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Letter to Editor

Assessment of Toxic Interaction of Metals in Binary Mixtures Using *Lepidium sativum* and *Spirodela polyrrhiza*

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Abstract

The toxicity of $CuSO_4 \cdot 5H_2O$, $K_2Cr_2O_7$, $ZnSO_4 \cdot 7H_2O$, and $Ni(NO_3)_2$ and their binary mixtures on *L. sativum* and *S. polyrrhiza* was determined. The type of toxic interaction at each tested metal combination was evaluated by a statistical approach on testing the null hypothesis of "additive toxicity" at $p<0.05$. The effects were defined as antagonistic, additive, or synergistic, in accordance with the sign of difference between the tested hypothesis and the value of the observed toxicity at tested combinations. In the majority of metal combinations, the interactions for *L. sativum* were of antagonistic nature (94%), and only 6% of additive. The 100% additive interactive effects were found in the metal mixtures for *S. polyrrhiza*. The antagonistic or additive interactive effects found in almost all metal ion mixture combinations confirms the presumption that the interaction between ions can be caused by competition for the same reaction center on cell membranes if these ions belong to the same group of Lewis acids.

Keywords: test-organisms, *Lepidium sativum*, *Spirodela polyrrhiza*, metal ions, binary mixtures, interaction effects

Introduction

Many chemical mixtures, where concentrations of individual chemicals commonly exist at levels not considered toxic, are often present in aquatic systems. However, it is reckoned that chemical mixtures where individual constituents are present at low, non-toxic concentrations may trigger toxicity due to additive or synergistic effects among the constituents [1, 2]. Understanding the rules for interactive toxic effects in mixtures is therefore necessary [3-5]. Most of the investigations in this area have been performed using animals, bacteria or algae [3-6]; however, the bioassays with vascular plants can also be used as one of the tools to detect the presence of hazardous chemicals in the environment, evaluate the effects of mixtures, and demonstrate the bioavailibility of contaminants to different species [7-9]. The scope of this research included the evaluation of the observed and predicted effects of metals' mixture on terrestrial plant *Lepidium sativum* and aquatic plant *Spirodela polyrrhiza* and their comparison with the prediction model used in describing the toxic interactions of metals in the mixture. The goals of this research are to determine the toxicity of $CuSO_4 \cdot 5H_2O$, $K_2Cr_2O_7$, $ZnSO_4 \cdot 7H_2O$, and $Ni(NO_3)_2$ salts and their binary mixtures at various concentrations on test-organisms, and to assess the toxic interaction effects using an additive toxicity model.

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Materials and Methods

Stock solutions of 1000 mg/L of $CuSO_4 \cdot 5H_2O$, $K_2Cr_2O_7$, $ZnSO_4 \cdot 7H_2O$ (Reachim, St. Petersburg, Russia), and $Ni(NO_3)_2$, (Merck, Darmstadt, Germany) were prepared from chemically pure substances in distilled water. The predominant species of Cr(VI) ion in our experiment was $HCrO_4^-$, because the pH of experimental solutions was 6.0 and concentrations of Cr(VI) were smaller than 0.1 M [10]. The test levels of binary metal mixture (MM) were chosen according to the single metal EC_{50} values, which were estimated by linear regression. 2-days of seed germination and root growth test with *Lepidium sativum* L. (*Brassicaceae*) and 14-days growth inhibition test with *Spirodela polyrrhiza* (L.) Schleid. (*Lemnaceae*) were conducted according to the modified Magone [11] methods. These bioassays have been widely described in our previous report [2]. The test solutions initially were adjusted to pH=6.0 with 0.1 M HCl or NaOH. Germination power of seeds and length of *L. sativum* roots in distilled water (control) were 96 ± 4% and 36.0 ± 2.8 mm, respectively. The plant amount of *S. polyrrhiza* in nutrition medium (control) was 39.3 units. The experimental set of each testing scheme consisted of 5 control samples and 5 or 10 replicates of the test sample. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, at $p<0.05$.

The type of metal interactions in each tested MM was evaluated by the statistical method suggested by Ince et al. [12]:

$$H\,(x+y)_i = Ax_i \cdot Ay_i / 100,$$

where: $(x+y)_i$ is the i^{th} test combination in the mixture in mass concentration units, Ax_i and Ay_i are estimated parameters (root length or plants amount in %) for each metal, recorded at the x_i and y_i singular concentration.

The interactive effects were called "antagonistic," "additive," or "synergistic" in accordance with the sign of the difference between the values of the calculated toxicity (H) and observed toxicity (T) at the 95% significance level. If the difference was positive and statistically significant (*t* test), the interaction was called "antagonistic," signifying that the toxicity of the MM was lower than additive toxicity. If the difference was negative and statistically significant, the interaction was called "synergistic," signifying that toxicity of the MM was higher than additive toxicity. If the difference was statistically insignificant (irrespective of its sign), the interaction was called "additive."

Results and Discussion

Single Metal Toxicity

Toxicity of single metals to *L. sativum* and *S. polyrrhiza* are reported in Table 1 as 2-day EC_{50} values (mg/l) and as 14-day EC_{50} values (mg/l) according to the relative growth of seedlings' root of *L. sativum* and plant amount of *S. polyrrhiza*, respectively. It was revealed that the toxicity of the test metals to *L. sativum* decreased in the following order: Cr(VI) > Cu(II) > Ni(II) > Zn(II) (Table 1), and the EC_{50} values for *S. polyrrhiza* can be ranged in the following sequence: Cu(II) = Cr(VI) > Ni(II) > Zn(II) (Table 1). All studied metals caused morphological changes in the culture of *S. polyrrhiza* (plants were smaller or they formed irregular colonies, some leaves lost chlorophyll). The effects of single metals on the above-mentioned plants had been widely analyzed in our earlier report [2].

Toxicity of Binary Mixtures

The toxic interaction of metal pairs in combinations $(x:y)_i$ were evaluated by calculating the value of "additive toxicity" (H_i) of the concerned mixture, using the individual values of *L. sativum* root growth of its metal components at x and y. All calculations are presented in

Table 1. Comparison of the Cu(II), Zn(II), Ni(II), and Cr(VI) toxicity to *L. sativum* and *S. polyrrhiza.*

Metal	*L. sativum*		*S. polyrrhiza*	
	•2-day EC_{50}, mg/l	CI, mg/l	••14-day EC_{50}, mg/l	CI, mg/l
Cations				
Cu(II)	7.6	6.3 ÷ 9.3	3.3	3.0 ÷ 3.7
Ni(II)	73.7	63.2 ÷ 85.4	4.5	3.7 ÷ 4.8
Zn(II)	149	143 ÷ 156	48.6	43.0 ÷ 55.2
Anions				
Cr(VI)***	1.8	1.5 ÷ 1.9	3.5	3.0 ÷ 4.2

CI – 95% confidence interval, • – 50% effective concentration according to the decrease in root length of *L. sativum*, •• – 50% effective concentration according to the decrease of plant amount of *S. polyrrhiza*, *** – the predominant species of Cr(VI) is HCO_4^-.

Table 2. It should be noted that although the root grew in all binary test levels, H_i is not reported for some of them. Some concentrations of Cr(VI) and Cu in the binary mixtures were much higher than their EC_{50} value. The root growth of *L. sativum* in these concentrations of single Cu(II) and Cr(VI) was inhibited. Due to this reason, the value of the null hypothesis (additive toxicity) could not be evaluated, and the interactive effects were not determined. The data reported in Table 2 show that no synergistic interactions were found. From the 50 studied cases, 14 cases were unpredictable (referred to as NC), 34 were found to be antagonistic, and two were additive. The facts that toxic effects of metal binary mixtures on organisms are often less pronounced than have to be in accordance with the hypothesis of "additive toxicity" are indicated in literature [12-14].

The predicted antagonistic interactions of Zn(II) + Cu(II) combinations at all levels (except for 0.01 + 0.01 mg/l) presume that Zn(II) may reduce the toxic effects of Cu(II) on *L. sativum* (Table 2). The same conclusion was made by some authors [12, 13] who tested the toxicity of the above mentioned metal pair on *Lemna minor*. The root growth of *L. sativum* in almost all combinations of Cu(II) + Ni(II) and Ni(II) + Cr(VI) was much better than expected. The calculation of the value of the null hypothesis indicated the antagonistic interaction of the metals. The root of *L. sativum* grew in some combinations of Ni(II) + Cr(VI) and of Ni(II) + Cu(II), in which Cr(VI) and Cu(II) concentrations were higher than its EC_{50} value for a single metal (Table 2). For combinations of Ni(II) + Cr(VI) and Cu(II) + Ni(II) at equal concentrations of each metal (5; 10 and 20 mg/l) the suppression of the inhibitory effects of Cr(VI) and Cu(II) (Table 2) was revealed. This allowed us to make a presumption that Ni may suppress the inhibitory effects of the above-mentioned metals to the root growth of *L. sativum*. Sresty and Madhava Rao [15] states that the inhibitory effects of Cu(II) and Cr(VI) may decrease due to Zn(II) and Ni(II); whose relatively low concentrations may induce a greater degree of plant cell vacuolization, increasing cell ability to reduce the cytotoxic effects of the metals. However, some authors [16, 17] propose that scilicet copper inhibited the binding and cellular uptake of zinc, which resulted in decreased toxicity of these metal mixtures to plants. Our earlier investigations [18] with alga *Nitellopsis obtusa* showed that according to the accumulation coefficient, Cr(VI) was the least accumulated metal in the cell wall in comparison with Cu, Cd, Ni, Zn, Pb, and Mn. However, carryover of Cr(VI) into the cell protoplast was rather high. The decrease in the uptake of Zn, Cu, N, P, Mg and other nutrients in plants due to excess of Cr was mentioned in some reports which were summarized in the review of Shanker and co-authors [19].

For comparison of the effects of binary mixture of metal on plants, the toxicity of combinations of Cu(II) + Ni(II) at equal concentrations of each metal on *S. polyrrhiza* was investigated (Table 3). Statistical analysis of the data showed that in all studied cases the toxic effects of the mixture of Cu(II) + Ni(II) on *S. polyrrhiza* were additive (Table 3).

Investigation established that for *L. sativum*, in the majority of metal combinations, the interactions were of antagonistic nature (94%), and only 6% were additive. The 100% additive interactive effects were observed in the metal mixtures for *S. polyrrhiza*. So, *L. sativum* was less sensitive to the impact of Cu(II) + Ni(II) mixture than *S. polyrrhiza*. Only in the lowest tested Cu(II) and Ni(II) concentrations (1 and 1 mg/l) were the additive interaction of heavy metals in the mixture determined for *L. sativum*. In all other tested combinations the antagonistic interactions of metals in the mixture were defined. Ince with co-authors [12] maintain that the prediction made with the data of the impact of various concentration of metal binary mixtures on *Lemna minor* (*Lemnaceae*) in 87% and 13% cases was of antagonistic and additive nature, respectively. Synergism was not found in this investigation. The additive and antagonistic metal interactions within the mixture are indicated in literature as the most frequently occurring in the investigations of the impact of metal mixtures on bacteria and plants [2, 13, 20]. However, the toxic effects of the same metal mixtures on animals may be synergistic and *vice versa* [14, 21].

A comparative evaluation of the results within the tested plants revealed that, because of biological differences affecting toxicant uptake, the impact of metals and their mixtures on aquatic plant *S. polyrrhiza* and terrestrial plant *L. sativum* differed. Mineral nutrition in the *S. polyrrhiza* takes place via the whole bottom surface of the plant, and the roots are more significant for the plant balance than for mineral nutrition [22]. The seed coats of vascular plants are especially selective-permeable, thus the uptake of toxicants into the seed is rather limited [23].

Metal interactions within organism are very complicated due to the multiplicity of metal ion reactions with the various functional groups of cell membranes. The first step of metal accumulation by organisms is rapid adsorption or binding to the surface. The second step is slow, diffusion-controlled transport into the cell interior. It is known that the interaction of metallic elements with living systems is dominated by the properties of metal ions as Lewis acids (electron pair acceptors) or complex anions as Lewis bases (electron pair donor) [24–26]. The theory of Lewis acids and bases is sometimes used as one of the theories discussing the interaction of metals [14, 21, 27]. Reactive sites of metals as Lewis acids or bases can be either oligosaccharides of biological membranes or proteins. According to Tomasik with co-authors [21] the oxygen atoms of membranes are hard basic sites for metal cations and the hydrogen atoms of the hydroxyl groups of saccharide units of membranes are hard acids. The hard bases are proteins with reaction centers at either nitrogen or oxygen atoms, as well as the sulfhydryl bridges and other S(II) atoms with soft reaction centers. In our case Zn(II), Cu(II) and Ni(II) are borderline heavy metal Lewis acids and anion complex of Cr(VI) is hard Lewis base. So it is likely that the interaction between ions of particular

Table 2. Observed (T) and calculated (H)* toxicity (± SE) of Cu(II) + Zn(II), Cu(II) + Ni(II), and Cr(VI) + Ni(II) mixtures to the root growth of *L. sativum* and predicted interaction of metals within the mixtures.

Metal, mg/l		Root growth,%		Difference(S=T–H)	Statistical significance (Y/N) at 95% confidence level	Interaction
		Observed	Calculated			
Cu(II)	Zn(II)					
0.01	0.01	89.6 ± 4.4	84.9 ± 3.6	4.7	Y	antagonistic
0.1	0.1	99.5 ± 0.4	81.4 ± 4.8	18.1	Y	antagonistic
0.5	0.5	104.9 ± 2.2	83.8 ± 2.6	21.1	Y	antagonistic
1	1	95.9 ± 2.8	86.2 ± 2.4	9.7	Y	antagonistic
1	5	108.4 ± 6.3	82.3 ± 3.3	26.1	Y	antagonistic
2	2	100.6 ± 2.6	78.5 ± 2.7	22.1	Y	antagonistic
2	5	110.1 ± 4.9	77.1 ± 2.9	33.0	Y	antagonistic
2	10	94.5 ± 5.6	72.2 ± 1.8	22.3	Y	antagonistic
5	1	112.3 ± 4.6	58.2 ± 3.4	54.1	Y	antagonistic
5	5	100.2 ± 1.7	55.7 ± 2.6	44.5	Y	antagonistic
5	10	98.2 ± 3.7	52.2 ± 2.4	46.0	Y	antagonistic
10	1	100.6 ± 3.6	39.1 ± 3.5	61.5	Y	antagonistic
10	2	100 ±6.3	38.4 ± 3.7	61.6	Y	antagonistic
20	2	101.8 ± 3.7	–	–	–	–
Cu(II)	Ni(II)					
1	1	83.8 ± 2.2	81.8 ± 3,.7	2	N	additive
1	5	100.4 ± 2.3	78.2 ± 1.6	22.2	Y	antagonistic
1	10	93.2 ± 5.2	77.1 ± 2.2	16.1	Y	antagonistic
1	20	95.3 ± 2.2	70.5 ± 3.3	24.8	Y	antagonistic
1	50	94.4 ± 1.6	50.3 ± 2.1	44.1	Y	antagonistic
5	5	95.7 ± 3.3	52.8 ± 3.9	42.9	Y	antagonistic
5	1	85.9 ± 3.6	55.4 ± 4.6	30.5	Y	antagonistic
5	10	103.6 ± 1.3	52.2 ± 1.9	51.4	Y	antagonistic
5	20	103.1 ± 3.7	47.6 ± 3.2	55.5	Y	antagonistic
5	50	89.3 ± 2.5	34 ± 1.9	55.3	Y	antagonistic
10	10	96.2 ± 1.6	34.9 ± 2.2	61.3	Y	antagonistic
10	1	78.7 ± 3.1	37.1 ± 1.8	41.6	Y	antagonistic
10	5	95.2 ± 2.4	35.4 ± 3.1	56.8	Y	antagonistic
10	20	101.7 ± 1.6	32 ± 2.4	69.7	Y	antagonistic
10	50	88.6 ±2.1	22.8 ± 2.1	65.8	Y	antagonistic
20	20	101.4 ± 2.4	–	–	–	–
20	1	81.4 ± 3.5	–	–	–	–
20	5	93.6 ±5.8	–	–	–	–
20	10	101.7 ± 6.3	–	–	–	–

Table 2. continued.

Metal, mg/l		Root growth,%		Difference(S=T–H)	Statistical significance (Y/N) at 95% confidence level	Interaction
		Observed	Calculated			
20	50	81.5 ± 2.4	–	–	–	–
Cr(VI)	Ni(II)					
1	1	93.6 ± 2.9	69.6 ± 2.8	24.0	Y	antagonistic
1	5	102.2 ± 2.5	66.4 ± 3.6	34.4	Y	antagonistic
1	10	81.8 ± 2.6	65.6 ± 3.2	16.2	Y	antagonistic
1	20	99.9 ± 1.1	59.8 ± 3.4	40.1	Y	antagonistic
5	1	69.1 ± 1.6	66.4 ± 3.6	2.7	N	additive
5	5	71.9 ± 5.1	4.7 ± 0.3	67.2	Y	antagonistic
5	10	86.5 ± 2.1	4.7 ± 0.3	81.8	Y	antagonistic
5	20	76.9 ± 2.0	4.3 ± 0.2	72.6	Y	antagonistic
10	1	71.3 ± 4.2	–	–	–	–
10	5	85.9 ± 2.8	–	–	–	–
10	10	81.7 ± 3.2	–	–	–	–
10	20	74.4 ± 2.8	–	–	–	–
20	1	72.5 ± 1.8	–	–	–	–
20	5	73.0 ± 5.8	–	–	–	–
20	10	77.9 ± 3.7	–	–	–	–
20	20	61.3 ± 3.2	–	–	–	–

* – according to Ince et al.[12]; H – toxic effect calculated in accordance with the theory of "additive toxicity"; T – observed toxic effect; S – difference between the observed and calculated toxic effects; Y – statistically significant; N– statistically insignificant; (–) – the value of null hypothesis could not be evaluated.

Table 3. Observed and calculated* toxicity (± SE) of Cu(II) + Ni(II) mixture to the growth of *S. polyrrhiza* and predicted interaction of heavy metals within the tested mixture.

Metal, mg/l		Plant growth,%		Difference (S=T–H)	Statistical significance (Y/N) at 95% confidence level	Interaction
Cu(II)	Ni(II)	Observed (T)	Calculated (H)			
0.01	0.01	91.5 ± 1.8	99.2 ± 2.6	–7.7	N	additive
0.1	0.1	79.2 ± 1.9	88.1 ± 2.5	–8.9	N	additive
0.5	0.5	67.1 ± 2.1	75.1 ± 1.9	–8	N	additive
1	1	58.8 ± 1.6	56.8 ± 3.4	2	N	additive
2.5	2.5	40.1 ± 0.9	36.0 ± 2.9	4.1	N	additive
5	5	28.9 ± 2.5	18.1 ± 1.5	10.8	N	additive

* – according to Ince et al. [12]; H – toxic effect calculated in accordance with the theory of "additive toxicity"; T – observed toxic effect; S – difference between the observed and calculated toxic effects; Y – statistically significant; N– statistically insignificant; (–) – the value of null hypothesis could not be evaluated.

atoms can be caused by competition for the same reaction center if these ions belong to the same group of Lewis acids or bases. The antagonistic or additive interactive effects founded in almost all metal ion mixture combinations confirm the above-mentioned presumption. It was found that the impact of metals on aquatic plant *S. polyrrhiza* and terrestrial plant *L. sativum* and the interactions of metals within their mixtures differed. So, forecasting the changes in the functioning of the ecosystem under exposure of various contaminants it is necessary not only to determine the biological impact caused by multicomponent mixtures, made in accordance with naturally found concentrations, but also to assess the joint effects in these mixtures. In addition, assessment for water quality made using mixture toxicity information for single–species biotest may be insufficient for a more biologically complex system; therefore, organisms of different phylogenetic levels and ontogenesis have to be involved in these investigations.

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