Quantifying the toxicity of mixtures of metals and metal-based nanoparticles to higher plants

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Chapter 4

Comparing three approaches in extending biotic ligand models to predict the toxicity of binary metal mixtures (Cu–Ni, Cu–Zn and Cu–Ag) to lettuce (*Lactuca sativa* L.).

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Abstract

Metals are always found in the environment as mixtures rather than as solitary elements. However, effect models such as biotic ligand models (BLMs) are usually derived for toxicity prediction of single metals. Our study aimed at predicting mixture toxicity of Cu-Ni, Cu-Zn and Cu-Ag combinations to lettuce (*L. sativa* L.) by combining BLMs with three toxicity indexes: the toxic unit, the overall amounts of metal ions bound to the biotic ligands and the toxic equivalency factor. The accumulation of metal ions at the biotic ligands was used to determine the toxic potency of metals alone or in combination. On the basis of parameters derived from toxicity assessment of individual metals, these three extended BLMs appeared to be all acceptable ($p<0.0001$) in assessing toxicity of diverse metal mixtures. The BLM-based approaches integrated competition between metal ions in assessing mixture toxicity and showed different predictive ability for each metal combination. The outcome of modeling suggested that the combined toxicity depends on the specific components of the metal mixtures. The best developed models assist in identifying the type of underlying toxic mechanisms of diverse metal mixtures in terrestrial plants.

Keywords: Metal mixtures; toxicity; lettuce; biotic ligand models; toxicity index

4.1 Introduction

Complex metal mixtures are often found in aquatic and terrestrial ecosystems, instead of individual metals only. Joint actions of metals will create more distinct effects compared to simple summation of the effects of individual metals to assess toxicity of metal mixtures for living organisms. By modeling relationships between metal exposure and bioavailability or toxicity, basic toxic information of typical combinations of metals could be gained as a baseline for risk assessment.

Toxic impacts of metal mixtures have been investigated often on the basis of metal concentrations (Borgmann et al., 2008) and were estimated using toxicity indexes such as the toxic unit (TU) (Marking, 1985) and the toxic equivalency factor (TEF) (Delistraty, 1997). However, it has been recognized that water chemistry, such as activities of common cations and pH may affect metal toxicity by competitive binding to biotic ligands (BLs) and by influencing metal speciation (Niyogi and Wood, 2004). Biotic ligand models (BLMs) as an integration of reactive species of metals and competitive binding to the BLs are commonly suggested as useful tools in quantifying toxic effects of metals to organisms (Paquin et al., 2002). BLMs are usually applied to predict the toxicity of single metals. How to extend BLMs for mixture toxicity assessment has just recently attracted the attention of researchers. Thereupon, it may be helpful to elaborate the mechanisms of metal joint toxicity by combining BLMs with toxicity indexes.

For most of the metals, ionic channels are often considered as the primary sites of action (Niyogi and Wood, 2004). It is therefore the BLMs were extended as additive models to predict toxicity of metal mixtures with known stability constants derived from single-metal BLMs in previous studies. Playle (2004) was one of the first who tried to build a multi-metal modeling framework by combining BLM with the TU concept. Using the BLM-based TU approach, it is assumed that single metals in the mixture bind to different target sites on the BLs. Thus, no competition would exist between individual metals in the mixture (Hewlett and Plackett, 1979). Experiments performed by Hatano and Shoji (2008) demonstrated the feasibility of this framework to estimate toxicity of Cu-Cd mixtures to duckweed *L. paucicostata*. Besides the competition of major cations, competition between metal ions may also influence the amount of ion binding to the BLs and consequently diminish or enhance the toxicity of metal mixtures to organisms. Thus, if two metals in the mixture compete for binding to the same target site on the BLs, the total amount of metal ions bound to the site of toxic action (i.e. the f_{mix} index), likely assists in assessing mixture toxicity (Jho et al., 2011). Additionally, if the individual metals in the mixture have different potencies, the BLM-based TEF method is preferred (Van den Berg et al., 1998) as shown by Le et al. (2013) in their research on lettuce. Currently, there are still considerable uncertainties regarding the combined approach that is most reliable to predict combined effects of specific metal mixtures.

A great number of trace metals such as Ag, Cu, Ni and Zn have been found to be released into the natural environment due to anthropogenic activities (Charles et al., 2013). The elevated levels of trace metals may produce negative effects on fauna and flora in the environment and may cause damage to human health either through the food chain or through direct uptake. In view of its high sensitivity to environmental stresses (Valerio et al., 2007), lettuce (*Lactuca sativa*) was selected as a bio-indicator in the present study. Standard testing protocols for lettuce have been recommended by EPA (1988) and OECD (2006). Thus, our paper aims at examining which BLM-based approach (i.e. TU, f_{mix}, and TEF) would be most accurate in assessing the combined toxicity of Cu-Ni, Cu-Zn, and Cu-Ag combinations to lettuce. Basic modeling parameters were gained from Ni-only, Cu-only, Zn-only, and Ag-only toxicological data in the presence of different concentrations of Ca^{2+}, Mg^{2+}, K^{+}, Na^{+} and different levels of pH.

4.2 Material and methods

4.2.1 Plant bioassays

Lettuce seeds (*L. sativa* L.) and seedlings were all cultured in hydroponic solution. The nutrient solution for the plant culture and the test medium was prepared according to the Steiner solution formula (Steiner 1961; Le et al., 2012). Seeds of lettuce were germinated in a climate room (15˚C, 80% humidity, 16:8 hours light: dark cycle) for 4 days on sterilized expanded perlite in Steiner solution. Then the seedlings were fixed in parafilm straps floating on the surface of glass beakers (10 cm height) with spiked medium. Four plants were put in each beaker. Beakers were placed in a large container with a layer of water inside to prevent excess evaporation of exposure media. After exposure, 5 ml medium of each treatment was acidified and preserved in a 4°C refrigerator for chemical analysis.

4.2.2 Metal exposure and analysis

Cu and Ni were added into the Steiner solution as nitrate salts since NO_3^- was assumed not to interfere with the performance of the Cu-selective electrode. The concentrations of added Ni ranged from 34 to 85 μM and the range of Cu-activities was from 0.8 to 21 nM. The activity of Cu^{2+} was checked using an ISE25 Cu-selective electrode (Radiometer analytical, France) and adjusted every other day to keep the Cu-activity constant as designed during the exposure. Solution pH was kept at 7.0 every other day using either HNO_3 or NaOH and checked using a 691 pH meter (Metrohm, Switzerland). Metal concentrations of the test medium and the Steiner solution were measured by flame atomic absorption spectroscopy (Perkin Elmer AAnalyst 100, US), reference analytes were found to be within 15% of the certified reference values. Speciation calculations were conducted by Windermere Humic Aqueous Model 7.0.1 (Centre for Ecology & Hydrology, UK) based on the measured concentrations in solution (Supplementary Material). Actual concentrations of the cations and anions in solution were calculated according to the Steiner solution formula applied in the study of Le et al. (2012). The pCO_2 was set at $10^{-3.5}$ atm since the hydroponic system was open to the ambient air.

4.2.3 Response measurements

The root length of seedlings was measured before and after 4 days of exposure as the distance from the transition point between the hypocotyls and the root to the root tip. Root elongation was reported to be a suitable and sensitive endpoint of toxicity for metal exposure (EPA 1988; OECD 2006). The root growth of 4 seedlings was averaged as lettuce root elongation at a given concentration. The relative root elongation inhibition (*REI*, %) was used to determine the toxic response of lettuce to Ni^{2+}/Cu^{2+}/Zn^{2+}/Ag^{+} and their mixtures in the present study:

$$REI = (1 - \frac{RG_S}{RG_C}) \times 100\% \quad (4\text{-}1)$$

In equation (4-1): RG_s = the average root growth of plants in the sample solution; RG_c = the average root growth of plants in the control solution.

4.2.4 Data analyses

The toxicological data (i.e. K_{MgBL} and f_{50M}) derived from exposure of lettuce to single metals were collected from previous studies and were summarized in Table S4.1 (Supplementary Material). The response data of Cu-Zn and Cu-Ag mixtures used for modeling were taken from the research of Le et al. (2013) in which the same test species was used and the exposure was executed under similar experimental conditions. The single toxicity of Cu^{2+}/Zn^{2+}/Ag^{+} to *L. sativa* L. was significantly inhibited only by H^{+}, and Ni^{2+} binding to the biotic surface was found to be Mg^{2+}-dependent (Le et al., 2013; Liu et al., 2014). Thus, it is assumed that metals investigated in this paper mainly enter the biological cells as ionic forms through major cations or protons transport sites. In other words, the modes of action (MoA) of Cu, Ni, Zn, and Ag are presumed similar, but their specific mechanisms are unknown. Based on this assumption, toxicity of Cu-Ni, Cu-Zn and Cu-Ag mixtures was predicted by combining the BLM concept with the TU, f_{mix} and TEF indexes.

In the BLMs, the interactions of the metals with the BLs are assumed to be purely competitive. The fraction *f* of the total number of biotic ligand sites bound by metal ions is considered as the key indicator of metal toxicity (Jho et al., 2011).

$$f = \frac{K_{\mathrm{MBL}} \times \{\mathrm{M}^{2+}\}}{1 + K_{\mathrm{MBL}} \times \{\mathrm{M}^{2+}\} + \sum K_{\mathrm{EBL}} \times \{\mathrm{E}^{n+}\}} \quad (4\text{-}2)$$

In equation (4-2): *K* = the binding constant for binding to the biotic ligand sites; M^{2+} = the metal ions of interest, namely Cu^{2+}, Zn^{2+}, Ag^{+} and Ni^{2+} in our case; E^{n+} = essential or major ions competing for binding to the BLs, namely H^{+} or Mg^{2+} in our case; { } = the chemical activity.

The Cu-Ni combination is used as an example to explain the development of binary-metal BLMs. Similar approaches can be applied to extending BLMs for Cu-Zn and Cu-Ag mixtures. If Cu^{2+} and Ni^{2+} bind to different specific transporters/sites on the biological membrane, which fits the assumption of TU approach (Khan et al. 2012), there would be no competition between Cu^{2+} and Ni^{2+} because of the different mechanisms of action (MOA). In that case, binding of these metal ions to the distinct target sites is only influenced by major cations, i.e. Mg^{2+} for Ni^{2+} and H^{+} for Cu^{2+}. Then equation 4-2 can be transformed:

$$f_{\mathrm{Cu}} = \frac{[\mathrm{CuBL}]}{[\mathrm{BL}]_{\mathrm{T}}} = \frac{K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\}}{1 + K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\} + K_{\mathrm{HBL}} \times \{\mathrm{H}^{+}\}} \quad (4\text{-}3)$$

$$f_{\mathrm{Ni}} = \frac{[\mathrm{NiBL}]}{[\mathrm{BL}]_{\mathrm{T}}} = \frac{K_{\mathrm{NiBL}} \times \{\mathrm{Ni}^{2+}\}}{1 + K_{\mathrm{NiBL}} \times \{\mathrm{Ni}^{2+}\} + K_{\mathrm{MgBL}} \times \{\mathrm{Mg}^{2+}\}} \quad (4\text{-}4)$$

Toxicity of Cu-Ni mixtures can be described as adding up the TU values of each metal:

$$\mathrm{TU} = \sum \mathrm{TU}_i = \frac{f_{\mathrm{Cu}}}{f_{50,\mathrm{Cu}}} + \frac{f_{\mathrm{Ni}}}{f_{50,\mathrm{Ni}}} \quad (4\text{-}5)$$

In equation (4-5): f_{50} = fraction of the biotic ligands occupied by metal ions at the 50% response level.

When Cu^{2+} and Ni^{2+} are assumed to act through similar mechanism of action (Van den Berg et al., 1998), both competition between metal ions and competition between cations for binding sites are supposed to affect the overall amounts of ions binding to the target sites.

$$f_{\mathrm{Cu}} = \frac{K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\}}{1 + K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\} + K_{\mathrm{NiBL}} \times \{\mathrm{Ni}^{2+}\} + K_{\mathrm{HBL}} \times \{\mathrm{H}^{+}\}} \tag{4-6}$$

$$f_{\mathrm{Ni}} = \frac{K_{\mathrm{NiBL}} \times \{\mathrm{Ni}^{2+}\}}{1 + K_{\mathrm{NiBL}} \times \{\mathrm{Ni}^{2+}\} + K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\} + K_{\mathrm{MgBL}} \times \{\mathrm{Mg}^{2+}\}} \tag{4-7}$$

Toxicity of Cu-Ni mixtures may be expressed:

$$f_{\mathrm{mix}} = f_{\mathrm{Cu}} + f_{\mathrm{Ni}} \tag{4-8}$$

If single metals in a mixture have dissimilar potency, the TEF index as an adjustment coefficient can be combined with the BLM for toxicity assessment of metal mixtures (Le et al. 2013). The value of TEF represents the comparative toxic potency for each metal in the mixture. Toxicity of a complex mixture can be expressed in terms of the toxic equivalent (TEQ). It is calculated by summing the products of concentration and TEF for each metal in the mixture (Delistraty, 1997). In the present study, Cu^{2+} was selected as the reference metal for standardization of toxicity of individual metals since Cu^{2+} has the highest stability constant (Table S4.1) and thus was assumed to be the most toxic metal in the mixtures. The values of TEF were equal to 1 and 0.63 for Cu and Ni respectively according to equation 4-9.

$$\mathrm{TEF}_{\mathrm{Ni}} = \frac{f_{50,\mathrm{Cu}}}{f_{50,\mathrm{Ni}}} \tag{4-9}$$

$$\mathrm{TEQ} = \sum f_{\mathrm{M}} \times \mathrm{TEF}_{\mathrm{M}} = f_{\mathrm{Cu}} \times \mathrm{TEF}_{\mathrm{Cu}} + f_{\mathrm{Ni}} \times \mathrm{TEF}_{\mathrm{Ni}} \tag{4-10}$$

Inhibition of lettuce root elongation (*REI*) was expressed using TU, f_{mix} and TEQ as follows:

$$REI = \frac{100}{1 + 10^{(x_{50} - x) \times \beta}} \tag{4-11}$$

In equation (4-11): β = the fitted parameter determining the slope of the dose response curve; x = the value of the toxicity index, i.e. TU, f_{mix} and TEQ at a given mixture concentration; x_{50} = the value of TU/f_{mix}/TEQ when 50% inhibition to root elongation is performed. Response data for Cu-Ni, Cu-Zn and Cu-Ag mixtures were fitted to the dose-response curves, using the software Origin 8.0725 (Origin Lab, UK).

The IC_{50} values of one of the metals in the binary mixture, expressed as activity, were plotted against activities of the other metal to further investigate the competition between metal ions in the mixture. The median inhibition concentration (IC_{50}) for each metal was also determined by means of equation 4-11.

The adjusted root mean square error (*RMSE*) was calculated for the three extended BLMs and used for model comparison:

$$RMSE = \sqrt{\frac{SS}{n-k}} \tag{4-12}$$

In equation (4-12): *SS* = residual sum of square; *n* = number of points; *k* = number of free parameters in the model. The lowest value of *RMSE* indicated the best modeling method. To quantify the statistical differences between each model, the bootstrapping method was used to estimate the distribution of differences between *RMSEs*. Five thousand samples (typically 1000 to 10000) were randomly resampled from each original dataset. Two-tailed *p* values were obtained multiplying the proportions of smallest differences close to zero by two. The calculations were conducted using Statistics Analysis System 9.2 (SAS Institute Inc., US).

4.3 Results

4.3.1 Toxicity of Cu-Ni mixtures

Observed toxic effects of the Cu-Ni mixtures plotted against BLM-based TU, f_{mix} and TEQ values are shown in Figure 4.1. Using these three models, increased values of TU, f_{mix} and TEQ significantly (p<0.0001, Table 4.1) correlated to the increasing root elongation inhibition of *L. sativa.* Although the difference with the TEF method (Table S4.2, Supplementary Material) was not statistically significant (p=0.10), the BLM-based TU approach was slightly better in interpreting toxicity of Cu-Ni mixtures because of the highest value of *Adj.* R^2 (0.86) and the smallest *RMSE* (10.54). Although the value of TU_{50} was manually calculated to be 1.23±0.02, considering the actual experimental error, this deviation from additivity of Cu-Ni mixtures was assumed to be not significant. The β values derived using the BLM-based TU approach for the three metal combinations were significantly different (Table 4.1) since their 95% CIs deviated significantly from each other.

Changes of 4 d IC_{50} values of Cu^{2+}/Ni^{2+} to *L. sativa* at various Ni^{2+}/Cu^{2+} activities are presented in Figure 4.2. Significant logistic correlations (p<0.01) revealed that the more Cu^{2+} was added, the lower the IC_{50} value of Ni^{2+}, and vice versa. The toxicity of Ni^{2+} increased with increasing activities of Cu^{2+} and the IC_{50} values of Ni^{2+} when exposed in a mixture with Cu were always lower than the corresponding values in single Ni experiments (3.03×10^{-5} M). A similar trend was observed in the relationship between Ni^{2+} activities and the IC_{50} of Cu^{2+}. A 43-fold reduction of IC_{50} of Cu^{2+} was observed when the activity of Ni^{2+} increased up to 3.4×10^{-5} M. The above results demonstrated that the increased activities of Ni^{2+} did not reduce the Cu-toxicity and vice versa which implied that Ni^{2+} and Cu^{2+} may be bound to different target sites on the BLs.

4.3.2 Toxicity of Cu-Zn mixtures

Statistically significant correlations (p<0.0001) between the three toxicity indexes and *REI* were obtained (Table 4.1) for the Cu-Zn combination. The strength of the correlations differed from *Adj.* R^2=0.58 to 0.73 (Figure 4.1). With the highest value of *Adj.* R^2 (0.73) and the lowest *RMSE* (15.15), the predictive power of the BLM-based f_{mix} model was significantly better than the BLM-based TU/TEF approaches (p<0.001, Table S4.2) in assessing toxicity of Cu-Zn mixtures. The TU_{50} (1.79) was calculated to be significantly higher than 1 since the 95% confidence interval of the estimated TU_{50} (1.71-1.88) exceeded unity significantly. This implied that the concentration-addition hypothesis was rejected at the 5% significance level and the Cu-Zn combination resulted in an antagonistic effect. The f_{mix50} of Cu-Zn mixtures (0.59) was similar to the values derived for both the Cu-Ag combination (0.62) and the Cu-Ni combination (0.58).

Logistic regressions (Figure 4.2) demonstrated that the IC_{50}s of Zn^{2+} decreased significantly upon increasing activities of Cu^{2+}, and vice versa (p<0.001). The elevation of Cu^{2+} activities resulted in a 45-fold reduction of the IC_{50} of Zn^{2+}. At lower activities of Cu^{2+} (< 3.38×10^{-8} M), the IC_{50} of Zn^{2+} was increased as compared to the value when Zn^{2+} operated alone (1.06×10^{-4} M). Cu^{2+} turned into the dominant cause of inhibition at higher activities because of the low IC_{50} of Zn^{2+}. Almost all the IC_{50}s of Cu^{2+} in the mixtures were higher than the values in the treatment of Cu^{2+} alone (2.60×10^{-8} M) except at higher activities of Zn^{2+} (>1.23×10^{-4} M). Thus, conforming to the assumptions of the BLM-based f_{mix} model, Zn^{2+} exerted an ameliorative effect on Cu-toxicity to lettuce and vice versa.

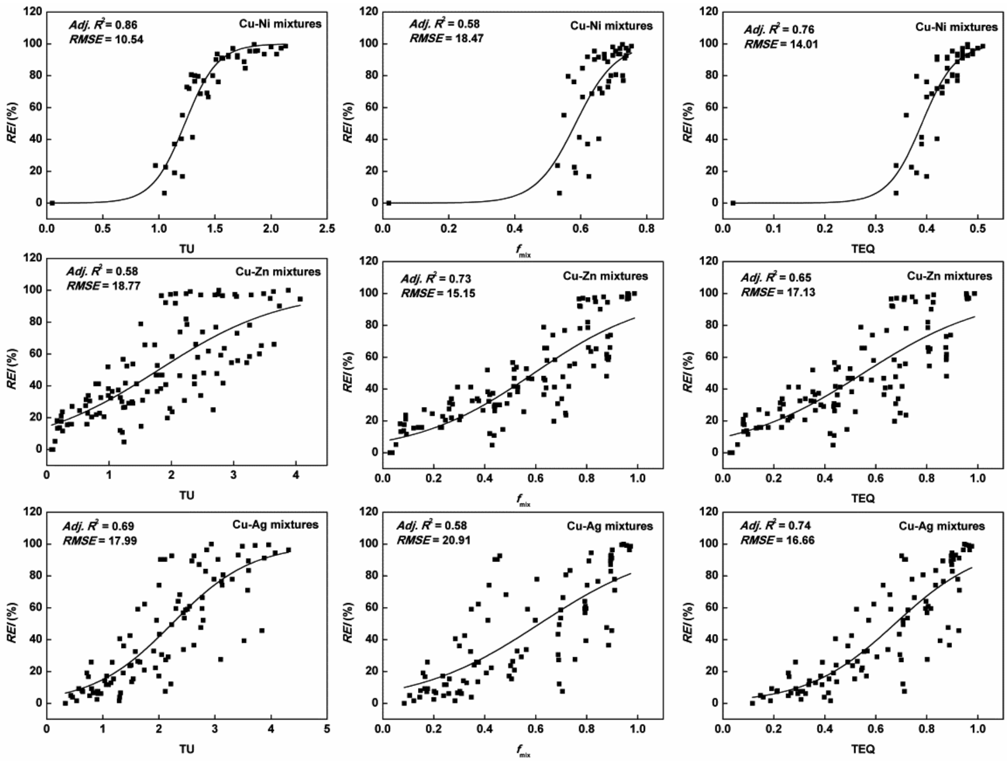

Figure 4.1 Dose-response relationships between root elongation inhibition (*REI*, %) to lettuce *L. sativa* and toxic indexes i.e. TU (first column), f_{mix} (second column) and TEQ (third column) for the mixture combinations Cu-Ni (first row), Cu-Zn (second row) and Cu-Ag (third row). The solid lines represent the logistic model fits (equation 4-11). R^2 indicates the coefficient of determination adjusted for the degrees of freedom for the measured and the predicted *REI*. *RMSE* indicates the adjusted root-mean-square error of the predicted *REI*.

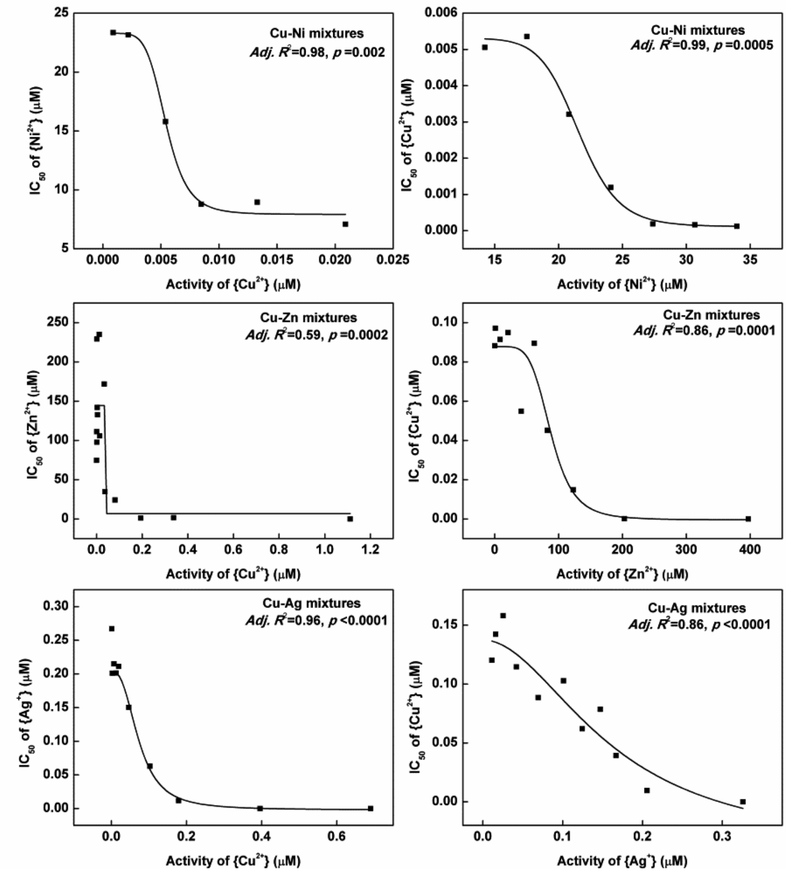

Figure 4.2 Relationships between the median inhibition concentrations (IC_{50}s) of Zn^{2+}/Cu^{2+}/Ag^{2+}/Ni^{2+} for *L. sativa* after 4 d exposures and the activities of Cu^{2+}/Zn^{2+}/Ag^{2+}/Ni^{2+} in the mixture. The first row shows impacts in Cu-Ni mixtures, the second row shows impacts in Cu-Zn mixtures and the third row shows impacts in Cu-Ag mixtures. The solid lines represent the logistic model fits. R^2 indicates the coefficient of determination adjusted for the degrees of freedom. *p* indicates the statistical significance level.

4.3.3 Toxicity of Cu-Ag mixtures

Toxic effects of Cu-Ag mixtures to lettuce were estimated by using the BLM-based TU, f_{mix} and TEQ indexes. Dose-responses curves are presented in Figure 4.1 and all correlations showed to be statistically significant (p<0.0001, Table 4.1). The highest *Adj.* R^2 (0.74) and the lowest *RMSE* (16.66) were obtained using the

BLM-based TEF in describing the combined toxicity of Cu-Ag mixtures. However, no statistically significant differences (p=0.15, Table S4.2) were found between using TU and TEF indexes. The TU_{50} value (2.23) for the Cu-Ag combination was likewise significantly higher than 1, which implied an antagonistic relationship between Cu^{2+} and Ag^{+}. The β values derived using the three models significantly deviated from each other at the 5% significance level.

As shown in Figure 4.2, a significant decrease of IC_{50}s of Cu^{2+}/Ag^{+} with increasing activities of Ag^{+}/Cu^{2+} was observed (p<0.0001). Addition of Cu^{2+} alleviated the toxicity of Ag^{+} due to the higher value of IC_{50}s as compared to single Ag^{+} exposure (1.34×10^{-7} M). Up to 1.03×10^{-7} M, the higher activities of Cu^{2+} resulted in root elongation inhibition again. Similarly, reduction of Cu-toxicity was observed with the addition of Ag^{+}. Thereupon, competition may occur between Cu^{2+} and Ag^{+} when lettuce was exposed to Cu-Ag mixtures in solution.

4.4 Discussion

4.4.1 Competitions and metal toxicity of binary metal mixtures

Overall, the results of this study showed that the three extended BLMs all succeeded to predict toxicity of Cu-Ni, Cu-Zn and Cu-Ag mixtures to lettuce. However, their predictive abilities varied for different binary-metal combinations, which indicated that the mixture toxicity is dependent on the specific composition of the metal mixture and the relative quantities of each metal presented in the mixture. The statistical difference between the BLM-based TU and TEF approaches was not significant in predicting toxicity of Cu-Ni and Cu-Ag mixtures, which differed from the finding for the Cu-Zn combination. In order to determine the most suitable model for each metal combination, the correlations between the IC_{50} values and the activities were developed to further explore the occurrence of competition. The increased values of the toxicity indexes (i.e. TU, f_{mix} and TEQ) reflected the increased toxicity of binary-metal mixtures to *L. sativa.* By combining the BLMs with the TU/f_{mix}/TEF approaches, the site-specific theory of ion binding provides explanations for competition between metals in the mixture.

Table 4.1 Model fit summary and coefficients of toxicity estimations for Cu-Ni, Cu-Zn and Cu-Ag mixtures using binary-metal BLMs.

Combinations	Methods	*Adj.* R^2	*RMSE*	*p*	*F*	x_{50} (95% CIs)	β (95% CIs)
Cu-Ni	TU	0.86	10.54	<0.0001	1117	1.23 (1.21-1.25)	2.98 (2.62-3.34)
	f_{mix}	0.58	18.47	<0.0001	350	0.58 (0.57-0.60)	7.17 (5.74-8.60)
	TEF	0.76	14.01	<0.0001	623	0.39 (0.38-0.40)	13.13 (11.23-15.02)
Cu-Zn	TU	0.58	18.77	<0.0001	474	1.79 (1.71-1.88)	0.43 (0.39-0.48)
	f_{mix}	0.73	15.15	<0.0001	759	0.59 (0.58-0.61)	1.87 (1.72-2.02)
	TEF	0.65	17.13	<0.0001	582	0.55 (0.53-0.57)	1.76 (1.59-1.92)
Cu-Ag	TU	0.69	17.99	<0.0001	393	2.23 (2.16-2.31)	0.61 (0.55-0.68)
	f_{mix}	0.58	20.91	<0.0001	278	0.62 (0.59-0.64)	1.80 (1.59-2.01)
	TEF	0.74	16.66	<0.0001	466	0.68 (0.66-0.69)	2.52 (2.28-2.76)

Adj. R^2: the coefficient of determination adjusted for the degrees of freedom for the measured and the predicted *REI* (%); *RMSE*: the adjusted value of the root-mean-squared error of the predicted *REI* (%); *p*: the statistical significance level; *F*: the value of *F* test; x_{50}: the value of TU/f_{mix}/TEQ when 50% inhibition to root elongation is performed by metal mixtures; β: the fitting parameter determining the slope of the dose response curve; 95% CIs: 95% confidence intervals.

The outcome of modeling plus the observations displayed in Figure 4.2 showed that the BLM-based TU model which only integrated competition attributable to the major cations, was the best predictive tool in explaining toxic effects of Cu-Ni mixtures to *L. sativa.* This indicated that Ni^{2+} followed a distinct pathway from Cu^{2+} in lettuce for uptake or translocation, which was consistent with the finding on *Blepharis aspera* (Nkoanea et al., 2007). However, some researchers pointed out that interactions occurred at the internal pathways. Both Cu and Ni were found to trigger oxidative stress in plants by generating reactive oxygen species (ROS) (Charles et al., 2013). On the other hand, no or little effect of Ni on the ionic balance was found in *D. magna* (Pane et al., 2003) which differed from the observed effects of Cu in *G. pulex* (Brooks and Mills, 2003). Thus, the significance of competition between Cu^{2+} and Ni^{2+} may be different due to diverse factors (Spurgeon et al., 2010), such as endpoint of assessment and test species.

The toxicity prediction of Cu-Zn mixtures was improved significantly when competition among metal ions was incorporated. This implied that Zn^{2+} and Cu^{2+} interacted at the organism level. This finding was consistent with the research of Luo and Rimmer (1995) on barley growth. The affinity for the same targets may be associated with a lack of binding preference of Zn^{2+}, which makes Zn^{2+} bind to structurally diverse ligands (Peijnenburg and Vijver, 2007). In addition, Bræk et al. (1976) found that in *P. tricornutum*, all divalent metal ions, including Cu^{2+} and Zn^{2+} act on a common site. Essential elements such as Cu and Zn exist within the plant as organometallic complexes, the remobilization potential of which were found to be similar from senescing tissues to the seeds (Cataldo and Wildung, 1978). Thus, possibly due to the occurrence of competition between Cu^{2+} and Zn^{2+}, the BLM-based f_{mix} method was found to be best in predicting toxicity of Cu-Zn mixtures to lettuce.

In toxicity prediction of Cu-Ag mixtures, although the difference between the BLM-based TU and TEF models was not statistically significant, the results of TU_{50} and competition exploration showed that the toxicity of Ag^{+} was reduced by addition of Cu^{2+}, and vice versa. This may imply that Cu^{2+} competed with Ag^{+} at the level of metal uptake, which was similar to the findings in aquatic animals (Niyogi and Wood, 2004). Moreover, Howe and Merchant (1991) also found that the presence of Cu^{2+} blocked the synthesis of the Ag^{+}-inducible components and made plant cells resistant to Ag^{+}. The different toxic potencies of Cu^{2+} and Ag^{+} to lettuce which were suggested by TEF approach may be due to their dissimilar valence numbers. The similar MOA of Cu^{2+} and Ag^{+} indicated that the necessity of elements may not be the only criterion in judging interactions between metals.

4.4.2 Application of estimated coefficients and models

Based on the assumptions of the extended BLMs, the coefficients obtained from simulations may also have the potential to be indicative of the underlying mechanisms of metal mixtures in solution.

According to the traditional method used for soil animals (Weltje, 1998), patterns of interaction between metals in a mixture may be obtained by rescaling the concentrations in terms of TUs. The TU_{50} values of Cu-Zn and Cu-Ag mixtures indicated that the deviations from additivity were statistically significant at the 5% significance level. In accordance with the null hypothesis of models, Cu^{2+} may compete with Zn^{2+}/Ag^{+} on the similar transport sites. Relationships between median inhibition concentrations of Zn^{2+}/Cu^{2+} and activities of Cu^{2+}/Zn^{2+} did demonstrate a protective effect of Zn^{2+}/Cu^{2+} on Cu^{2+}/Zn^{2+} to lettuce. The impacts at lower activities of Cu^{2+}/Zn^{2+} on Zn^{2+}/Cu^{2+} were found to be different from the impacts at higher metal activities. This may imply that the interactions were dose-level dependent. Similar trends were found in this study for Cu-Ag mixtures. This is in agreement with the general finding that antagonism is the predominant response in modeling toxic effects of metal mixtures to organisms in the environment (Vijver et al., 2010). Another explanation for the discovered antagonism may be due to the overestimated prediction made by the conservative concentration addition model. Therefore, the BLM-based TU_{50} seems to be useful in determining interaction patterns for binary-metal mixtures. As the interaction in BLM was assumed to be competition, the interactive strength became a measure of magnitude of antagonism.

The similar fractions of the total number of biotic ligands occupied by mixture ions to cause 50% inhibition of root growth (f_{mix50}) may be indicative of similar sensitivities of lettuce to the three binary-metal mixtures studied. Significant differences of β values were found among diverse metal combinations and different modeling methods. Plackett and Hewlett (1952) explained that observed dissimilarities in concentration-effect curves resulted from differences in transport or metabolic pathways from exposure level to the actual target within organisms.

The significant dependence of mixture toxicity on the TEQ (Cu-equivalents) values across various metal combinations indicated the practicality of the BLM-based TEF approach in assessing toxicity of metal mixtures as for dioxin-like chemicals. The value of TEQ, which was the sum of weighted potency of each component in the mixture, represented the magnitude estimate of relative potency (Birnbaum and DeVito, 1995). The ranking of TEQ_{50}s was found to be Cu-Ni<Cu-Zn<Cu-Ag. According to the binding constants of single metals, Ag^+ was supposed to be most toxic among the three metals added to Cu. The biggest TEQ_{50} value of Cu-Ag mixtures may be attributed to relatively strong competition between Cu^{2+} and Ag^+ when 50% root elongation inhibition was induced (Le et al., 2013). Unlike classes of organic chemicals, it is difficult to classify metals due to their different toxic effects on various plant species. The individual TEF values are associated with the standard metal selected, which may consequently influence the toxicity or TEQ of a mixture (Safe, 1998). Thus, the utility of TEF/TEQ values to compare the toxic load of metals and their mixtures in terrestrial plants remains to be determined.

The additivity models developed in this study are also applicable to predict toxicity of complex mixtures consisting of more than two metals if the binding affinities of metal components are known. Based on the combination-specific modeling results, the BLM-based TU approach is recommended as a good first approximate estimation of toxic effects of metal mixtures since it is relatively conservative and simple to implement.

4.5 Conclusions

In summary, the present study supported the BLM concept that the fraction of the total amount of BLs occupied by metal ions was a good indicator determining mixture toxicity with consideration of environmental impacts. The three extended

BLMs based on known stability constants of single metals successfully accounted for the toxicity of metal mixtures to lettuce. The predictive power of combining BLM principles and the TU/f_{mix}/TEF indexes differed for the specific combination of metal mixtures. The incorporated ion-ion competition and toxic potency of individual metals gave more accurate toxicity assessment for specific metal mixtures. However, due to a limited understanding of metals mechanisms in terrestrial plant species, it is difficult to straightforwardly give a best approach in predicting toxicity of all possible metal mixtures. Thus, we suggest using the BLM-based TU method for the general risk assessment of new metal combinations. By comparing the performance of the three extended BLMs, the best model obtained is likely indicative of the underlying mechanisms of toxicity of metal mixtures.

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Supplementary Materials

S4.1 Chemical composition of Steiner solution

The concentrations of Mg, Ca, K, Na and Zn in the Steiner solution were measured using FAAS. The values were averaged to be 1.674, 2.103, 5.662, 1.251, 0.002 mM respectively, and used for speciation calculation of mixture ions in WHAM 7.0.1.

S4.2 Selection of conditional stability constants and fraction of the total number of BLs occupied by metal ions

Mixture toxicity prediction in the present study was based on the known stability constants and the fraction of the total number of biotic ligands occupied by metal ions at the 50% response level which derived from the BLMs for single metals in previous studies. The value of K_{HBL} (log scale) used for modeling in this study was set as a constant value for lettuce *L. sativa* since the affinity constants of H^+ binding for diverse organisms in the aquatic system were found to be constant around 6 (Verschoor et al., 2012).

Among the conditional stability constants, Cu^{2+} has the highest binding affinity to the biotic ligands when compared to Ag^+, Zn^{2+}, and Ni^{2+}, i.e. log K_{CuBL} (7.4) > log K_{AgBL} (6.39) > log K_{NiBL} (5.10) > log K_{ZnBL} (4.0).

Table S4.1 Binding constants of Cu, Zn, Ag and Ni to lettuce (*Lactuca sativa*) and the fraction of the total number of biotic ligands of lettuce occupied by metal ions at the 50% response level (f_{50M}).

Metal ions	log K_{MBL}	log K_{HBL}	log K_{MgBL}	f_{50M}	Sources
Cu^{2+}	7.40	6.27	-	0.36	Le et al. (2012)
Zn^{2+}	4.00	6.27	-	0.42	Le et al. (2012)
Ag^{2+}	6.39	6.27	-	0.22	Le et al. (2012)
Ni^{2+}	5.10	-	2.86	0.57	Liu et al. (2014)

S4.3 Model comparisons using Bootstrapping

In our case, the BLM-based models are non-nested by only changing the toxic indicators (i.e. TU, f_{mix}, and TEQ). Thus, the traditional statistical hypothesis testing (such as F test) cannot be used to compare models. Bootstrapping method which was introduced in 1979 by B. Efron (1979) was chosen to determine the relative likelihood of two models for each combination. The constructed two-sided *p* values were used to interpret the significance of differences between usages of two models.

Table S4.2 Model comparisons by using Bootstrapping.

Combinations	TU versus f_{mix} (*p* values)	TU versus TEF (*p* values)	f_{mix} versus TEF (*p* values)
Cu-Ni	<0.001*	0.10	<0.001*
Cu-Zn	<0.001*	0.03*	<0.001*
Cu-Ag	0.014*	0.15	<0.001*

*: statistically significant difference at the 5% significance level.