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The use of *Anodonta cygnea* as an indicator of heavy metal contamination in Anzali wetland

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Abstract
Background: Wetlands are among the most important ecosystems and areas of life that are ecologically and economically important in the world and susceptible for heavy metal contamination. The elements in water and sediment may be easily and in large quantities available to animals in aquatic ecosystems. This study aimed to investigate the level of heavy metal contamination in *Anodonta cygnea*.
Methods: *Anodonta cygnea* sampling was carried out in the summer 2023. A total of 35 specimens were collected randomly by fishing net. Following the laboratory analyses, the concentration of aluminum (Al), arsenic (As), copper (Cu), cobalt (Co), cadmium (Cd), iron (Fe), manganese (Mn), magnesium (mg), lead (Pb), nickel (Ni), tin, vanadium (V) and zinc (Zn) were determined by ICP-OES.
Results: Target hazard quotient (THQ) values of Mn were above 1. The p_i index showed that *A. cygnea* is slightly contaminated by Pb. Analyses of the metal accumulation showed that *A. cygnea* bivalve was moderately contaminated by Zn and severely contaminated by As. The results showed no significant relationship between the total weight of the organism and concentration of Al, Fe, and Ni.
Conclusion: Aquatic organisms, including bivalves, do not possess an advanced excretory system, therefore the excretion of metabolites and pollutants is a is a slow process. once the concentration of heavy metals in the aquatic environment increases, they become easily available to aquatic organisms and enter the biological system. In this study, the absorption rate of metals was higher than its excretion or removal by the excretory system which results in their accumulation in various tissues and organs of the organism.
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Introduction

Anzali wetland is considered as one of the most important freshwater wetlands in Iran. This wetland located in the southwest of the Caspian Sea (near Anzali Port) in Guilan province. The area of this wetland is estimated to be 193 km^2 (1). The development of industries, the excessive increase in population, the development of agricultural areas, and excessive use of fertilizers and pesticides have caused industrial and urban wastes and agricultural effluents rich in heavy metals enter the aquatic ecosystems (2). Human activities in and near coastal waters and along the rivers entering the sea carry large amount of pollution agents including heavy metals into the sea (3).

In recent decades, with the growth of population followed by the expansion of industries and agriculture, many pollutants have been introduced into the environment, one of these pollutants of great importance, is heavy metals, which have been introduced into aquatic ecosystems and results in their pollution (4,5). Although heavy metals naturally occur on earth surface, however they tend to increase due to human activities such as urban, industrial, and agricultural waste disposal, mining and consumption of fossil fuels, etc (6-8).

Heavy metals are very stable and due to their toxicity and bioaccumulation potentials are considered one of the most important pollutants and with tendency to accumulate in the body of large array of living organisms, including aquatic animals through food chains (9-11). The concentration of heavy metals in aquatic ecosystems is determined by measuring their concentration in water, sediments and various tissue of the target organisms.

The elements in water and sediment may be easily and in large quantities available to animals, especially aquatic organisms. An increase in the concentration of harmful substances in water and sediment in river and sea environments leads to an increase in the volume of these substances in the body tissues of aquatic organisms. If this process continues, it will cause biological changes in aquatic animals, and through the food chain, it will lead to the aggravation of pathogenic factors in humans (12-14). Bivalves are one of the most vital components of aquatic ecosystems, and considered suitable species for studying living conditions in water bodies owing to their rich diversity and different responses to stressors, which make them most practical and economical candidate to evaluate and monitor biological health status of water bodies and human impacts on water quality of the ecosystem being studied (15,16).

Invertebrates due to their position at the base of the energy pyramid and food chains play an essential role in the process of energy transfer and food supply for other invertebrates and higher vertebrates. Some invertebrate groups i.e., bivalve mollusks are of extreme economic and ecological importance (17). Bivalves are organisms prone to accumulation of contaminants have always attracted the interests and attention of researchers. These organisms are very important biological indicators heavy metal contamination (18,19).

Feasibility of sampling and identification, abundance and availability throughout the year, and reflection of environmental pollution are among those characteristics which entitle bivalves to be used as biological indicators (20). One of the most important bivalves in the Anzali wetland is *Anodonta cygnea*, which can serve as bioindicator of pollution in this water body. Numerous studies in the field of heavy metals using different species of bivalves have been conducted around the world (21-23). The core idea is that the edible consumption of bivalves is not very common in some countries, thus little investigations have been performed concerning these species as pollution bioindicators. Considering the importance of Anzali wetland as one of Iran's most important freshwater wetlands, it is imperative to determine and manage pollution in general and heavy metal pollution in particular using biological indicators. This study aimed to determine heavy metal pollution in the Anzali wetland with the pollution index of the bivalve (*A. cygnea*).

Materials and Methods

Sampling was done in the summer season in 2022 at three sampling areas (″ N 49° 17′14″ E, ″ N 49° 17′ 52″ E, ″ N 49°″ 19′ 11″ E). A total of 35 samples were taken randomly with a fishing net (Figure S1).

Anodonta specimens were taken at sampling area transferred to the laboratory for biometric measurements. The mean length, width, and height were determined by a digital caliper with a precision of 0.01 mm and the total weight was determined with a digital scale with 0.01 g precision. By the end of biometric measurement, the two shells were opened and the soft tissue was removed and placed in the Petri dish (6).

Each of the samples was placed separately in a Petri dish and dried in an oven at 105°C for 48 hours until a constant weight was achieved. The dried tissues were ground into fine powder. For chemical digestion 1 g of each powdered sample (all 5 tissues separately) was weighed with a digital balance (precision of 0.001 g) and added into a 100 mL Erlenmeyer flask. For acid digestion of samples 10 ml of 65% nitric acid (HNO_3, Merck, 65%) was added to each of the tissues and kept at laboratory temperature for 5 hours for digestion.

Whatman 40 filter paper was used to filter the digested samples. Then the volume of each sample in the flask reached to 25 mL by addition of double distilled water. By the end of this step the concentration of elements was measured by Perkin- Elmer Opti 8300 ICP-OES (Inductively coupled plasma - optical emission spectrometry) spectrometer using the standard solution of Geostats Australia, based on the protocol of the device for animal tissues (24,25). Argon gas was used in this device.

Assessment of the level of heavy metal pollution

The Nemerow index (Pc) is the main integrated multifactorial assessment for water pollution (Eq. 1) (26).

$$Pi = Ci / Si \tag{1}$$

First, the pollution index (P_i) is calculated, then, the maximum index value (P_{max}) and the mean value (P_{avr}) are used to calculate the combined index. Both *Pi* and *Pc* are used to assess the level of heavy metal contamination in edible bivalves. The pollution index is as follows:

where P_i is the only pollution index that refers to multiples of any other index, C_i is the measured concentration of heavy metals, and S_i is the standard value of any heavy metal.

When $Pi < 0.2$, the pollutant is within the normal background value range; When $0.2 \leq Pi \leq 0.6$, slightly polluted; When $0.6 \leq Pi \leq 1$, moderately polluted; When $Pi \geq 1$ heavily polluted.

The standard limit values of each element are presented in Table S1.

It is a convenient and effective method to evaluate heavy metal pollution with the Nemerow index, which includes a simple mathematical process and basic physical concepts (27).

P_C is in the form of formula (Eq. 2) (2).

where Pi max is the maximum value of P_i and P_{iavr} is the average value of P_i.

$$P_c \sqrt{\frac{Pimax^2 + Piavr^2}{2}} \tag{2}$$

The classification standard of P_i and P_c is shown in Table S2.

Health risk assessment of heavy metals

The concentration of heavy metals in *A. cygnea* was used

Table 1. Reference dose in bivalves (30,31)

RFD (mg·kg $^{-1}$ ·bw·d $^{-1}$)	Fe	Mn	Cu	Zn	As	Cr	Cd	Pb	Hg
Element	0.8	0.14	0.5	0.3	0.0003	0.003	0.001	0.035	0.0057

to calculate the daily intake of heavy metals (EDI) and the target hazard quotient (THQ) to assess the health risk of exposure to heavy metals. EDI was calculated with the following formula (Eq. 3) (28):

$$EDI = \frac{EF \times ED \times FIR \times C}{WAB \times TA} \qquad (3)$$

where *EF* represents the exposure frequency (365 d·a-1), *ED* is the duration of exposure (70 a), equivalent to the average human lifespan, *FIR* is the food consumption rate (40 g/day), *C* is the metal content in seafood (mg·kg^{-1}), *WAB* represents the average human body mass (60 kg), and *TA* is the average time of exposure to non-carcinogenic sources (365 d·a-1 × ED).

THQ represents the ratio between exposure and reference dose (Table 1), and the equation is expressed as follows (Eq. 4) (29):

$$THQ = \frac{EDI \times 10^{-3}}{RFD} \qquad (4)$$

Results

The mean and standard deviation of metal concentrations of metals in *A. cygnea* are listed in Table 2. The concentration of elements follows the following pattern (Figure S2):

Mn > Mg > Fe > Zn > Al > As > Cu > Sn > Pb > Co > Ni > V > Cd

EDI represents the daily intake of heavy metals by selected bivalve consumption as listed in Table 3. The highest amount of metal intake was related to Al metal (25.92) and the lowest one was related to Cd metal (0.11).

THQ is a non-carcinogenic health risk estimation method and an integrated risk index, which has been widely used to assess the risk of various pollutants (32).

THQ values for individual metals from selected bivalve consumption are shown in Table 4. The average THQ values of all metals in all samples were below 1, while the THQ values of Mn were determined above 1 (2.89), indicating that there is a potential health risk in the long term (Table 4).

Table 5 shows the pollution index (P_i) of heavy metals in *A. cygnea*.

According to Table 6, the accumulation of Cu and Cd were within the normal background value range. The amount of Pb in *A. cygnea* showed little contamination. Analyses of the metal concentration showed that the animal was moderately contaminated by Zn and severely contaminated by As.

Table 6 shows the Nemerow index (Pc) of heavy metals. Therefore, it can be said that the amount of Cu and Cd was at a safe level. The concentration of Pb and Zn was at the levels responsible for slight pollution while the level of As resulted in severe contamination of the *A. cygnea*.

Table 2. Mean ± SD concentration of metals (ppm) in *Anodonta cygnea* tissue

Element	Mean ± SD	Min-Max
Al	55.54 ± 49.99	11.41-290.58
As	6.07 ± 3.72	3.28-26.62
Cd	0.11 ± 0.04	0.047-0.22
Co	0.58 ± 0.21	0.27-1.16
Cu	5.52 ± 1.12	3.71-8.11
Fe	609.28 ± 278.38	243.81-1732.66
Mg	769.28 ± 105.36	599.63-1029.66
Mn	2897.33 ± 1006.69	1438.5-5231.89
Ni	0.51 ± 0.34	0.065-1.67
Pb	1.03 ± 0.54	0.24-2.59
Sn	1.30 ± 7.55	0.03-44.75
V	0.35 ± 0.19	0.15-1.15
Zn	113.22 ± 36.09	72.86-225-75

Relationship between total weight and accumulation of metals in Anodonta cygnea

The results showed that there was a significant relationship between the total weight of *A. cygnea* and Al, Fe, and Ni metals ($P < 0.05$), while there was no significant correlation between the total weight *A. cygnea* and As, Co, Cu, Mg, Mn, Pb, Sn, V, and Zn ($P > 0.05$) (Figures 1-3).

There was a significant relationship between the total length of *A. cygnea* and the accumulation of Al and Fe ($P < 0.05$). There was no significant relationship between the total length of *A. cygnea* and the concentrations of As, Cd, Co, Cu, Mg, Mn, Ni, Pb, Sn, V, and Zn ($P > 0.05$) (Figures 4-6).

Discussion

In recent decades, as a result of human activities, there have been ever growing concerns about environmental pollution. Heavy metals such as lead, cadmium, and arsenic are among the most hazardous pollutants. In general, heavy metals are divided into three groups of essential metals, such as copper, zinc, and iron, possibly essential metals such as cobalt, vanadium, and unnecessary or toxic metals such as mercury, lead, and cadmium (33).

The U. S. Environmental Protection Agency lists metals such as aluminum, beryllium, cadmium, chromium, copper, mercury, nickel, lead, selenium, and antimony as potentially toxic metals and metals such as silver, barium,

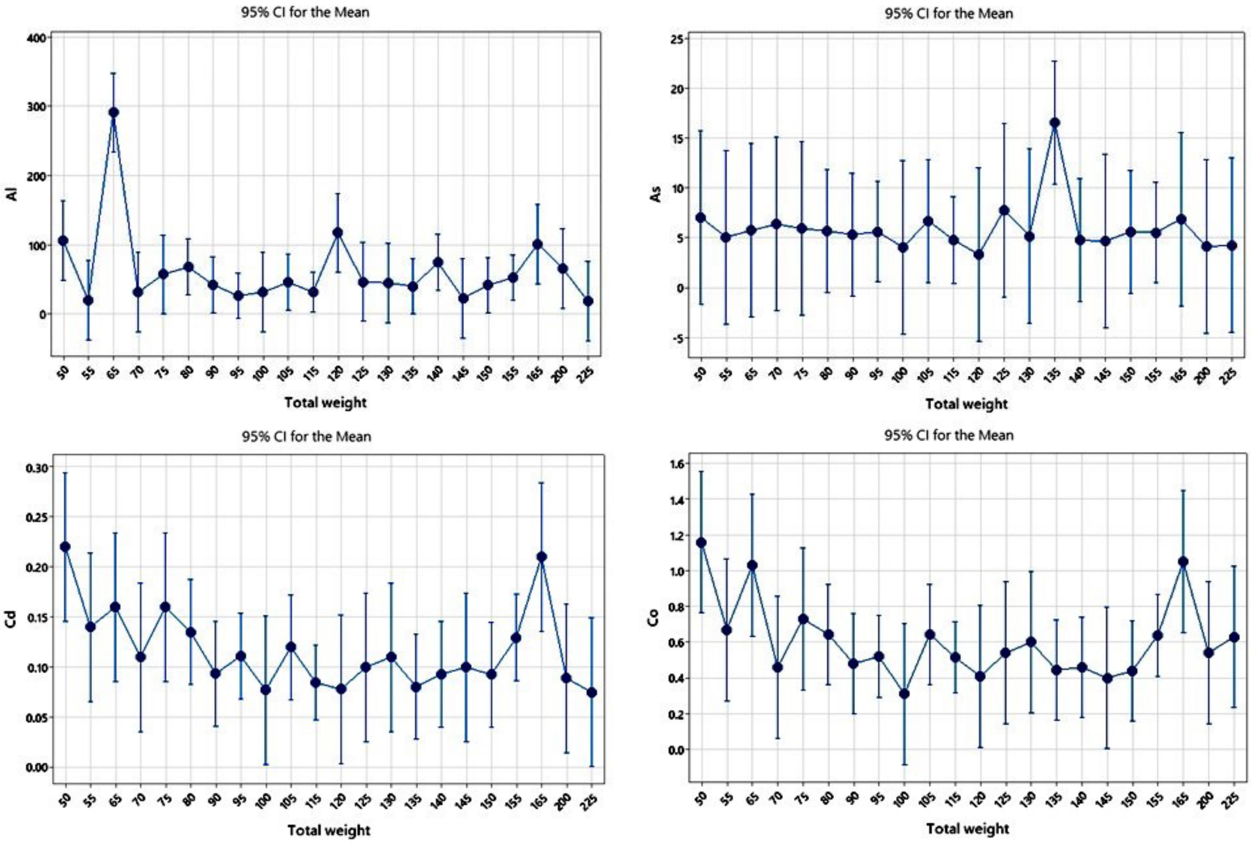

Figure 1. Relationship between total weight and accumulation of metals (Al, As, Cd, Co) in *A. cygnea*

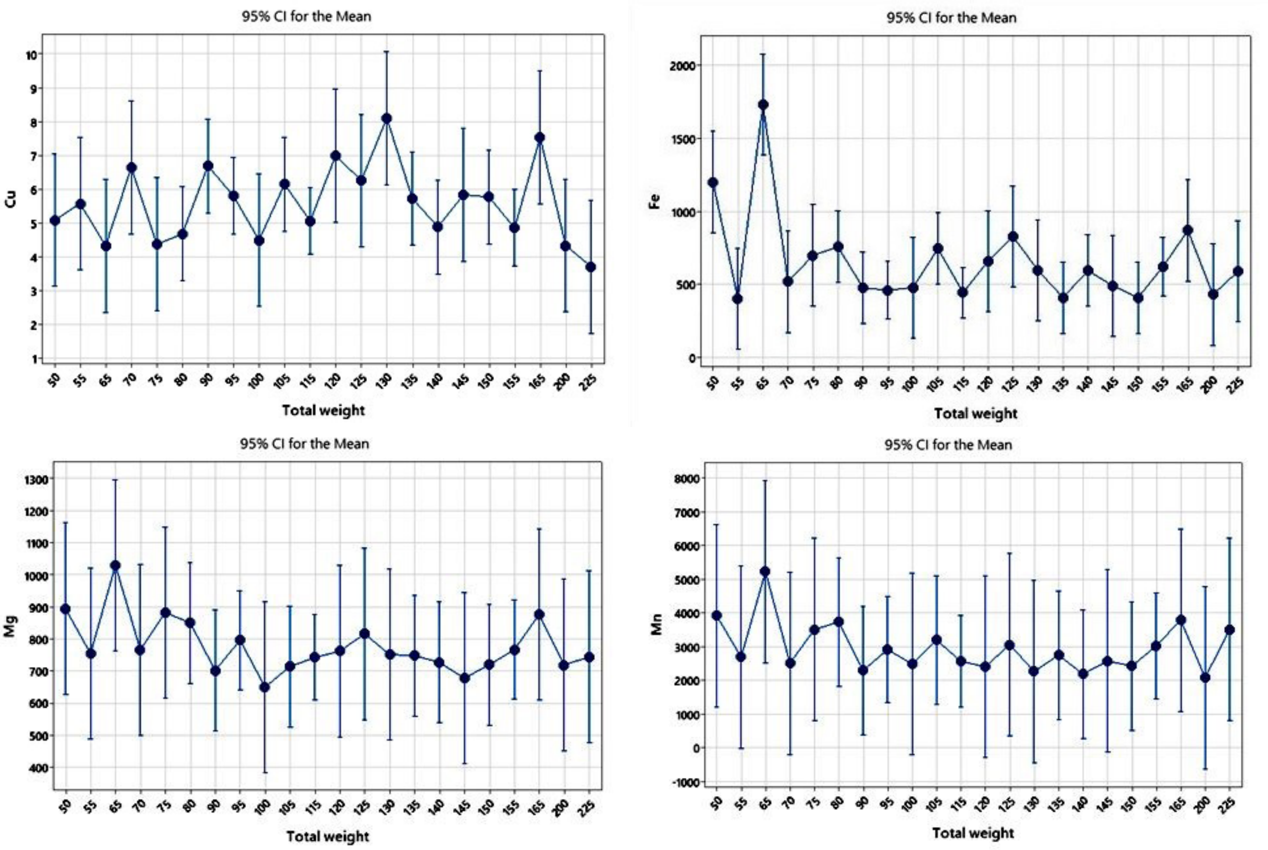

Figure 2. Relationship between total weight and accumulation of metals (Cu, Fe, Mg Mn) in *A. cygnea*

Table 3. Estimated daily intake (EDI) of heavy metals in *Anodonta cygnea*

EDI												
Al	**As**	**Cd**	**Co**	**Cu**	**Fe**	**Mg**	**Mn**	**Ni**	**Pb**	**Sn**	**V**	**Zn**
25.92	28.36	0.11	0.58	5.52	60.91	7.69	28.97	0.51	1.03	1.30	0.35	11.3

Table 4. Target hazard ratio (THQ) of heavy metals in *Anodonta cygnea*

THQ				
Zn	**Pb**	**Mn**	**Fe**	**Cu**
0.17	0.41	2.89	0.35	0.51

Table 6. Index (Pc) of heavy metals in *Anodonta cygnea*

As	Cu	Cd	Pb	Zn
6.21	0.087	0.138	1.72	1.190

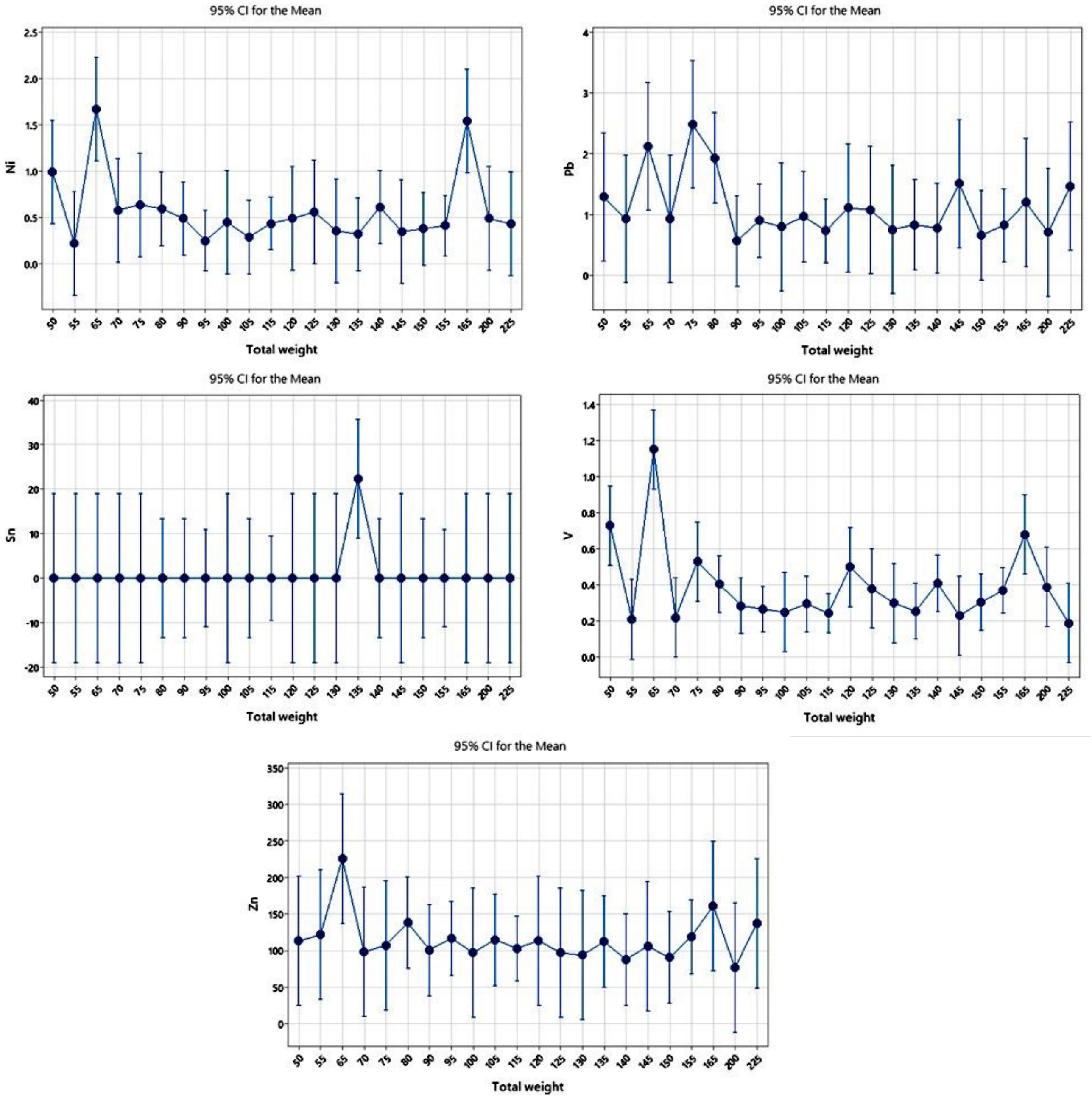

Figure 3. Relationship between total weight and accumulation of metals (Pb, Sn, Zn, V, Ni) in *A. cygnea*

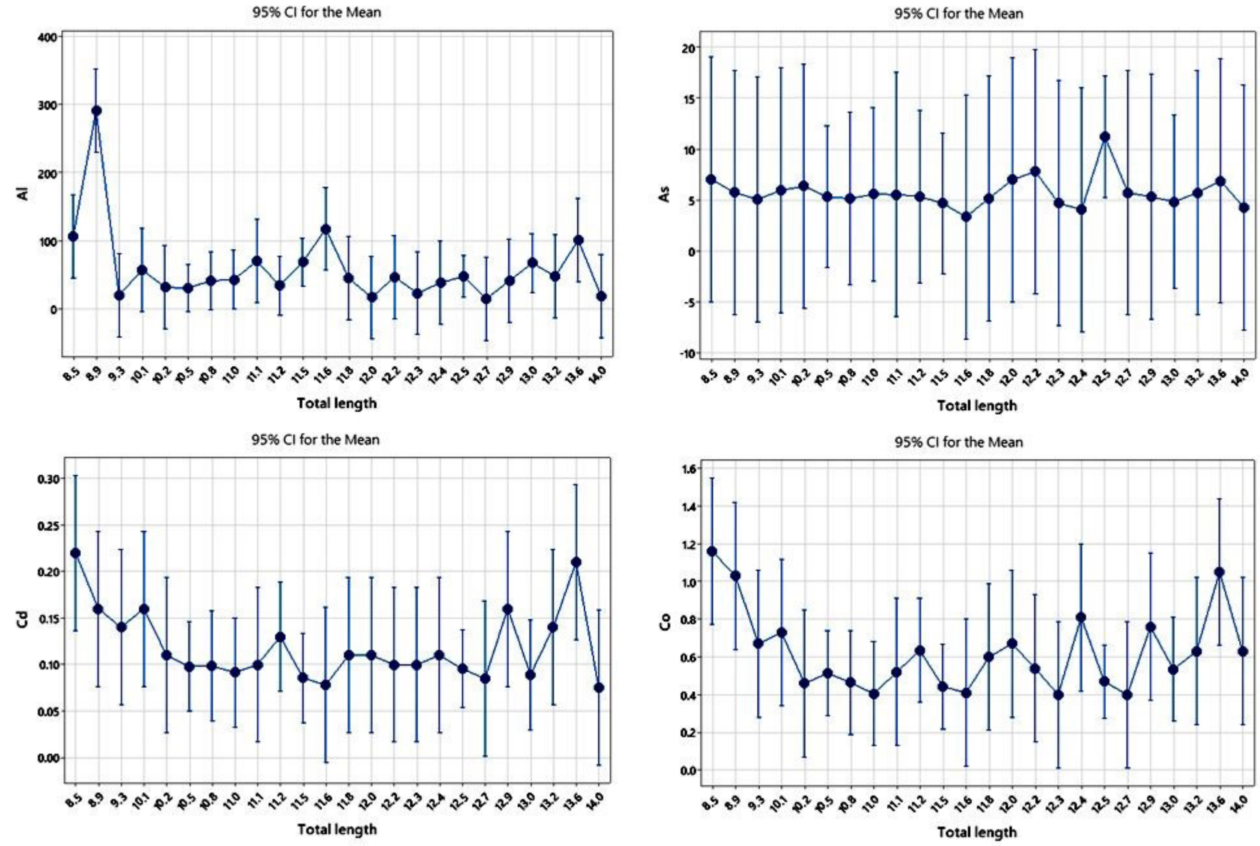

Figure 4. Relationship between total length and accumulation of metals (Al, As, Cd, Co) in *A. cygnea*

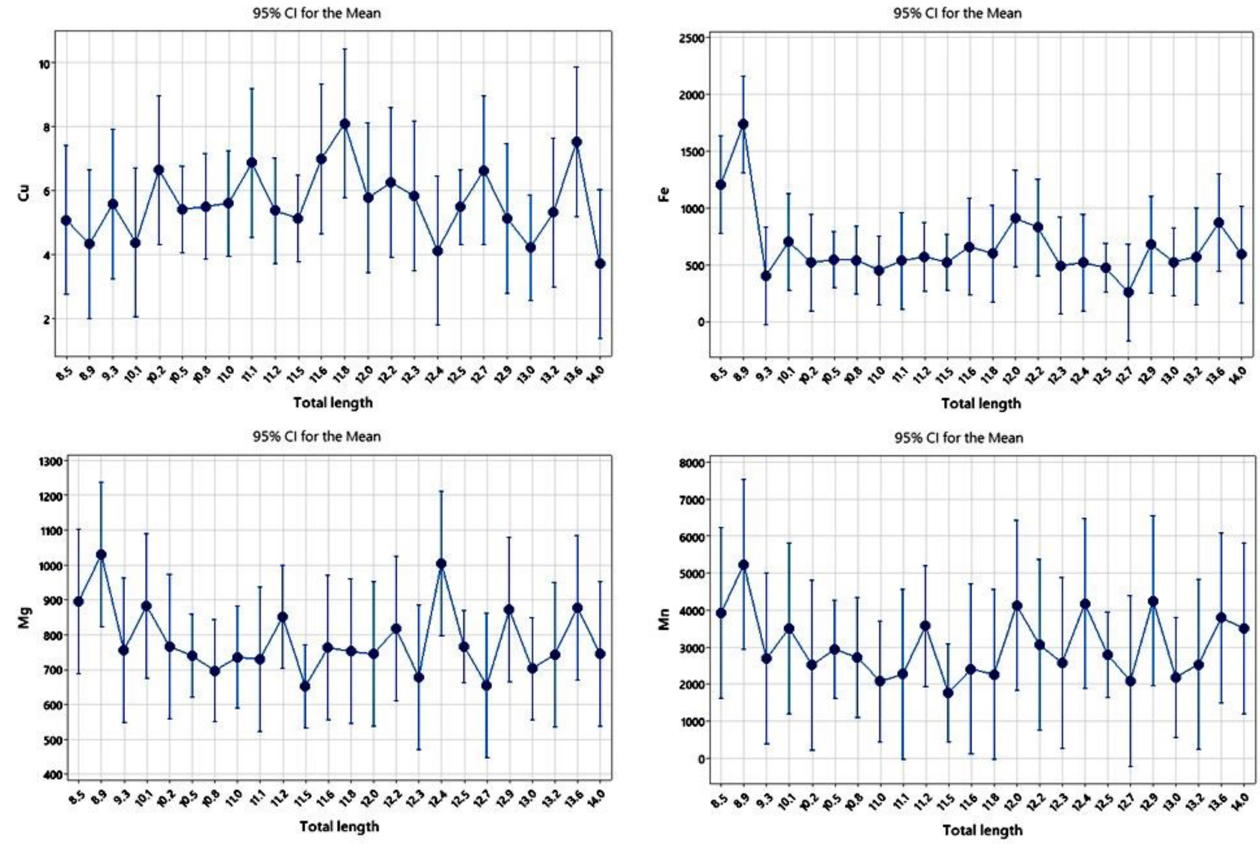

Figure 5. Relationship between total length and accumulation of metals (Cu, Fe, Mg, Mn) in *A. cygnea*

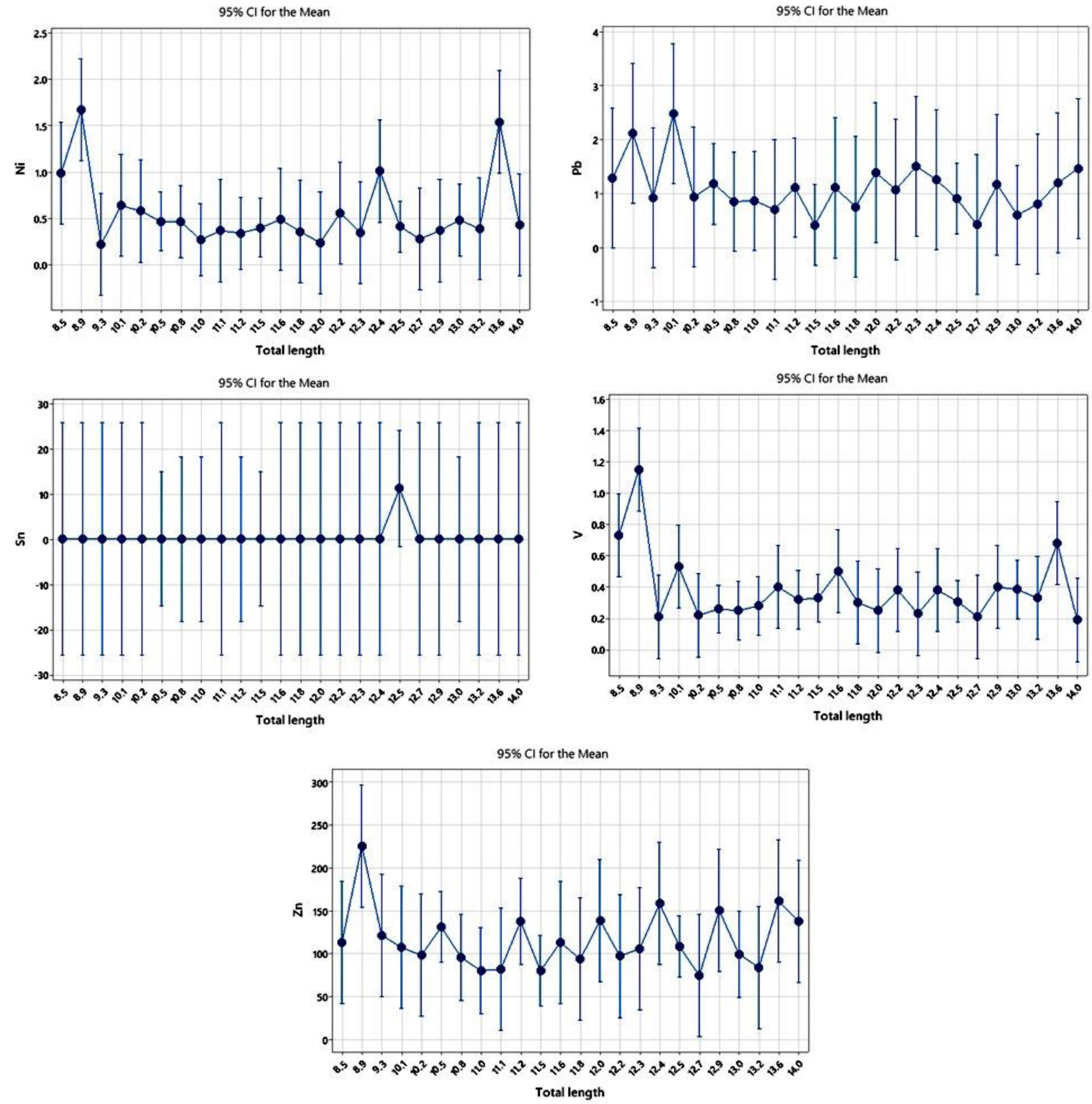

Figure 6. Relationship between total length and accumulation of metals (Zn, Ni, V, Sn, Pb) in *A. cygnea*

Table 5. Pollution index (Pi) of heavy metals in *Anodonta cygnea*

As	Cu	Cd	Pb	Zn
15.12	0.11	0.05	0.68	0.75

cobalt, manganese, molybdenum, sodium, thallium, iron, and zinc classified as metals with lower toxicity (34).

There is an optimal concentration for potential and toxic metals and essential metals that are needed in small amounts to carry out chemical processes and body functions. Unnecessary metals are toxic at any concentration, and as their concentration in the body increases, the ability of the immune system to deal with them decreases, While the elements that are necessary for the human body also appear toxic in high amounts (35-37). Metal poisoning can lead to destruction or disruption of the blood and cardiovascular systems (38).

Heavy metals are among the most important pollutants due to their toxicity to humans and the environment. These pollutants have been highly considered due to their non-degradability and high toxicity. Heavy metals are among systemic poisons and can cause death due to specific effects on nerves and carcinogenesis. These toxins cause adverse effects in humans by disrupting the nervous system and affecting neurotransmitters and the immune system.

Aquatic organisms, including bivalves, do not have advanced excretory system, and the process of excretion occurs at a slow speed, so when the concentration of these metals in the aquatic environment increases, they become readily available to organisms and the rate of entry surpasses the speed of its removal by the excretory system, which in turn results accumulation of the pollutant in various body tissues and organs. The binding of heavy metals to metallothionein and the distribution of heavy metals in different tissues such as oysters are among the strategies used by these organisms to regulate the concentration of metals in their bodies (39).

Zarkami and Kia have studied the effects of various physico-chemical parameters such as increase in factors such as water depth, water flow speed, water turbidity, electron conductivity, orthophosphate, and nitrate on anodonta in Anzali wetland (40). Fluctuations of these parameters may decrease the probability of the presence of individuals of the species while increase in dissolved oxygen concentration and total hardness may increase the probability of the presence of the species in the wetland.

Ganjali and Mortazavi examined the accumulation of cadmium and lead in *A. cygnea* and determined the content of cadmium and lead in sediments surface, soft tissue, and shell of *A. cygnea* (41). The results showed that the shell of *A. cygnea* can be used as an accurate case for the biomonitoring of cadmium and lead due to the higher sediment accumulation coefficient and the lower variation coefficient values in the shell compared to the soft tissue, which is consistent with the results of the present study.

Conclusion

Although bivalves usually consume less food, they can be used as an indicator to express the pollution of aquatic ecosystems. This study showed that the Anzali wetland is becoming polluted with some metals. The entry of several polluted streams into this wetland poses many risks for its inhabitants. The annual survey of pollution in this wetland may reveal the trend of its pollution, which is a great help for the management of this area. It is suggested that in addition to examining metals in bivalves, sediments and other aquatic organisms should also be examined simultaneously so that a better comparison can be made.

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Authors' contributions

Data curation: Mehdi Bibak.
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Funding acquisition: Javid Imanpour Namin.
Investigation: Javid Imanpour Namin.
Methodology: Mehdi Bibak.
Project administration: Simin Khojasteh Noshari.
Resources: Simin Khojasteh Noshari.
Software: Mehdi Bibak.
Supervision: Javid Imanpour Namin.
Validation: Mohammad Forouhar Vajargah.
Visualization: Mohammad Forouhar Vajargah.
Writing – original draft: Simin Khojasteh Noshari.
Writing – review & editing: Mehdi Bibak.

Competing interests

The authors declare that they have no conflict of interests.

Ethical issues

This is a part of the Master's thesis of Simin Khojasteh Noshari which has been approved and registered under 33280 index number in the research system of the University of Guilan (The confirmation document has been sent by email).

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Supplementary file

Supplementary file 1 contains Figures S1-S2 and Tables S1 and S2.

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