the pH of tissues and hemolymph decreases and causes specific physiological changes.[30] In a study by Abdel-Halim *et al.*,[13] the LDH activity was observed to increase, which indicate a physiological response to the hypoxia-induced stress following the exposure of *Helix aspersa* to heavy metals. In addition, a significant increase has been reported in the LDH activity in the land snails (*Eobania vermiculata*) exposed to methomyl.[27]

In the current research, the activity of ALP in the snails exposed to sewage was significantly lower compared to the controls. ALP is a membrane glycoprotein, which catalyzes the hydrolysis of phosphate monoesters and is essential to protein synthesis.[29] The activity of ALP depends on three metal ions, including two Zn^{+2} ions and one Mg^{+2} ion in the active site of the enzyme.[31] The active site of ALP has a serine base.[29] As a result, the competition between the metals present in the ions or interaction of other pollutants with the serine base at the active site of the enzyme may have inhibited the ALP activity.

Previous findings have denoted the reduced activity of ALP in the liver of the snails (*Oncomelania hupensis*) exposed to the herbal extract of *Pulsatilla chinensis,* which was used as a molluscicide.[29] Furthermore, the reduced pH of hemolymph and tissue due to the increased lactic acid in the snails exposed to sewage may disrupt the ALP activity.[30] Similar results have been reported in the land snail (*E. vermiculata*) exposed to carbamate pesticides.[27]

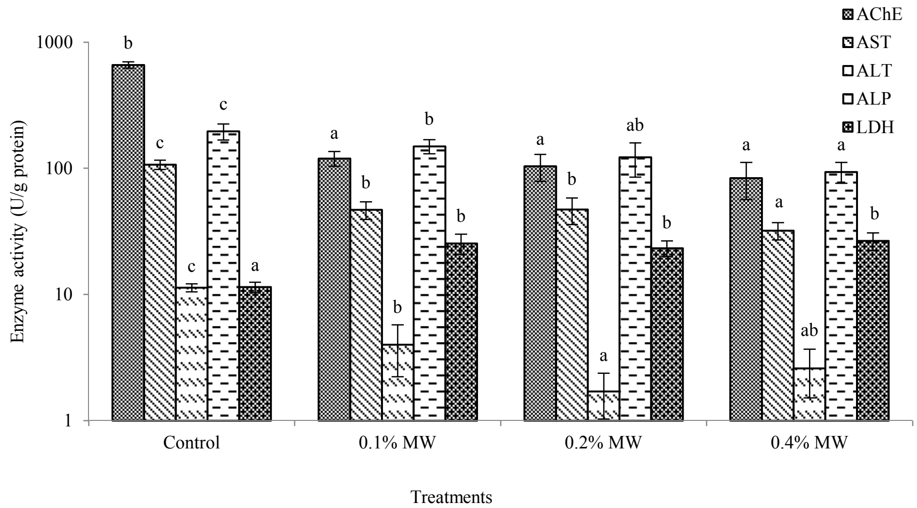

Fig. 1. Enzyme activities in entire body of *galba truncatula* exposed to 0.1%, 0.2%, and 0.4% of municipal wastewater for 14 days

In the current research, the activities of catalase and G6PDH decreased significantly compared to the controls ($P<0.05$). In contrast, the activity of GPx significantly increased (Figure 2). The activity of G6PDH in the snails exposed to sewage was significantly lower than the controls. Increased reactive oxygen species (ROS) could inhibit the activity of G6PDH, which in turn decreased the synthesis rate of nicotinamide adenine dinucleotide phosphate (NADPH), preventing oxidative stress in mollusks.[32] Therefore, the reduced activity of G6PDH could diminish the storage of glutathione in the cells of the *G. truncatula* that were exposed to sewage. Reduced activity of G6PDH leads to oxidative stress and cell apoptosis.[33]

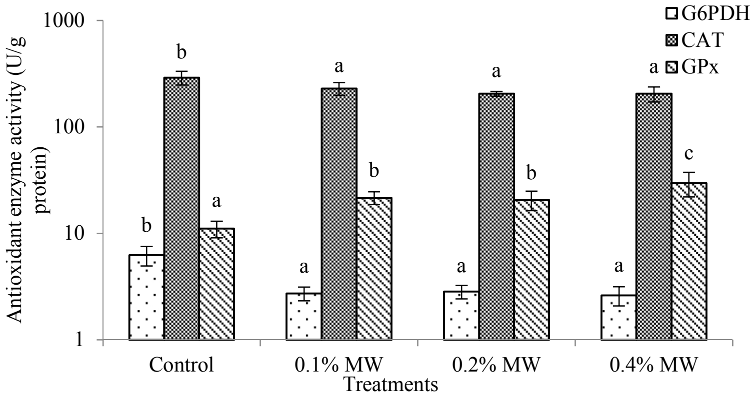

Fig. 2. Antioxidant enzyme activities in entire body of *galba truncatula* exposed to 0.1%, 0.2%, and 0.4% of municipal wastewater for 14 days

Peroxide radicals are converted into hydrogen peroxide by mitochondrial superoxide dismutase (Mg^{+2} SOD) and cytosolic superoxide dismutase (Cu^{+2}/Zn^{+2} SOD). Hydrogen peroxide is decomposed into water and oxygen by catalase in peroxisomes and by glutathione peroxidase in the cytosol and mitochondria.[34] Despite the role of GPx and catalase in eliminating hydrogen peroxide,[12] the activity of catalase significantly decreased in the snails exposed to sewage, while the activity of GPx increased significantly. The increased activity of GPx and reduced activity of catalase could be attributed to the changes in the gene expression of these enzymes in response to increased H_2O_2.[34] The reduced activity of G6PDH and synthesis rate of NADPH may affect the reduction of catalase activity.[35]

Previous studies have reported the reduced activity of catalase in the freshwater snails (*Physa acuta*) exposed to abamectin.[26] Similar results have also been proposed in the mud snails (*Amphibola crenata*) exposed to waterborne cadmium.[15] As long as the reduced glutathione (GSH) in cells is at a normal level, the GPx activity in response to H_2O_2 increases. However, with the decreased cell reserves of GSH, the activity of GPx has been reported to decline as well. The increased activity of GPx has also been reported in the *T. pisana* and *H. aspersa*[13] snails exposed to pollutants.[14]

After exposure to municipal sewage for 14 days, the glycogen level decreased significantly compared to the controls in the present study (Table 3). Glycogen breakdown and increased glucose are among the well-known mechanisms to provide energy in mollusks in stressful environments.[30] The energy is used to cope with the cytotoxicity induced by municipal sewage. Another study in this regard denoted the reduction of glycogen in tissues and increased level of hemolymph glucose in the freshwater snails (*Bulinus truncates*) exposed to glyphosate herbicide,[11] which is in line with the results of the present study. Similarly, the reduction of tissue glycogen has been reported in the mud snails (*Amphibola crenata*) exposed to waterborne cadmium.[15]

Table 3. Biochemical parameters in entire body of *galba truncatula* exposed to 0.1%, 0.2%, and 0.4% of municipal wastewater for 14 days

Biochemical Parameters	Control	0.1% Municipal wastewater	0.2% Municipal wastewater	0.4% Municipal wastewater
Glycogen (mg/g tissue)	19.30±1.95^{c}	11.99±2.12^{b}	10.82±1.65^{ab}	8.65±1.33^{a}
Total antioxidant (µM/g protein)	713.93±122.09^{c}	484.88±52.08^{b}	358.72±60.12^{a}	349.86±43.44^{a}

In the current research, the antioxidant capacity of the soft tissues of the snails exposed to sewage for 14 days had a significant reduction ($P<0.05$). On the other hand, MDA levels significantly increased in *G. truncatula* (Table 3). The antioxidant defense system (enzymatic and non-enzymatic) is an appropriate biochemical biomarker to assess the impacts of environmental pollutants on mollusks. In the present study, the reduced total antioxidant capacity of cells was considered to be a significant physiological response of the antioxidant defense system to the increased production of free radicals after the exposure of *G. truncatula* to municipal wastewater.

According to the findings of the current research, the decreased antioxidant capacity of the cells and increased ROS in the *G. truncatula* exposed to municipal sewage led to the significant increase of the MDA level, which demonstrated the increased rate of lipid peroxidation. Consistently, previous findings have also denoted increased MDA levels in freshwater snails (*P. acuta,*[26] *L. luteola,*[12] *B. alexandrina,*[11] *T. pisana,*[14] and *H. aspersa*).[13] In addition, similar behaviors have been reported in the mud snail (*Amphibola crenata*) exposed to waterborne cadmium.[15]

According to the present study, the bioaccumulation of cadmium and lead increased in the soft tissues of the snails in response to exposure to higher concentrations of sewage (Figure 3). The main organs that absorb waterborne heavy metals in aquatic organisms are the gills, skin, and gastrointestinal tract, and metal-contaminated feed is considered to be the main route of this pollution.[3] The current research indicated that the higher ratio of sewage increased the bioaccumulation of heavy metals (e.g., lead) in the soft tissues of *G. truncatula*. The bioaccumulation of cadmium was observed to increase in the snails exposed to municipal sewage after 14 days compared to the controls.

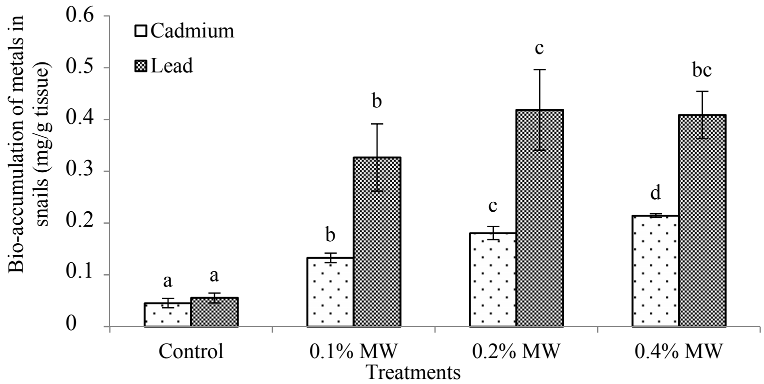

Fig. 3. Bioaccumulation of cadmium and lead in soft tissues of *galba truncatula* exposed to 0.1%, 0.2%, and 0.4% of municipal wastewater for 14 days

The increased bioaccumulation of cadmium and lead in the snails exposed to urban wastewater may be due to the reduced synthesis of metallothionein and dysfunction of metal detoxification. Reducing the gene expression of metallothionein could affect the detoxification of cadmium[36] and lead in the snails exposed to municipal sewage. Moreover, municipal sewage influences the physiology of the cell membrane, thereby altering the ability to absorb heavy metals. In a study in this regard, Radwan *et al.* reported a significant correlation between the bioaccumulation of heavy metals and oxidative stress in *Theba pisana.*[14] The recommended maximum limits of cadmium and lead in wastewater sludge by the Environmental Protocol Agency (EPA) are presented in Table 4.[37]

Table 4. Recommended maximum limits of EPA for cadmium and lead in wastewater sludge

Heavy metal	Concentrations (mg/Kg)
Cd	39
Pb	300

Conclusion

According to the results, the discharge of untreated municipal sewage into the Maroon River adversely affects the health of *G. truncatula*. Significant changes were observed in the biochemical parameters and enzymatic activity of the soft tissues of the snails with the increased concentrations of pollutants. Additionally, the total antioxidant level and catalase activity significantly decreased in the soft tissues. However, MDA levels and GPx activity were observed to increase in the soft tissues of the snails exposed to various concentrations of municipal sewage.The free radicals that are produced during xenobiotic detoxification in urban sewage might give rise to lipid peroxidation, oxidative stress, and biochemical changes in the soft tissues of snails. Furthermore, the presence of heavy metals (e.g., cadmium and lead) may be hazardous to water ecosystems and affect the health of aquatic organisms. Therefore, it could be concluded that the bioaccumulation of cadmium and lead increases following exposure to sewage.

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Ethical Considerations

Neither the present study nor any other studies mentioned in our research contain human participants. All the applicable international, national, and/or institutional guidelines for the care and use of animals were observed in this research project.

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