Accumulation of Heavy Metals in Freshwater Organisms: Assessment of Toxic Interactions[*]

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Heavy metals are continuously released into the terrestrial environment by natural sources and human activities. The uptake and accumulation of heavy metals by plants promotes a mechanistic understanding of the biological significance of particular metal concentrations and distributions in biota. The toxicity of chromium, zinc, copper and cobalt ions and their binary mixtures are studied at varying test levels using duckweed as the test organism. The accumulation of metal ions are determined by atomic absorption spectroscopy. The type of toxic interactions in binary mixtures is assessed as 'synergistic', 'antagonistic' and 'additive' by a statistical approach.

Key Words: metal accumulation, heavy metal interactive effects, duckweed

Introduction

Diverse industrial wastes have aggravated the problem of water pollution. This problem becomes complex because of the non-degradability of inorganic pollutants like heavy metals[1]. Metals have received particular attention among other non-degradable toxic chemicals because of their adverse effects on aquatic life forms[2,3]. To control water pollution, the immediate problems have to be solved by adopting alternative technologies to chemical-specific tools which suit low capital availability and minimum manpower. There has been considerable interest in using aquatic plants for removal of various pollutants, including heavy metals, from water bodies because of their fast growth rate and simple growth requirements, which are favorably compared to those of fish[4,5]. Moreover, aquatic plants are particularly important in heavy metal pollution studies, since the analysis of these plants can give an indication of the state of water environment to which they have been exposed[6].

The common duckweed, *Lemna minor,* is potentially useful as an indicator of pollution because of its ability to integrate and rapidly monitor the pollutants' variations in the water. Moreover, they tolerate unstable environmental conditions and exhibit high sensitivity to heavy metal toxicity[7]. The majority of published data concerning the heavy metal removal potential by aquatic plants is focused on single metal

effects. However, aquatic organisms in natural systems are exposed to mixtures of metals, which may substantially add to, multiply or suppress the effects of single components.

The purpose of the present study is to assess the interactive metal accumulation effects in binary combinations as 'additive', 'antagonistic', or 'synergistic'. The aim of this study was to evaluate metal enrichment ability and toxicity to assess the feasibility of using duckweed as an indicator of metal contamination in aquatic ecosystems.

In this study, the method that is followed is composed of two major parts: Part 1 involved testing of binary combinations of Zn+Co, Zn+Cu, Zn+Cr, Cu+Cr, Cu+Co and Cr+Co at various concentration levels, and Part 2 involved assessing toxic interactions by statistical testing of the difference between metal accumulation in binary mixtures and in single components.

Experimental

Reagents and Supplies

Duckweed plants were subcultured from original stocks, maintained in our laboratory since 1988. Continuous illumination of stock and test cultures was made with fluorescent tubes of Philips TLD 36W/54 having an intensity of 40 μEm^{-2}s^{-1} at plant level. Several different test protocols are available for duckweed, as reported by Huebert et al.[8], ASTM[9] and Cowgill and Milazzo[10]; the method used in this study was based on static conditions. Duckweed plants were subcultured from an original stock in full-strength Jacob culture medium[11]. Sixty-four milligrams (wet weight) of bright green and healthy duckweeds were measured out and rinsed carefully with distilled water. Duckweeds were then placed in 200-mL of metal test solution contained high-quality glass jar and covered with aluminum foil to exclude side lighting and with a watch glass to prevent evaporation. The test solutions were adjusted to pH 6.0-6.5 with 0.1 M KOH or HCl. Temperature was kept at 25-27°C. On the seventh day of frond incubation, plants were washed three times with distilled water and weighed. The experimental set for each binary mixture consisted of control samples and five replicates of the test sample. Consequently, plants were dried at 80°C and digested in 3 mL of concentrated HNO$_3$. Metal accumulation in the plant body was measured by a flame atomic absorption spectrometer (FAAS), Varian SpectrAA Model 250 Plus. An air-acetylene flame was used. Working conditions for the metal ions, i.e., wavelength, concentration ranges and typical sensitivity values, are as follows, respectively:

Cu (324.7 nm), 2-8 mg/L, 0.04 mg/L

Zn (213.9 nm), 0.4-1.6 mg/L, 0.009 mg/L

Co (240.7 nm), 3-12 mg/L, 0.066 mg/L

Cr (357.9 nm), 2-8 mg/L, 0.055 mg/L

Samples were diluted to a suitable final volume with double-distilled water prior to FAAS measurements. Cr determinations were made with a flameless atomic absorption spectrometer equipped with a carbon rod atomizer (CRA), Varian Techtron Model 1200. Interference in the air-acetylene flame from Cu, Mg and Ca has been reported and the extent of interference is strongly dependent on the flame stoichiometry. Also, Co and Fe have been found to cause depression of the Cr signal[12]. In the CRA technique, samples were diluted accordingly to suit the working conditions of 0.01-0.1 mg/L. Standards were prepared from 10 mg/L Cr and diluted with 20% NaCl to prepare 0.025, 0.05 and 0.1 mg/L concentration levels.

Binary test levels were selected with respect to the previously reported data of the EC$_{50}$ (mg/L) of

each metal component[13], i.e., the effective toxicant concentration to induce 50% growth inhibition. Binary metal combinations were prepared basically in two main groups. Group I consisted of sets with varying equimolar concentrations of the metal ions within the range of EC_{50} of each metal component. Group II consisted of sets with combinations where the concentration of one metal ion was kept constant while the other one was varied. Stock solutions of 1000 mg/L of $CoCl_2$ and $K_2Cr_2O_7$ were prepared from reagent grades, and the dichromate solution was acidified with sulfuric acid to maintain a relatively stable Cr (VI) species. Stock solutions of 1000 mg/L of zinc and copper were prepared from high purity metals by dissolving them in 1:1 (v:v) HCl (12 M) and 1:1 (v:v) HNO_3 (16 M). Metal accumulation in the plant body was reported as mg of metal per gram of dry plant weight.

Statistical Modeling

The interactive metal effect was asssessed by comparing the metal accumulation (MA) in single components (x,y) at the ith test level and at the concentration $(x+y)_i$ in binary mixtures where x and y are the concentration of the first and second metal ions, respectively. Statistical testing involved the following scheme:

i-calculating the MA_{diff}, defined as $MA_{diff} = MA_{(x+y)i} - MA_{(x,y)i}$ (1)

ii-calculating the standard error $(SE)_{diff}$ [14] of MA_{diff} and the estimated t value for each test level, defined as

$$SE_{diff} = \{[SE_{(x+y)i}]^2 + [SE_{(x,y)i}]^2\}^{1/2} \tag{2}$$

$$t_{estimated} = MA_{diff} / SE_{diff} \tag{3}$$

iii-comparing the $t_{estimated}$ in equation 3 with the tabulated t value (Student's t) to determine if the MA_{diff} is statistically significant at 95% confidence level

iv -assessing the type of binary interaction at each test level

If the difference was positive and statistically significant, the interaction was called 'synergistic', implying that the metal accumulation in the binary mixture was higher than the additive interaction. If the difference was negative and statistically significant, the interaction was called 'antagonistic', implying that the metal accumulation in the binary mixture was lower than the additive interaction. If the difference was statistically insignificant (irrespective of its sign), the interaction was called 'additive'.

Results and Discussion

Toxicities of single test metals to duckweed were reported as EC_{50} (mg/L) and the toxicity of the test metals were found to increase in the order Cu > Cr = Zn > Co as estimated previously by N.H. İnce et a.l[13]. The observed metal accumulations in single components and in binary mixtures at each level are shown in Tables 1 to 6. Results are based on the average of five replicate measurements. Interactive effects of the metal pairs at the combinations $(x+y)_i$ were evaluated by calculating the value of 'MA_{diff}' of the mixture under consideration and the predicted interactions are given in the last columns of Tables 1 to 6. The observed data for the binary mixtures of Co+Cr, Cu+Cr and Zn+Cr showed that the interactive effects of Cr on Co, Cu, and Zn were of 'antagonistic' nature at all test levels. This implied that when the two metal ions were applied together, Cr interfered with the action of the other, e.g., either Co, Cu or Zn accumulated in smaller quantities than their corresponding single component counterpart. On the other hand, the interactive effects of Co, Cu, and Zn on Cr accumulation were 'synergistic' in nature, e.g., Cr uptake was much higher

in binary mixtures than when it was applied alone. The data for the Cu+Zn mixture showed that the the effects of Cu on Zn accumulation was 'antagonistic'. The results of the experiment with Cu+Co and Zn+Co mixtures exhibited 'antagonistic' effects also, e.g., Co accumulation decreased in both of the binary mixtures. However, different types of interactive effects were evident when the effects of Co on Cu and Zn accumulation were evaluated. In the case of Cu and Co mixture, the effect of Co on Cu accumulation was 'antagonistic' and for the mixture of Zn and Co, the effect of Co on Zn accumulation was 'synergistic'. Clearly, the observed interaction types for the binary mixtures showed that they were always the opposite of each other except for the Zn+Co mixture, i.e., 'antagonism' was evident for both of them. 'Additive' interaction was found to be rare, e.g., existed only in Cu+Cr at 2+5 mg/L and 2+20 mg/l levels and was not observed for any of the other binary mixtures at the given test levels. There are many examples in the aquatic toxicology literature of both additive and synergistic toxicity[15]. However, examples of antagonism are not common.

Table 1. Metal accumulation and interactive effect in Cu+Co

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Cu+Co	Cu	Co	Cu	Co	
2+2	0.64±0.08	4.63±0.23	0.64±0.02	0.07	ANT[b]
5+5	ND[c]	6.20±0.19	3.77±0.10	0.90±0.02	ANT
10+10	ND	7.66±0.16	5.86±0.14	4.39±0.12	ANT
1+10	0.46±0.05	7.66±0.16	0.68±0.03	5.08±0.16	ANT
2+10	0.64±0.08	7.66±0.16	0.80±0.03	4.78±0.10	ANT
1+20	0.46±0.05	8.69±0.27	0.89±0.04	7.29±0.28	ANT
2+20	0.64±0.08	8.69±0.27	1.12±0.06	7.21±0.21	ANT

[a]Interactive effect = effect of Cu on Co

[b]ANT = antagonistic

[c]ND = not determined, *L. minor* did not survive

Table 2. Metal accumulation and interactive effect in Co+Cr

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Co+Cr	Co	Cr	Co	Cr	
2+2	4.63±0.24	0.27±0.04	1.42±0.07	0.54±0.02	ANT[b]
5+5	6.20±0.19	0.31±0.03	2.53±0.13	1.66±0.03	ANT
10+10	7.66±0.23	0.41±0.03	2.89±0.09	2.38±0.07	ANT
15+15	8.04±0.16	0.58±0.06	3.25±0.11	3.25±0.09	ANT
20+20	8.69±0.17	0.74±0.08	1.60±0.06	3.89±0.12	ANT
10+5	7.66±0.23	0.31±0.03	3.92±0.09	1.72±0.12	ANT
10+15	7.66±0.23	0.58±0.06	2.88±0.08	3.01±0.08	ANT
10+20	7.66±0.23	0.74±0.08	1.60±0.05	3.49±0.15	ANT
20+5	8.69±0.17	0.31±0.03	5.17±0.15	1.62±0.04	ANT
20+10	8.69±0.17	0.41±0.03	4.02±0.12	2.12±0.07	ANT
20+15	8.69±0.17	0.58±0.06	3.01±0.09	2.42±0.08	ANT

[a]Interactive effect = effect of Cr on Co

[b]ANT = antagonistic

Table 3. Metal accumulation and interactive effect in Cu+Cr

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Cu+Cr	Cu	Cr	Cu	Cr	
1+1	0.46$\pm$0.05	0.25$\pm$0.07	0.22$\pm$0.02	0.39$\pm$0.01	ANT[b]
2+2	0.64$\pm$0.08	0.27$\pm$0.04	0.49$\pm$0.03	0.68$\pm$0.08	ANT
5+5	ND[d]	0.31$\pm$0.03	1.89$\pm$0.08	1.52$\pm$0.05	ANT
10+10	ND[d]	0.41$\pm$0.03	6.61$\pm$0.15	2.29$\pm$0.10	ANT
15+15	ND[d]	0.58$\pm$0.06	8.80$\pm$0.15	3.49$\pm$0.09	ANT
2+5	0.64$\pm$0.08	0.31$\pm$0.03	0.64$\pm$0.05	1.65$\pm$0.08	ADD[c]
1+20	0.46$\pm$0.05	0.41$\pm$0.03	0.27$\pm$0.06	2.14$\pm$0.07	ANT
2+20	0.64$\pm$0.08	0.41$\pm$0.03	0.61$\pm$0.04	2.54$\pm$0.16	ADD

[a] Interactive effect = effect of Cr on Cu

[b] ANT = antagonistic

[c] ADD = additive

[d] ND = not determined, *L. minor* did not survive

Table 4. Metal accumulation and interactive effect in Zn+Cr

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Zn+Cr	Zn	Cr	Zn	Cr	ANT[b]
1+1	2.26$\pm$0.16	0.25$\pm$0.07	1.32$\pm$0.08	0.50$\pm$0.04	ANT
2+2	3.72$\pm$0.22	0.27$\pm$0.04	1.46$\pm$0.10	1.02$\pm$0.05	ANT
5+5	6.80$\pm$0.24	0.31$\pm$0.03	2.99$\pm$0.08	2.32$\pm$0.10	ANT
10+10	7.86$\pm$0.20	0.41$\pm$0.03	3.30$\pm$0.12	4.32$\pm$0.10	ANT
15+15	8.04$\pm$0.23	0.58$\pm$0.06	4.95$\pm$0.10	5.14$\pm$0.12	ANT
5+10	6.80$\pm$0.24	0.41$\pm$0.03	1.27$\pm$0.11	1.15$\pm$0.05	ANT
5+15	6.80$\pm$0.24	0.58$\pm$0.06	3.26$\pm$0.16	1.46$\pm$0.09	ANT
10+5	7.86$\pm$0.20	0.31$\pm$0.03	4.12$\pm$0.09	1.81$\pm$0.96	ANT
10+15	7.86$\pm$0.20	0.58$\pm$0.06	4.38$\pm$0.07	3.27$\pm$0.10	ANT

[a] Interactive effect = effect of Cr on Zn

[b] ANT = antagonistic

The interactions of dissolved metals with biological surfaces such as cell membranes can affect the transport, chemistry, bioaccumulation, and toxicity of metals. Biological surfaces are the most important substrate for metal binding in lakes and, in some cases, dissolved metal concentrations are controlled by adsorption to settling biological surfaces. The interactions that occur at biological surfaces in natural waters are very complicated. Reactions of metal ions with the various surface functional groups (sulfhydryl, amino, carboxyl, hydroxide, oxide) are numerous and difficult to quantify individually. Studies on the metal accumulation by aquatic organisms indicate that this is a two-step process consisting of rapid adsorption or binding to the surface, followed by slow, diffusion-controlled transport into the cell interior. Transport to the interior of the cell may be either by diffusion of the metal ion across the cell membrane or by active transport by a carrier protein. Such differences complicate the development of general relationships between the aqueous chemistry of metals and their toxicological properties. Among the factors affecting

the bioaccumulation of heavy metals by aquatic organisms, solution conditions, the nature of the metal ion (correlations involving metal ion radius or charge-radius function) and the nature of the aquatic organisms are of primary importance[16].

Table 5. Metal accumulation and interactive effect in Zn+Co

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Zn+Co	Zn	Co	Zn	Co	
5+5	6.80+ 0.11	6.20+0.14	4.79+0.23	2.27+0.15	ANT[b]
10+10	7.86+0.20	7.66+0.16	5.38+0.31	2.87+0.22	ANT
1+10	2.26+0.16	7.66+0.16	1.24+0.12	4.40+0.18	ANT
5+10	6.80+0.24	7.66+0.16	3.55+0.16	3.09+0.08	ANT
1+20	2.26+0.16	8.69+0.27	1.08+0.021	6.00+0.23	ANT
5+20	6.80+0.24	8.69+0.27	2.69+0.19	3.83+0.17	ANT
20+10	8.34+0.34	7.66+0.16	6.39+0.20	3.69+0.10	ANT
10+20	7.86+0.22	8.69+0.27	7.11+0.22	3.91+0.14	ANT
20+20	8.34+0.34	8.69+0.27	6.40+0.21	0.70	ANT

[a]Interactive effect = effect of Co on Zn

[b]ANT = antagonistic

Table 6. Metal accumulation and interactive effect in Cu+Zn

Metal Pair at (x+y), mg/L	Observed Metal Accumulation in single components, mg/g		Observed Metal Accumulation in binary mixtures, mg/g		Interactive Effect[a]
Cu+Zn	Cu	Zn	Cu	Zn	
2+2	0.64+0.08	3.72+0.22	0.34+0.02	1.75+0.05	ANT[b]
10+10	ND[c]	7.86 +0.20	1.76+0.08	1.47+0.03	ANT
1+5	0.46+0.05	6.80+0.24	1.20+0.06	2.50+0.11	ANT
2+5	0.64+0.08	6.80+0.24	0.52+0.03	2.17+0.09	ANT
1+10	0.46+0.05	7.66+0.16	0.35+0.02	3.05+0.14	ANT
2+10	0.64+0.08	7.66+0.16	0.64+0.05	3.59+0.15	ANT
1+20	0.46+0.05	8.34+0.34	0.41+0.03	5.50+0.21	ANT
2+20	0.64+0.08	8.34+0.34	0.61+0.04	4.83+0.15	ANT

[a]Interactive effect = effect of Cu on Zn

[b]ANT = antagonistic

[c]ND = not determined, *L.minor* did not survive

It can be concluded that duckweed is sensitive to the variations in metal concentration in binary mixtures and is capable of high metal enrichment at test levels. Nevertheless, the nature of the metal ion affects the uptake mechanism. The proposed model can be used safely to predict the type of interactive effect in metal accumulation once the metal ions are identified. However, in natural systems metals are not always freely available for uptake; a proportion may be bound in dissolved complexes. Thus, alterations of the physiochemical conditions of the environment (pH, redox potential, organic and inorganic ligands, T, etc.) can strongly influence the relative proportions of the metal ions that can be taken up. Evidently, the

implementation of bioindicator studies to assess the accumulation of metals and other pollutants in biota must be evaluated under well-defined physiochemical environmental conditions.

Acknowledgments

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