

Tissue Specific Accumulation and Histopathological Alterations of Zinc and Chromium and Their Effects on Clearance Rate In Swan Mussel, *Anodonta cygnea*

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Abstract: In freshwater ecosystems, heavy metals are of the main pollutants and exposure to over-tolerable levels of them can lead to destructive damages in aquatic biota. In the present study, the impacts of exposure to heavy metals, zinc (Zn) (essential element) and chromium (Cr) (non-essential element) on freshwater mussel, *Anodonta cygnea* (Linea, 1876) were investigated. For this purpose, clearance rate (CR), tissue specific accumulation and histopathological changes in two filtering organs includes gill and mantle were determined for a period of 18 days post exposure. Obtained results showed that basal levels of Zn in mantle and gill were significantly ($P < 0.05$) greater than Cr. The accumulated levels of Zn was significantly ($P < 0.01$) higher than Cr and cumulative capacity of both metals in gill were higher ($P < 0.05$) than mantle. Furthermore, exposure to $125 \mu\text{g l}^{-1}$ of Zn and Cr was lead to significant decrease in clearance rate ($P < 0.0001$), so that the longer the exposure time, the greater the reduction in clearance rate. Mantle and gill histopathology showed remarkable changes in mussels exposed to metals, but no difference was observed between changes caused by each metals. According to these results, studied ecotoxicological markers can simultaneously be used in biomonitoring heavy metals pollution in freshwater ecosystems.

Key words: *Anodonta cygnea* • Heavy Metals • Bioaccumulation • Clearance Rate • Histopathology • Mantle • Gill

INTRODUCTION

Heavy metals are one of the important groups of pollutants in aquatic ecosystems. They can be divided into two groups: essential (with defined biological functions) and non-essential (without biological roles) [1]. Zinc (Zn) and chromium (Cr) are two essential and non-essential heavy metals respectively that are of main contaminants in freshwater environments [2, 3]. These elements in concentrations higher than physiologically tolerable levels have detrimental effects on aquatic biota [4-8]. Natural concentrations of them in freshwaters are lower than $0.1 - 50 \mu\text{g l}^{-1}$, but in polluted waters by anthropogenic origin reach 4 mg l^{-1} [9].

Among aquatic biota, bivalves are desirable organisms for biomonitoring purposes [10]. Since these organisms are in direct contact with polluted parts of water and sediments of their habitats and can accumulate high levels of heavy metals in soft parts of their body [11], they can provide strong evidences about occurrence of pollution in waters through cellular and physiological

responses. These invertebrates are sensitive indicators of chemical pollution due to their filtration activity [10].

Long-term exposure of bivalves to heavy metals can increase susceptibility to diseases and may cause histopathological malformations [12]. In bivalves, mantle and gills have respiratory and feeding functions [13] and significant potential for accumulation of heavy metals and other pollutants [14]. These organs are composed of ciliated epithelium, connective and muscular tissues and rich vessels of haemolymph [13]. Clearance rate is a physiological parameter that is used to investigate the effects of pollutant exposure on bivalves. This parameter reflects the performance of filtering organs, especially gills [15]. Many studies have been conducted to investigate heavy metal impacts on clearance rate of bivalves that showed negative effects of heavy metal exposure on clearance rate [16-22]. Also, histological studies showed that pathological changes can be used in morpho-functional assessment of different tissues and organs when exposed to heavy metals or other contaminants [23-26].

In present study, different levels of ecotoxicological assessment of heavy metal exposure in swan mussel, *Anodonta cygnea*, in exposure to Zn and Cr during 18 days were studied. Investigated levels included tissue specific accumulation in mantle and gill, histopathological alterations of them and clearance rate as a physiological parameter.

MATERIALS AND METHODS

Collection and Maintenance of Bivalves: Specimens of *Anodonta cygnea* were collected in October 2011 from the Tajan River estuary, Mazandaran province, Iran (36°48'46''N, 53°6'57''E). After transmission to laboratory, specimens were hold in fiberglass tanks for two weeks in order to adaption to laboratory condition. During the study, Water temperature and pH maintained at $16 \pm 0/5^\circ\text{C}$ and $7/1 \pm 0/2$, respectively. Bivalve specimens were not fed during study.

Exposure Plan: According to previous studies [4, 19,21] and also the natural concentrations of Zn and Cr, bivalves were exposed to $125 \mu\text{g l}^{-1}$ of both metals for 18 days. Before exposure, bivalves were sampled for determining the basal levels of Zn and Cr. 45 bivalve specimens were divided into 3 groups (two metal exposed groups; n=18 and a control group; n=9). In control group clearance rate and histopathological status were investigated. In metal exposed bivalves, clearance rate was measured and tissue samples from gill and mantle were obtained for histopathology and determination of metal content 9 and 18 day post exposure.

Clearance Rate Measurement: For measuring clearance rate, 3 bivalve specimens were sampled from each tanks and transferred to glass containers filled with 5 l of water. After 30 min (for acclimatization), 10 ml of *Chlorella vulgaris* algal suspension was added to glass containers to give a concentration of 18-20 algal cells per ml. Then, the water of glass containers were sampled at 15 min intervals for 2 hours and density of algal cells in water samples were determined using Neubauer lam. After counting algal cell density, clearance rate of bivalves was calculated using following equation [15]:

$$\text{CR} = V (\ln C_0 - \ln C_n) / w.t$$

Where CR: clearance rate ($\text{l min}^{-1}\text{g}^{-1}$ AFDW); V: volume of water (l); C_0 : initial cell count; C_n : cell count after 15 min; W: weight (g AFDW); and T: time interval (min).

There were three replicates at each time for each treatment in order to clearance rate measurement.

Metal Accumulation: In order to obtain dry mass of bivalve specimens, they were placed in oven for 48 h at 75°C . Then, dry masses were weighted and again were placed in oven for 1 h at 500°C to obtaining ash free dry weight (AFDW) [15]. Digestion of dry tissue samples of gill and mantle was performed according to Fukunaga and Anderson [27]. Metal concentration analysis was done using ICP mass spectrometry.

Histopathology: The valves of each sampled specimen were opened using a scalpel for cutting abductor mussels in anterior and posterior regions. Then, a mass with volume of about 0.5 cm^3 of target organs (mantle and gill) was cut and fixed in Bouin's solution (saturated picric acid: 75parts, 40% formaldehyde: 25parts and glacial acetic acid: 5 parts) for 48h and then stored in 70% ethanol. After dehydration [28], the fixed samples were embedded in paraffin and cut ($5\text{-}7 \mu\text{m}$ sections) with a microtome. Thereafter, tissue sections were stained with haematoxylin and eosin and stained tissues were observed under a light microscope (Leica MS5).

Statistical Analyses: Statistical analyses were performed using SPSS version 17.0. All results are presented as means \pm S.D. Significant differences between groups and in different times were determined using one way ANOVA. Level of significance was determined as $P < 0.05$.

RESULTS

Tissue Specific Accumulation: The levels of heavy metals in control and metal-exposed bivalves are presented in figure 1. Levels of Zn in mantle and gill were higher than Cr and there was no difference between Zn levels in mantle and gill, but Cr content of gill was significantly ($P = 0.01$) higher than mantle.

In ninth day post exposure, the levels of two elements in investigated organs, with exception of Zn level of mantle, were significantly ($P < 0.003$) more than control levels and the amount of Zn in mantle and gill were higher than Cr. maximum metal content was related to gill for Zn and Cr. In day 18 post exposure, there was no significant difference between levels of Cr and Zn in gill when compared with levels in ninth day, but levels of Cr and Zn in mantle were significantly ($P < 0.035$) higher

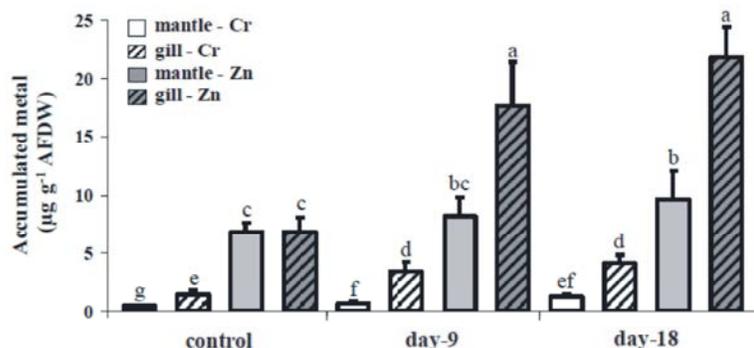


Fig. 1: Accumulated metal content in mantle and gills of metal-exposed bivalves. Error bars represent standard deviation.

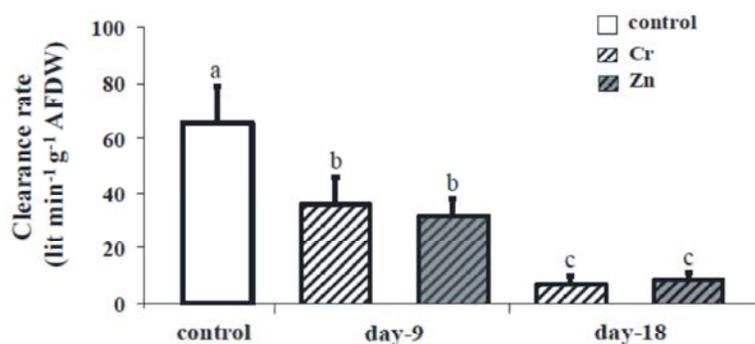


Fig. 2: Clearance rate (CR) of control and metal exposed bivalves. Error bars represent standard deviation.

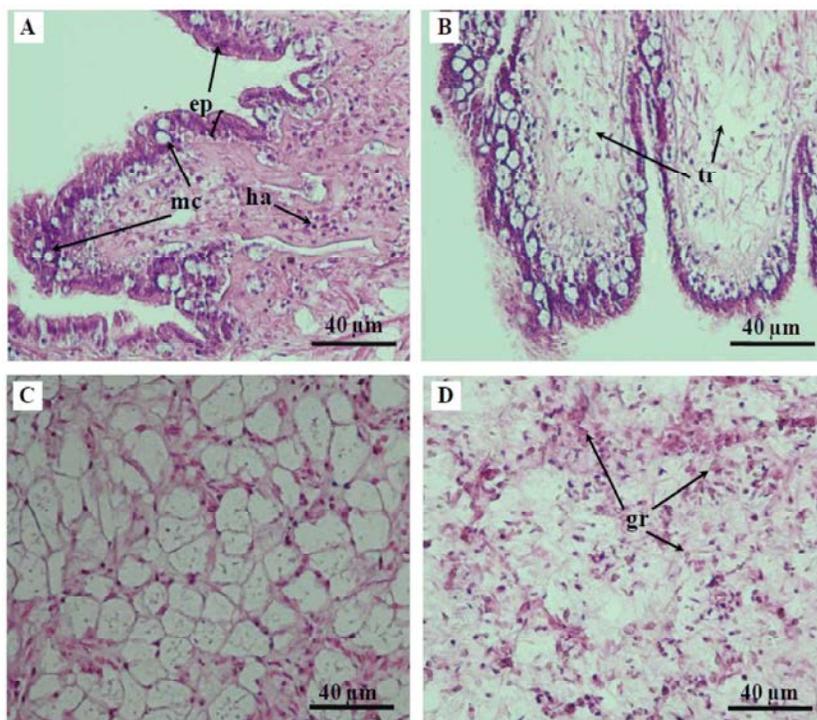


Fig. 3: Micrographs of transverse sections (5-7 µm thickness, stained with H and E) through mantle. control (A and C) and metal-exposed (B and D) specimens. ep: epithelium; ha: haemocyte; mc: mucous cell; tr: tissue rupture; gr: granuloma.

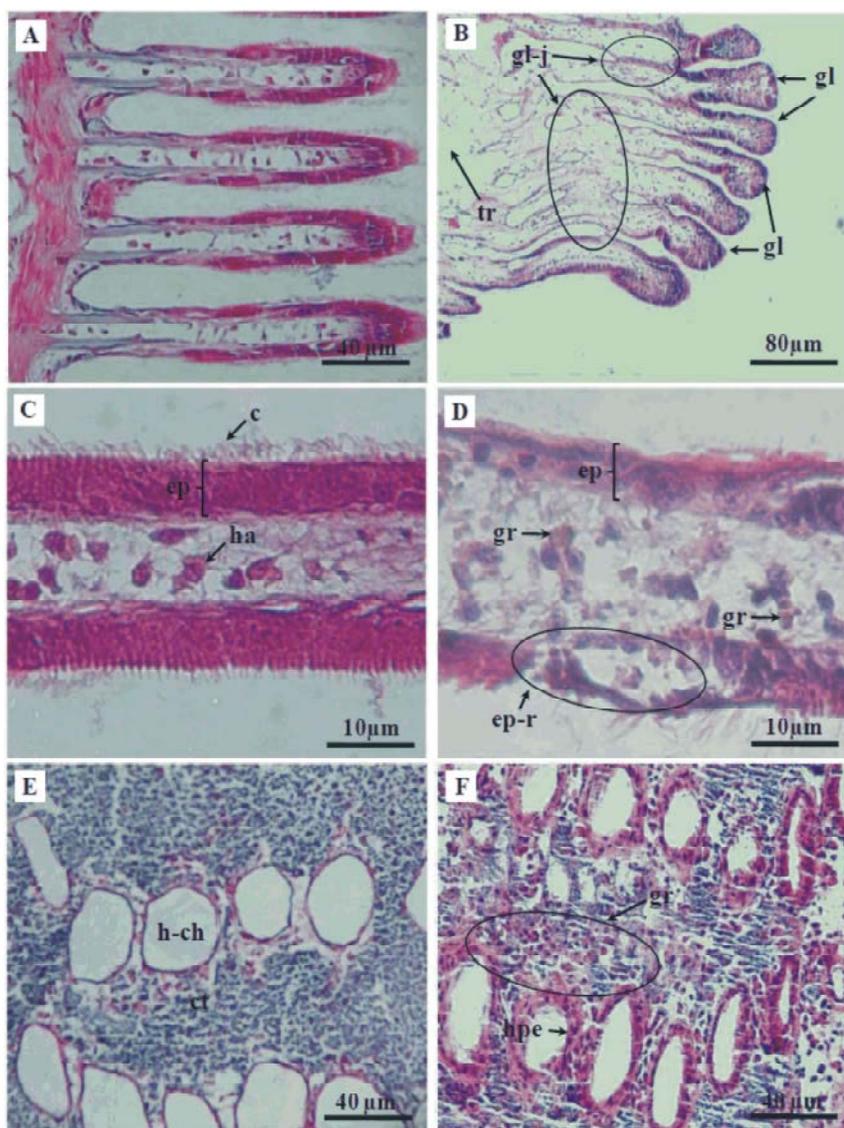


Fig. 4: Micrographs of transverse sections (5-7 μm thickness, stained with H and E) through gill. control (A, C and E) and metal-exposed (B, D and F) specimens. gl: gill lamellae; gl-j: junction of adjacent gill lamellae; tr: tissue rupture; c: cilia; ep: epithelium; ha: haemocyte; gr: granuloma; ep-r: epithelium rupture; h-ch: haemolymph channel; ct: connective tissue; hpe: hyperplasia.

than ninth day levels. Generally, increases of metals content in gill were much more than mantle and the increase of Zn was higher than Cr in each organ.

Clearance Rate: Metal exposure decreased clearance rate (CR) (Figure 2). Maximum CR was observed in control group that was equal to 65.83 ± 13.28 ($1 \text{ min}^{-1} \text{ g}^{-1} \text{ AFDW}$). CR of metal exposed bivalves following 9 days exposure, was significantly ($P < 0.000$) lower than control group CR. Indeed, in day 18, calculated CR of bivalves showed significant ($P < 0.000$) decreases than ninth day CR, as well

as, control group. There was no difference between CR of two metals by bivalves in days 9 and 18 post exposure.

Histopathological Alterations: Histology of transverse sections of mantle and gill of metal-exposed bivalves showed that there was no difference between histopathological alterations caused due to exposure to any of two metals.

Normal histological status of mantle was observed in control group of bivalves (Figure 3.A and C). Different layers of epithelial cells, subepithelial (connective) and

muscular tissues were distinctively existed. Mucous cells in subepithelial layer were dispersed uniformly.

In transverse sections of mantle of metal-exposed bivalves, clear signs of histopathological damages were occurred (Figure 3.B and D). The width of outer epithelium was decreased and relative number of mucous cells was increased in subepithelial layer. Granuloma (appearance of cells with yellow to brown color) was occurred in connective tissue. In connective tissue, disintegration of regular structure of cells was occurred and tissue rupture was observed.

Histological condition of gill in control group of bivalves is showed in figure 4.A, C and E. The epithelium of gill lamellae was healthy and the length of them was normal and equal. The connective tissue at the base of gill lamellae was integrated. In inner parts, Haemolymph channels were observed in a regular fashion and the spaces between these channels were composed of connective tissue.

Exposure to heavy metals was lead to histopathological damages in gill (Figure 4.B, D and F). The lengths of gill lamellae were changed and clubbing of their shape was occurred and Hypoplasia of epithelial cells was observed. In inner parts, swelling and hyperplasia of the haemolymph channels epithelium were observed. In many cases, tissue rupture in connective tissue and atrophy of haemolymph channels structure were observed.

DISCUSSION

Gill and mantle are two important organs of bivalves that involved in respiration and feeding of organism. Our results showed that accumulated contents of Zn in mantle and gill were higher than Cr. Different studies showed that there were different bioaccumulation trends for essential and non-essential metals [18,22, 29]. Differences between accumulated contents of different heavy metals in organs are related to different regulating systems of metals that include uptake, storing, detoxification and excretion processes of these metals [29]. In several studies, there was reported that metal accumulation potential of gills are higher than other organs in mollusks [17,22, 30]. Stewart [31] reported that maximum accumulation of cadmium during 80 days exposure of freshwater mussel, *Pyganodon grandis*, has been observed in the gill. On the other hand, other studies mentioned that accumulation potency of gill is lower than digestive gland [32, 33]. In bivalves, mantle has an important role in bioaccumulation of metals [13]. In this study we found lowest concentration of Zn and Cr in mantle. This result was

supported by the results of Metian *et al.* [34], as they reported that accumulation of Zn in mantle in comparison with other organs were lower, but with increase of exposure duration, metal content of mantle was relatively increased than gills. Basal levels of Cr in mantle and gill were much lower than Zn and this situation again, are related to this fact that Zn is an essential element and is more needed by organism than Cr for biological functions. In general, it can be said that accumulation of different heavy metals in different organs does not follow the same trend and are depending on the type of metal and target organs are different.

Obtained data about clearance rate (CR), obviously showed the significant effect of exposure duration period on CR of Bivalves, as longer period of exposure, greater decrease in CR. Decrease in CR as a result of exposure to copper is reported by Shi and Wang [20]. In contrast, it was reported that CR of *P. perna* following 18 days exposure to Cu has no difference from CR of control group [19]. These conflicting results may be due to different experimental conditions or different metal concentrations. Indeed, decrease in CR may be due to exposure to sub-lethal concentrations of heavy metals [17, 18]. The other reason of CR decrease in exposure to heavy metals can be attributed to valve closure response of bivalves [16]. Our Results showed that there was no difference between CR of polluted bivalves in relation to type of metal, while, Azarbad *et al.* [21] reported that decrease of CR of bivalves was greater in exposure to non-essential metals than exposure to essential metals.

Histopathology of gill and mantle of metal-exposed bivalves showed clear signs of damages that were not observed in control group. Haemocytes and other lipopigment cells are important signs of sorption and storing of toxic elements [33]. Appearance of these types of cells (or granuloma) was observed in mantle and gill in this study. Similar to our results, Changes in length and shape of gill lamellae were reported in different studies [14, 22]. The present results showed swelling and hyperplasia of epithelial cells of haemolymph channels. This damage was reported by Watermann *et al.* [36] due to heavy metal exposure. Hypoplasia of epithelium of gill lamellae was reported in previous studies [22]. Chakraborty *et al.* [14] reported appearance of hyperchromatic anaplastic cells in water channels in gill filaments due to exposure to arsenic, while this alteration was not observed in the present study. Relative increase in mucous cells number in subepithelial layer of mantle may be a response to decrease of epithelium width of this organ. Al-Subiai *et al.* [22] reported tissue rupture as a histopathological damage due to heavy metal exposure. This type of alteration was observed in mantle and gill.

CONCLUSION

With accordance to the results of present study, it can be said that in swan mussel, *Anodonta cygnea*, heavy metal accumulation potential of gill is higher than mantle. In other hand, we can say that sensitivity of mantle in exposure to heavy metals is higher than gill; because despite of higher accumulation of Zn and Cr in gill than mantle, there was no difference between types and severity of histopathological damages in these organs. Accumulation of heavy metals and it's histopathological damages in mantle and gills, which are involved in filtering activity of organism, consequently can lead to decrease in clearance rate of organism as a physiological response to stress of heavy metal exposure. Generally, using different levels of ecotoxicological markers simultaneously are offered as reliable tools for biomonitoring of chemical pollutants in aquatic environments.

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