Original Article

Toxicity and Bioconcentration of Cadmium and Copper in *Artemia Urmiana* Nauplii

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ABSTRACT

Background: *Artemia urmiana* are small crustaceans that because of its non-selective filter feeder pattern potentially may absorb high level of heavy metals through their living environment. In this study, the effects of different levels of cadmium and copper on survival, catalase activity and metals bioconcentration rates in *A. urmiana* nauplii have been investigated.

Methods: The research was carried out in February 2012 at University of Tehran, Tehran, Iran. First experiment was conducted in nine concentrations with six replication, then LC_{50} and probable interactions between experimental metals were evaluated. In the second experiment, concentrations of metals absorbed by *Artemia* and catalase activity were measured based on the acute toxicity indices, including NOEC, LOEC and LC_{50} at individual and mixed concentrations.

Results: The toxicity of copper sulphate (LC_{50}= 29.87) was 2.5 times greater than cadmium chloride (LC_{50}=79.08) and the toxicity interaction between cadmium and copper was synergistic. The rate of copper uptake in *Artemia* was higher than cadmium and increased concentration of heavy metals significantly decreased the bioconcentration factor. Comparison of mixed and individual concentrations showed that cadmium significantly decreased copper uptake, while it seems that cadmium bioconcentration was improved consequently. Biochemical analysis showed that the catalase activity was affected undesirably in different individual and mixed concentrations; however, these changes were not significant.

Conclusion: *A. urmiana* nauplia seems to be highly resistant toward cadmium and copper in their culture medium and demonstrated excessive potential for uptake of heavy metals from their rearing environment.

Keywords: *Artemia Urmiana* Nauplii, Bioconcentration, Catalase Activity, Heavy Metal, Survival.

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INTRODUCTION

Due to the industrial and agricultural developments and the improvement in the standard of living in recent decades, the application of heavy metals in variety of industrial and agricultural fields has been expanded [1]. Heavy metals from mining, combustion and industrial products can enter into the aquatic environment through atmospheric deposition and agricultural, industrial and municipal wastewater discharges [2, 3]. Industrial using of cadmium and copper, in particular, has been increased over the last century and seems to have reached to their peaks during the last 20-30 years [4, 5]. The increased concentration of heavy metals and their mixture are found in natural aquatic ecosystems, so their combined effects on living organisms has become serious concern. There are many investigations on the impact of individual pollutants on *Artemia* species [6-9], but a few researchers have considered their mixed effects [10, 11].

Organisms in the environment are continuously exposed to a variety of natural and anthropogenic stressors. If these stressors are present at a high level or for a long period, they will eventually influence the organism's physiological integrity (oxidative stress), thus the overall fitness would be decreased concomitantly [12, 13]. Plenty of physical and chemical stressors in nature (such as heavy metals) can enhance the production of reactive oxygen species (ROS). Some ROS is produced through natural processes of cellular metabolism, but antioxidant defense of the organism usually can control the harmful effects of these free radicals. However, external factors, including pollutants may intensify the

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ROS production and subsequent oxidative stresses may occur through biochemical disorders [13, 14]. The impact of free radicals can be counterbalanced by generating antioxidant enzyme systems such as superoxide dismutase, catalase and glutathione family proteins as detoxifying agents toward lipid peroxidation [15].

Many estuarine and marine species are able to absorb and accumulate metals from water or feed. In context of heavy metal uptake, there are many studies on several fish, oyster and large crustacean species [16-23], but little information is available regarding metal uptake capacity in planktonic crustaceans [24-26]. *Artemia urmiana* is an endemic species of *Artemia* in Iran [27] and in spite of high economic and ecological importance, there is no information about its toxicological aspects.

In this study, the effect of cadmium and copper on survival rate of *A. urmiana* nauplii was evaluated. We also assessed the interactive effect of these metals on metal uptake and catalase activity in *A. urmiana* nauplia.

MATERIALS AND METHODS

Cyst Hatching, Nauplia Rearing and Preparation of Stock Solutions

The research was carried out in February 2012 at Department of Fisheries, University of Tehran, Tehran, Iran. Cysts of *Artemia* were hatched under standard conditions [28]. Newly hatched nauplia were transferred into clean beakers and after consuming the yolk sac, they were fed with baker's yeast three times a day. The larvae were used for toxicity assay at 24 h post-hatch. At the same time, stock solutions of cadmium chloride and copper sulphate were prepared (200 mg/L). Filtered saline water (35 ppt) was used for preparing the stock solutions and all nominal concentrations were prepared by dilution.

Toxicity Assays with Experimental Heavy Metals

A preliminary test was designed in six concentrations to determine the toxicity ranges of cadmium chloride and copper sulphate (Merck, Germany) [29, 30]. The toxicity test was performed in a multi-well plate, each containing 5mL toxicant solution and 10 neonates (24 h post-hatch nauplia) [31]. Metal-free group was also considered as a control. All toxicity assays, including the control, were carried out in six replications. The multi-well plate was incubated at 25±1 °C without light for 24 h (salinity of 35 ppt) [32]. The tests were considered acceptable only if the mortality in the control wells did not surpass from 10%. Following the incubation, the number of dead animals for each metal concentration was counted and the percentage of the mortality was evaluated. Neonates were considered dead if they did not display any movement during 10 s of continuous observation [33].

Interactive Toxicity of Experimental Heavy Metals

In this part of experiment, the interactive toxicity of cadmium chloride and copper sulphate was investigated in mixed concentrations. The toxicity experiment was carried out using nine different concentrations of both metals in triplicates. The model followed in this study was based on the theory of probability. If P_1 was the inhibition rate caused by a specific concentration of chemical A_1 and P_2 the inhibition rate caused by a certain concentration of chemical A_2, Subsequently, the theoretically expected additive inhibition rate, when the concentrations of two chemicals are applied together, is shown by the following equation [34]:

$$P(E) = P_1 + P_2 - P_1P_2/100$$

Copper and cadmium uptake at different individual and mixed concentrations

With regard to the obtained results from the Probit Analysis, three concentrations in the range of NOEC, LOEC and LC_{50} for each metal were considered. For mixed concentrations, LOEC and NOEC were applied and therefore, LC_{50} was utilized only for individual concentrations. 250 ml from each metal concentration was poured into a beaker (salinity of 35 ppt) and 1000 individuals of 24 h old *Artemia* neonates were added to each beaker. A group without any metal was used as control treatment. Beakers were incubated for 24 h at 25±1 °C under a photoperiod of 16:8 (light: dark). The experiment was carried out as static and all concentrations, as well as the control, were performed in triplicates. After the incubation period, the samples were filtered and washed with distilled water to remove any non-absorbed metal by the animals. Filter paper (Whatman filters of acetic cellulose, metal free No. 40) containing the *A. urmiana* was dried and then placed in a furnace at 400 °C for 6 h. The subsequent ash was dissolved with a mixture of HNO3: HCl (1:1) and the solutions were filtered again under reduced pressure to separate the ash particles.

Consequently, each sample was diluted with distilled water up to 25 mL. The amount of metal was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Versamus, Australia). Based on the average weight of one *Artemia* neonate (which was determined to be 5µg) and Assuming the amount of metals in each solution measured by ICP-AES were absorbed by 1000 *Artemia* organisms, the quantity of metals absorbed by each animal and the bioconcentration factor (BCF) were determined subsequently [34, 11].

Enzyme Assay

This experiment was carried out in the same manner as detailed in previous section. After incubation, nauplii were filtered and stored in -80 °C until the analysis. Afterwards, samples were homogenized in phosphate buffer (1:4), centrifuged at 10000 rpm and supernatants were separated. The catalase activity was measured spectrophotometerically described by the Aebi [35]. The total protein was determined according to the Bradford method [36]. Catalase activity has been reported as µmol/min/mg protein.

Statistical Analysis

The toxicity data generated in this study were statistically analyzed by Probit Analysis using SAS (v 9.1). Analysis of variance was performed as a one way ANOVA and for the comparison of the means, Duncanmultiples range tests was applied. *t*-test was also used for comparison between two groups.

RESULTS

Toxicity Evaluation of Experimental Heavy Metals

Analysis of mortality data showed that copper sulphate had a greater toxicity for *Artemia* nauplii compared to cadmium chloride. Regression curves revealed a different pattern of mortality for each experimental metal ($P< 0.01$). While copper sulphate followed a logarithmic, on the other hand, an exponential mode was observed for cadmium chloride (Figure 1). Results from Probit Analysis also showed that the LC_{50} was 79.08 and 29.87 mg/l for cadmium chloride and copper sulphate, respectively.

Interactive Toxicity of Experimental Heavy Metals

Based on results, the observed mortality rates (in mixed concentrations) were significantly higher than those which estimated by the model (Figure. 2). The mortality of Artemia in the most mixtures was higher than 80% and it was higher than the theoretically expected mortality for all the concentration combinations tested. The theoretically expected mortalities ranged between 40 and 75%. There was a significant difference between the expected and observed values. Therefore, synergistic toxicity effects of cadmium and copper were confirmed.

Copper (Cu) and Cadmium (Cd) Uptake at Different Individual and Mixed Concentrations

An increase in each metal concentration led to increase in metal uptake by nauplia (Figure. 3). There was not no significant difference in uptake of Cd at various concentrations, while it were observed for Cu.

There was no apparent uniform approach in heavy metal uptake by *Artemia* neonates. In the case of Cd, by increasing the concentration to the LOEC (24 mg/l), the amount of absorbed Cd was raised similarly. On the other hand, despite to increasing three times higher concentration than LOEC, the metal uptake did not show significant accretion in LC_{50} (79.08 mg/l). A similar pattern was observed for the Cu. With an increase in the concentration of heavy metals, the bio concentration factor was decreased significantly (Figure.4).The quantity of Cd and Cu uptake at mixed concentration is demonstrated in Figure 5. The Cu uptake was significantly higher than Cd in all mixtures. On the other hand, the highest significant absorption of Cu was occurred when the lowest concentration of Cd was met in mixtures (see mixture A in Figure. 5). Accordingly, as can be seen in Figure. 6, there was no significant difference between absorbed Cd at individual and combined concentrations, whereas the uptake of Cu at mixed was decreased when compared to each individual concentration ($P< 0.05$).

Enzyme Assay

Results showed that in spite of the variation in enzyme activities, there was not any considerable difference between both experimental heavy metals ($P>0.05$). As can be seen in Figure. 7, enzyme activity enhanced after an increase in the concentration of metals from NOEC to LOEC, but in LC_{50}, the catalase activity was decreased compared to control ($P> 0.05$). In the case of mixed, the rate of catalase activity was less than individual concentrations, although there was no significant difference among the various levels.

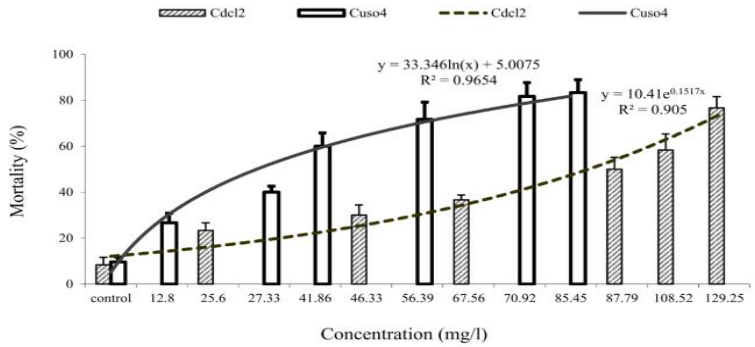

Figure 1. Comparison of mortality percentage in *A. urmiana* nauplii exposed to cadmium chloride and copper sulphate. Fitted curves, demonstrated the regression between concentration and mortality percentage for each metal ($P< 0.01$). Note to the different mortality patterns for two metals.

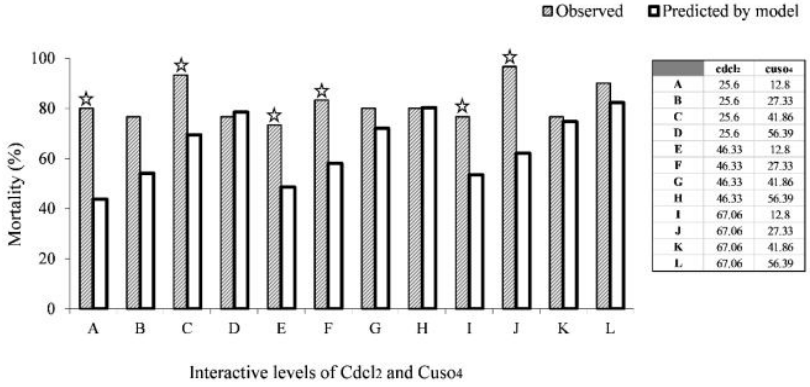

Figure 2. Comparison between observed and predicted mortality of *A. urmiana* nauplia using the model, in mixed concentrations (mg/L) of cadmium chloride and copper sulphate. (☆) Indicate significant difference between observed and predicted mortality at the same concentration ($P< 0.05$).

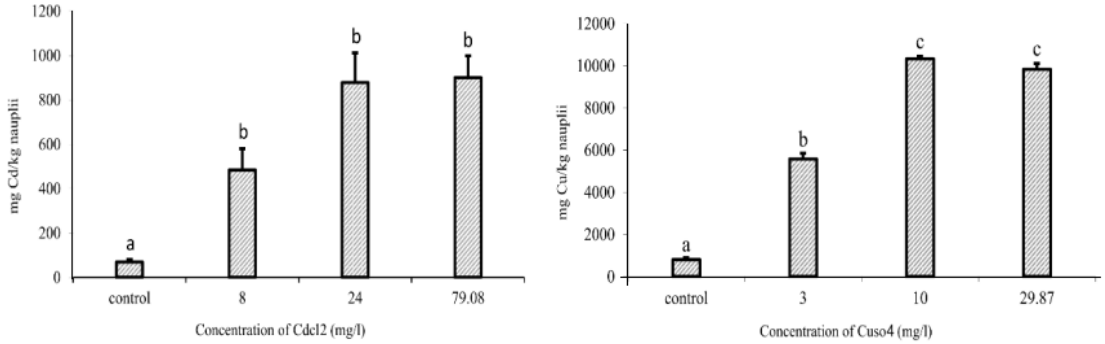

Figure 3. Cadmium and copper uptake at different concentration of cadmium chloride and copper sulphate in *A. urmiana* nauplii. Different letter shows significant differences among treatments ($P<0.05$).

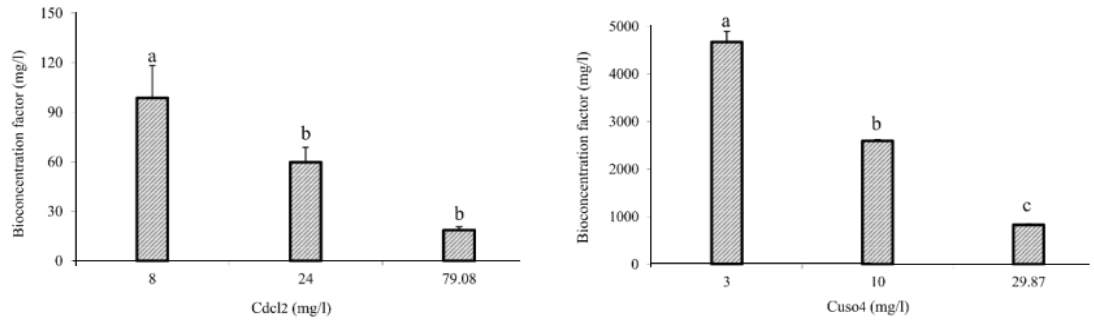

Figure 4. Bioconcentration factor of cadmium and copper in *A. urmiana* nauplii. Different letter shows significant differences among treatments ($P< 0.05$).

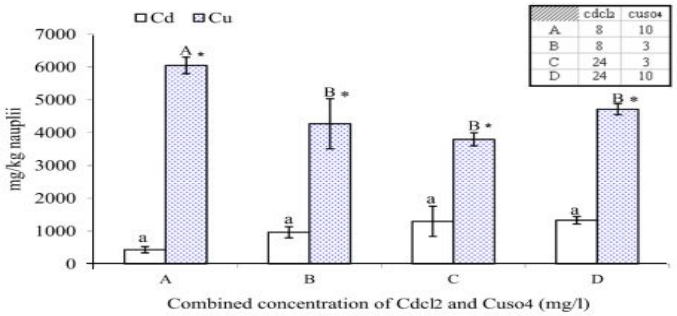

Figure 5. Cadmium and copper uptake at different cadmium chloride and copper sulphate mixed concentrations in *A. urmiana* nauplii. Different capital and small letter shows significant difference among mixtures for Cu and Cd respectively. Asterisk shows significant difference between Cd and Cu at same complex ($P< 0.05$).

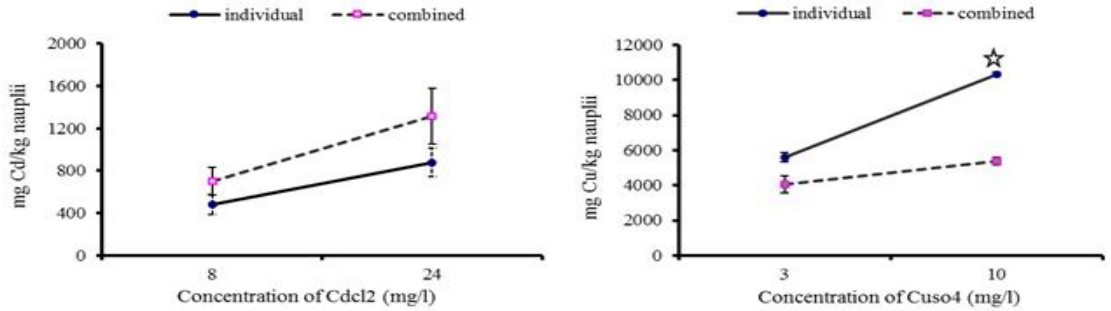

Figure 6. Comparison of cadmium and copper uptake quantity between individual and mixed concentrations in *A. urmiana* nauplii. . (☆) Indicate significant difference between levels ($P< 0.05$).

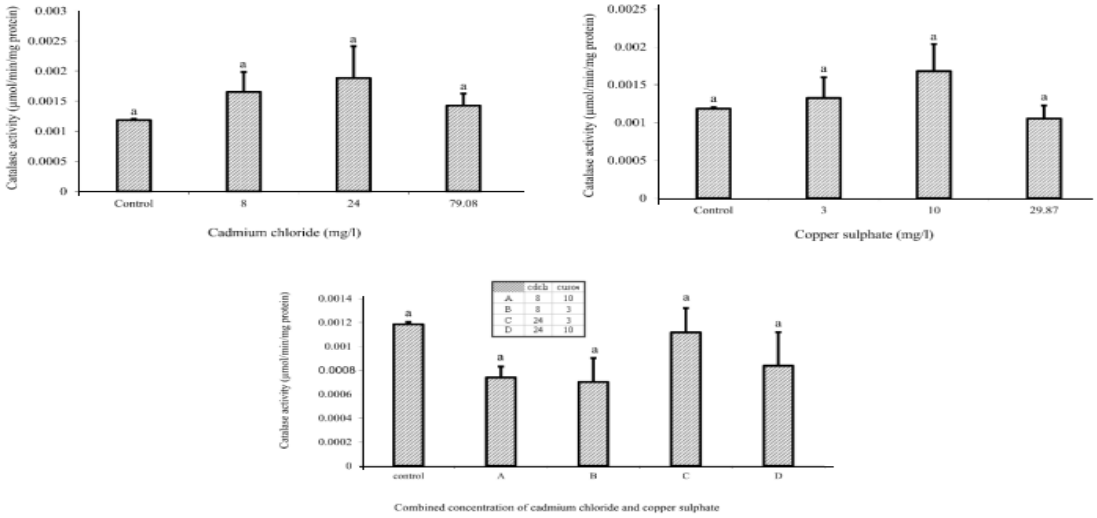

Figure 7. The effect of cadmium chloride and copper sulphate on catalase activity of *A.urmiana* nauplii ($P>0.05$).

DISCUSSION

With respect to other aquatic crustaceans, *Artemia* is more resistant toward a variety of pollutants [37, 38]. For example, toxicity of Cd for *Daphnia magna* was four times greater than *A. fransiscana* [39], but concerning the sensitivity of heavy metals, intra and even inter-species differences has been confirmed among several species of *Artemia* [40]. The results of this study are in agreement with the data reported by other researchers. Hadjispyrou et al. [11] assessed the toxicity of cadmium chloride in *A. fransiscana* and reported the LC_{50} of 155.5 mg/l, whereas in this study the LC_{50} was 79.08 mg/l, showing that

A. urmiana may be a more sensitive species to Cd when compared to *A. fransisana*.

Most aquatic crustaceans have a high susceptibility to the Cu [24, 41]. In this investigation, high toxicity of Cu for *A. urmiana* was recognized. When comparing the LC_{50} for cadmium chloride (79.08 mg/l) and copper sulphate (29.87), it seems that copper sulphate had 2.5 times greater toxicity than cadmium chloride. Similarly, a rise in Cd concentration resulted in a moderate exponential mortality rate, however in the case of Cu; the mortality curve had a higher slope and following a logarithmic pattern (Figure 1). This result supports the conclusion that Cu is more toxic than Cd for this animal. Supporting to our results, more inhibition rate of Cu compared to Cd and zinc on hatching of *A. fransiscana* cysts has been reported [42]. Generally, *Artemia* has a relatively high metallothionein synthesis suggested a reason for lower vulnerability (compared with other aquatic planktonic crustaceans) to heavy metals, especially cadmium [43].

The interactive effect of a large number of heavy metals is synergistic in a mixture [36]. Results from the current study also reflect such evidence. Statistical comparisons between observed and expected (by model) mortality rates in mixed concentrations confirmed that the interactive effects of two metals were synergistic (Figure. 2). The synergistic effect of metal mixtures of cadmium-tin and chromium-tin have been reported for *A. fransiscana* [11]. The interaction between Cd and Cu was synergistic in young crab (*Chasmagnathus granulata*) which is in agreement with current research [44].

Based on bioconcentration test, the amount of absorbed Cu by *A. nauplii* was much higher than Cd (Figure. 3). The quantity of Cu uptake in the blue swimmer crab (*Portonus palagicus*) and greasy back prawn (*Metapenaeus bennettae*) was more than cadmium, selenium and lead [45]. Cu is accumulated in the body due to its essential role in many species of molluscs and crustaceans. Results through bio concentration rate assessment of Cd and Cu in the isopod *Exosphaeroma gigas* also proposed that the amount of absorbed Cu was three times higher than Cd [1]. However, in contrast to our results, the measured bio concentration of Cd was higher than Cu in estuarine crab (*Chasmagnathus granulata*) [46]. This was due to non-biological characteristics of Cd, which suggests that intra-species variation should be taken into account.

With an increase in salinity, the toxicity of Cd would be decreased [43]. A rise in the salinity was followed by lower Cd toxicity and accumulation in *A. fransiscana* [47]. The tendency of the metal to form complexes with the chloride ion in saline environments makes the metal less available from solution and may largely explain the inverse relationship between Cd accumulation and salinity in the marine environment. Unlike Cd, Cu ions tend to form strong complexes with organic ligands and therefore salinity has no substantial effect on its bioavailability [48]. On the other hand, pH has been considered as a major factor in Cu bioavailability. The quantity of absorbed Cu was boosted when pH increased from 5.5 to 8.5 in *A. fransiscana* [49]. Concerning the assayed pH (7.7-7.9) and salinity (35 ppt) in this experiment, additional uptake of Cu with respect to the Cd is accommodated to the previous studies.

The magnitude of accumulated Cd and Cu at the individual was more than the mixed concentrations (compare Figure. 3 and 5). Since the interaction between the two experimental metals was evaluated as synergistic, it can be expected that the level of absorbed metals in mixed condition must be lower than individual concentrations. The influence of the two metals on their absorption by *A. nauplii* was considerable and it seems that Cd had a greater effect (Figure.6). Despite the increase in Cd uptake in mixed compared to individual concentrations, no significant difference was observed, but the quantity of Cu at mixed concentrations was decreased respect to individual ones. Based on this comparison, Cd had a negative effect on Cu absorption, while with probable increment in Cd bioavailability, Cu lead to an improvement in Cd bioconcentration. This conclusion is supported by Chen and Lru [50]. They investigated the effect of solitary and mixed concentrations of copper, cadmium, iron, nickel, cobalt, lead, manganese and zinc on absorption of these metals by *A. salina* nauplii and revealed that zinc and Cu, which were ranked in order, had assigned the most absorption by nauplii as compared with other metals. Mixed concentrations of nickel-cobalt-copper-cadmium showed no difference to individual concentrations of each metal, but incorporation of the mixture of iron-cadmium-

manganese had a negative effect on their absorption in contrast to solitary concentrations. Negative relationships between the uptake rate of Cd and zinc in the green mussel (*Perna viridis*) and manila clam (*Ruditapes philippinarum*) have also been reported [50].

With an increase in every metal, the bioconcentration factor was significantly reduced. Poldoski [51] assessed the bio concentration factor of Cd in *Daphnia magna*. During four days, the bioconcentration factors were 363.6 and 198 at concentrations of 0.22 and 1.01 µg Cd/l, respectively. In our study, at concentrations of 4.9 and 14.72 mg Cd/l (8 and 24 mg $CdCl_2$/l), the bioconcentration factors were assessed 98.67 and 59.69, respectively. A decrease in the bio concentration factor with an increase in concentration generally has been verified by several authors as an acceptable theory [11, 50, 52].

As mentioned, heavy metals are pollutants, which can intensify ROS production in the cells. Catalase is one of the most important enzymes in the elimination of active hydroxyle radicals [53]. Therefore, induction in enzyme production can be expected with an increase in metals concentration. Pan and Zhang [54] also reported the stimulation of catalase in a marine crab (*Charybdis japonica*) exposed to Cd. Due to the deadly nature of LC_{50} and because of the production of an enormous amount of free radicals, which can surpass from the animal's threshold, antioxidant defense system may repressed and catalase activity declined accordingly (Figure. 7).

CONCLUSION

Cu had a greater toxicity than Cd and the interaction between two metals was synergistic. It seems that *A. urmiana* potentially tend to more absorption capacity for Cu than Cd, However, Cd had a negative impact on Cu absorption when both metals used as a mixture. Generally based our finding, *A. urmiana* nauplia were highly resistant to increasing concentrations of Cd and Cu in its culture medium and demonstrated high potential for uptake of heavy metals from their rearing environment.

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