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

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Chapter 3

Statistically significant deviations from additivity: What do they mean in assessing toxicity of mixtures?

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Abstract

There is increasing attention from scientists and policy makers to the joint effects of multiple metals on organisms when present in a mixture. Using root elongation of lettuce (*Lactuca sativa* L.) as a toxicity endpoint, the combined effects of binary mixtures of Cu, Cd, and Ni were studied. The statistical MixTox model was used to search deviations from the reference models i.e. Concentration Addition (CA) and Independent action (IA). The deviations were subsequently interpreted as 'interactions'. A comprehensive experiment was designed to test the reproducibility of the 'interactions'. The results showed that the toxicity of binary metal mixtures was equally well predicted by both reference models. We found statistically significant 'interactions' in four of the five total datasets. However, the patterns of 'interactions' were found to be inconsistent or even contradictory across the different independent experiments. It is recommended that a statistically significant 'interaction', must be treated with care and is not necessarily biologically relevant. Searching a statistically significant interaction can be the starting point for further measurements and modeling to advance the understanding of underlying mechanisms and non-additive interactions occurring inside the organisms.

Keywords: Metal mixtures; lettuce; statistically significant; biologically relevant; reproducibility

3.1 Introduction

Industrial discharges, consumer wastes and the usage of plant protection products or sewage sludge bio-fertilizers may all lead to metal contamination in soil. Metals can be easily adsorbed in soils (Yang et al., 2009) and be accumulated in plants which may result in a threat to the health of the plant itself and consumers in the food chain. In the natural environment, plants are often exposed to multiple metals simultaneously rather than a single metal (Backhaus et al., 2000). Many metals listed individually within the safe range of industrial permits are extremely toxic to certain species and even more so when present in combination (Wong et al, 1987). Thus, to maintain healthy and functioning ecosystems, it is necessary to improve the understanding of combined effects of multiple metals on terrestrial plants.

Since current testing is cost- and time-consuming, computational models are developed to help predict toxicological responses and understand the toxicity mechanisms of mixtures. The most frequently used predictive tools for assessing mixture toxicity disregarding interactions are Concentration Addition (CA) (Loewe and Muischnek, 1926) and Independent Action (IA) (Bliss, 1939) if the constituents making up the mixture are known. The CA model is used for chemical mixtures for which a similar mode of action is assumed, whereas the IA model is used to predict effects of compounds with a different mode of action (Bliss, 1939). It has been argued that concentration addition should be a more suited default model in risk assessment of chemical mixtures because of its conservatism in most cases (Cedergreen et al., 2008). In addition, it is suggested that dissimilarly acting chemicals rarely exist in complex organisms (Faust et al., 2003). However, the sites or the modes of action are ambiguously defined at the biochemical level and can be dose dependent (Cedergreen et al., 2008). In most cases, the CA and the IA models are used only based on their mathematical connotation as the toxicity mechanisms of metals are still greatly unknown. The conceptually unrelated CA and IA models are single-time point approaches which make them suitable to make predictions for mixture effects based on standardized toxicological tests. Therefore, the CA and the IA models were both used in this research. An elaborate description of these two approaches can be found for example in the papers by Altenburger et al. (2000) and Jonker et al. (2005).

Predicting mixture effects becomes a challenge when a mixture is composed of interacting chemicals that synergize or antagonize the effects of each other. Accurately determining chemical interactions is not only conducive to adequately describing the relationship between exposure and effect, but also greatly aids risk assessments for chemical mixtures and further studies for underlying mechanisms of chemical toxicity. Synergistic interactions may cause severe effects on organisms (Johnson et al., 2013) which attract the attention of toxicology scientists and policy makers in finding synergism for naturally occurring mixtures. The reference models (i.e. CA and IA) are frequently extended to explore the presence of interaction between mixture components and to explain the variation in assessing mixture toxicity (Jonker et al., 2005; Le, 2012). Statistically significant deviations from the

predictions of reference models are usually interpreted as interactions. The strongest interactions often occur in binary mixtures and the interactive effects may become minor with an increased number of mixture components (Warne and Hawker, 1995; Lydy et al., 2004). Thereupon, experiments in this study were carried out with binary metal mixtures as a foundation for explaining joint effects of complex mixtures. The standardized framework described by Jonker et al. (2005) was applied to analyze the toxicity data of metal mixtures, a detailed description of which was given in our section 3.2.4.

By using the above approach, it is possible to assign a statistically significant deviation from the reference model (at a point in time). Nevertheless, some issues remain unresolved, for instance why the deviation occurs and how to interpret a statistically significant deviation as a toxicologically relevant interaction. It was shown that these statistically significant interactions show poor reproducibility (Cedergreen et al., 2007). Therefore, finding statistically significant interactions should be the starting point for further research on biology-related interactions but not the endpoint or the conclusion. Deviations from additivity can be caused by uncertainties in the measurements or the limited power of predictive tools instead of 'real' interactions between mixture components. Normally, the larger the sample size used, the more likely it represents a biologically relevant meaning of effects reflected in statistical significance (EFSA, 2011). Therefore, besides significance tests, a comprehensive experiment containing five independent experiments on the terrestrial plant *Lactuca sativa* L. was designed to explore the overall toxicity of Cu-Cd, Ni-Cd and Cu-Ni mixtures and to systematically examine the uncertainty of interactions between metal components.

The present study aimed at exploring whether the mixture components of Cu-Cd, Ni-Cd and Cu-Ni combinations would interact in a way (antagonism or synergism, etc.) that affected the toxicity of each other. Reproducibility of deviations from the reference models in assessing the overall toxicity was tested by repeating mixture toxicity experiments at different concentration levels or ratios. The overall toxicity was evaluated by measuring the combined effects of binary metal mixtures on root growth of lettuce, *Lactuca sativa* L.

3.2 Materials and Methods

3.2.1 Test chemicals and experimental design

In accordance with the seven heavy metals of greatest environmental concern, i.e., Cd, Cu, Cr, Hg, Pb, Ni and Zn (Han et al., 2002), Cd as a serious pollutant in the pedosphere through human activities was selected to be one of the test element and the essential elements (i.e. Ni and Cu) were chosen as the other components in the test mixtures. A comprehensive series of acute toxicity tests were designed for lettuce exposed to Ni, Cd, Cu and their binary mixtures. Metals were added into the nutrient solution in the form of nitrate salts (Sigma-Aldrich, >99%, Japan). The concentration of added NO_3^- was neglectable as compared to the nitrate concentration in the nutrient solution (68 times higher) at which no negative effect was observed on lettuce growth. In order to examine the reproducibility of interactions between mixture components at relatively low levels of input, the experiments of Ni-Cd and Cu-Ni mixtures were repeated three times across different exposure levels. To reduce the variation due to non-simultaneous toxicity tests, control groups with nutrient solution alone were conducted every week with mixture treatments in the same climate chamber. Independent experiments with different concentration ratios of Ni-Cd and Cu-Ni mixtures were separated into two groups. In the first group, the metal concentrations in the binary mixtures were set to be evenly distributed on both sides of the median effect concentrations (EC_{50}s) of each metal. The EC_{50}s for Cu, Ni, and Cd were estimated based on the results of pilot experiments for single metals. In the second group, the concentrations of Ni and Cd were slightly reduced and the concentrations of Cu were slightly increased to check if the statistically significant deviations are reproducible. Detailed spiked concentrations of Cu-Cd, Ni-Cd, and Cu-Ni mixtures are illustrated in Figure 3.1. In previous studies (Le, 2012; Liu et al., 2014a), it has been shown that the free-ion activities were the dominant metal species for the single toxicity of Cd, Cu and Ni to lettuce. Thus, taking bioavailability into account, the estimated free-ion activities were used instead of the measured total concentrations to express exposure of lettuce seedlings to metals in this study.

The pH levels of the test medium were checked using a 691 pH meter (Metrohm, Switzerland) and kept at 7.0±0.02 every other day by the addition of either HNO_3 or

NaOH. The activity of Cu^{2+} was checked using a Cu-ion selective electrode (Radiometer analytical, France). The metal concentrations in the nutrient solution for lettuce and in the test medium were determined by flame atomic absorption spectroscopy (FAAS, Perkin Elmer AAnalyst 100, US). Calibration standards (Sigma-Aldrich, Germany) and a reagent blank were analyzed after every 20 samples. Speciation calculation was conducted using the Windermere Humic Aqueous Model 7.0.1 (Centre for Ecology & Hydrology, UK) based on the measured concentrations by FAAS, the measured activities by Cu-ISE and the total concentrations calculated from a formulation of the nutrient solution (Liu et al., 2014a). As the hydroponic system was open to the ambient air, the pCO_2 was set at $10^{-3.5}$ atm. The pH value was set as 7.0 and the temperature was set as 15° C. Since the tests were conducted under controlled conditions in a laboratory nutrient solution, Al and Fe(Ⅲ) concentrations were considered negligible (Farley and Meyer, 2015) in the WHAM calculation.

3.2.2 Test organism and exposure

As recommended by the US Environmental Protection Agency (1988) and the Organization for Economic Cooperation and Development (2006), lettuce (*Lactuca sativa* L.) was selected as a bio-indicator for assessing the toxicity of metal mixtures. Seeds of lettuce were purchased from a commercial company (Horti Tops, Holland). Steiner solution, the preparation of which is shown in Supporting Information 3.1, was chosen as the nutrient solution for culturing lettuce since it has been shown to be well suited for plant growth (Steiner, 1961; Liu et al., 2014a). Seeds were germinated in a climate room at a temperature of 15°C, a humidity of 80%, a light intensity of 117 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and under a 16:8 h light: dark cycle for 4 d on expanded perlite. After germination, seedlings with taproot lengths beyond 3 cm were selected and fixed to parafilm strips floating on the surface of glass beakers (100 ml) containing the metal-spiked test medium. For each beaker, 4 seedlings were planted. All the beakers were put in a large container with a layer of water to prevent excessive evaporation. Five ml of medium of each treatment with one drop of 65% nitric acid was preserved after exposure at 4°C for chemical analysis.

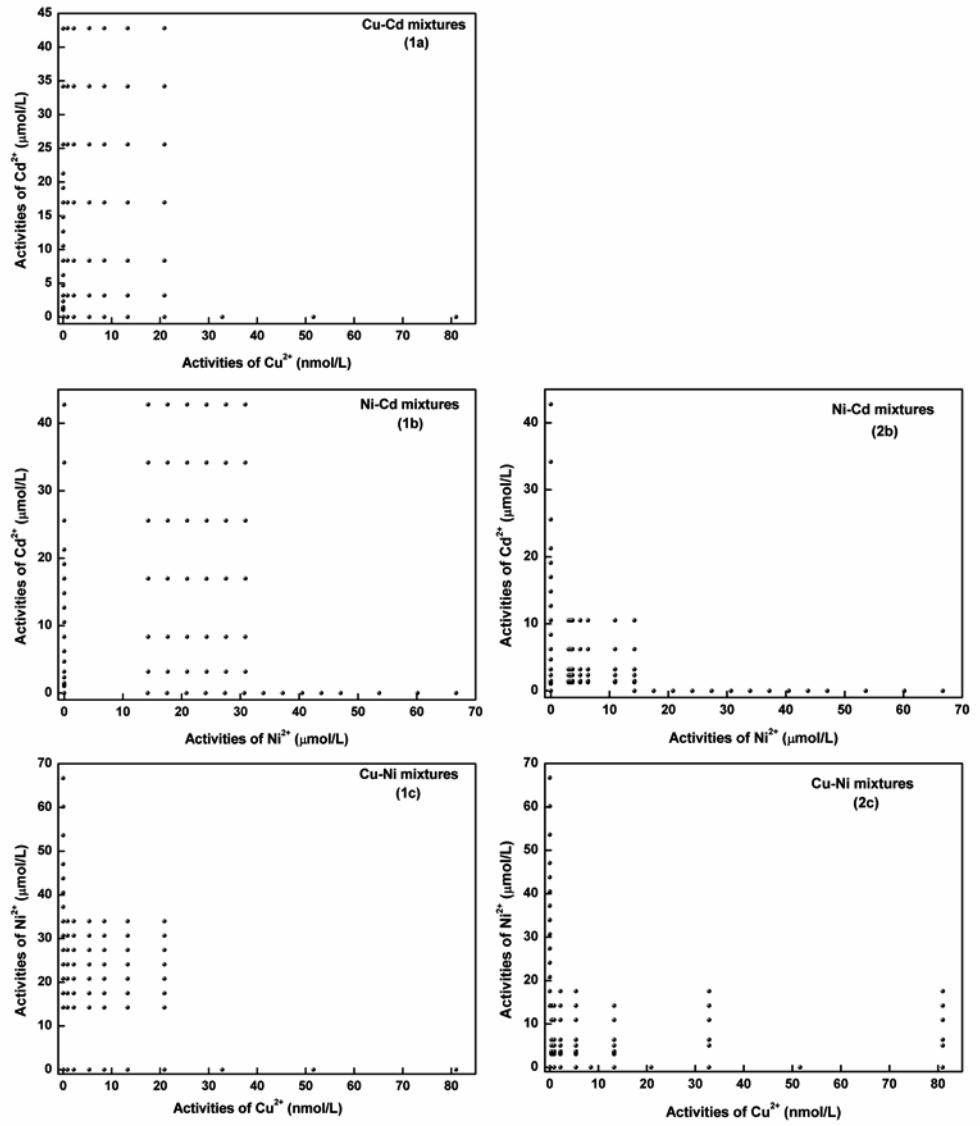

Figure 3.1 Set up of experiments for Cu-Cd, Ni-Cd, Cu-Ni mixtures expressed as free ion activities.

3.2.3 Toxicity determination

Relative root elongation (*RRE*, %) was chosen to be the toxicological endpoint of lettuce to exposures of Cu, Cd, Ni and their binary mixtures due to a relatively higher sensitivity of seedlings than seeds (Pfleeger et al., 1991) and the influence of non-simultaneous toxicity testing already considered in the formula. The length of

the root was measured before and after 4 d exposure, from the transition point between the hypocotyls and the root to the root tip (EPA, 1988). The root growth of 4 seedlings was averaged at a given treatment. The *RRE* was determined as follows

$$RRE = RG_S / RG_C \times 100\% \quad (3\text{-}1)$$

where RG_s: the averaged root growth of plants in the sample solution, cm; RG_c: the averaged root growth of plants in the control solution, cm.

3.2.4 Data analysis

To analyze the combined effects of Cu-Cd, Ni-Cd and Cu-Ni mixtures, two standard 'additivity' models were used as the reference model, i.e. concentration addition (CA) and independent action (IA). On the basis of CA concept, the relative contributions of mixture components to the overall toxicity can be added in the form of toxic units (TUs) (Jonker et al., 2005) as represented in equation (3-2). Strict concentration addition occurs when the toxic unit value of a mixture (TU_{mix}) equals one.

$$TU_{mix} = \sum_{i=1}^{n} c_i / EC x_i \quad (3\text{-}2)$$

where c_i: the concentration of individual chemical *i* in the mixture with *n* chemicals, free-ion activity was used to express c_i; $EC x_i$: the effect concentration of individual chemical *i* that results in the same effect (*x*%) as the mixture, free-ion activity was used to express $EC x_i$; TU_{mix}: a dimensionless ratio, the sum of each quotient or toxic unit (TU).

Based on the concept of independent action, the dose-response relationship of metal mixtures can be expressed as equation (3-3) by multiplying the non-response of each component in the mixture at a given exposure concentration:

$$Y = u_{max} \prod_{i=1}^{n} q_i(c_i) \quad (3\text{-}3)$$

where *Y*: the biological response; u_{max}: the maximum biological response; $q_i(c_i)$: the probability of non-response of individual chemical *i* in the mixture with *n* chemicals.

The deviation patterns of Cu-Cd (1a), Ni-Cd (1b) and Cu-Ni (1c) mixtures from 'additivity' were quantified by the freely downloadable software named the MixTox Model, provided by the Centre for Ecology & Hydrology (CEH). The programming

was conducted by the use of Visual Basic functions and the Solver program in Microsoft Excel. The reference models and the deviation functions (synergism/antagonism, dose ratio-dependent, and dose level-dependent) were all fitted to the toxicity data of Cu-Cd, Ni-Cd and Cu-Ni mixtures using the maximum likelihood method while minimizing the sum of squared residuals (*SS*). The median effect concentration (EC_{50}) and the slope of dose-response relationships (β) of single metals were calculated in an excel spreadsheet using the log-logistic function provided by CEH as well and used as initial values for mixture toxicity modelling. The statistical significance of the improved model-fit from additional parameters was quantified by the chi-square (χ^2) tests. In this study, a value of $p(\chi^2)$ lower than 5% was considered to indicate a statistically significance. Four types of deviation patterns were classified using the MixTox model, i.e. strict 'additivity' basically no deviations from the reference models (CA or IA), synergism/ antagonism (S/A) deviation, dose ratio-dependent (DR) deviation, dose level-dependent (DL) deviation. Since the deviation models of DR and DL were not nested, the comparison between these two models was not achieved using the chi-square (χ^2) tests. A detailed description of these mixture models is shown in the Supporting Information 3.2. The 2b and the 2c subsets of Ni-Cd and Cu-Ni mixtures were also entered into the MixTox model to check whether the statistically significant deviations from 'additivity' were reproducible.

3.3 Results

3.3.1 Background chemical analysis

The concentrations of Mg, Ca, K, Na and Zn in the Steiner solution were measured to be 1.67 ± 0.02 mM, 2.10 ± 0.02 mM, 5.66 ± 0.06 mM, 1.25 ± 0.02 mM, and 0.002 ± 0.0002 mM (n=16) respectively by FAAS.

3.3.2 Mixture toxicity modeling

The toxicity data of Cu, Ni, Cd and their three binary mixtures are shown in Table S3.1 which also includes dose-response curves of individual metals and relationships between observed effects and estimated effects of metal mixtures analyzed using the MixTox model. The fitting results of various mixture models are

demonstrated in Table 3.1 for all the datasets. The values of R^2 are shown to describe the goodness of fit of the reference models and the nested deviation functions in the MixTox model. The values of p (χ^2) are shown to indicate the statistically significant level.

Generally, the combined effects of Cu-Cd, Ni-Cd and Cu-Ni mixtures to *L. sativa* were equally well explained by the CA- and the IA-based mixture models. The predictive ability of the mixture models differed when assessing the toxicity of different datasets of Ni-Cd mixtures. Fitting of the CA- or the IA-based models to the first dataset of Ni-Cd mixtures explained 72%-80% (Table 3.1) of the variation in observed effects on lettuce. However, at the lower concentrations of Ni and Cd (Figure 3.1) in the second dataset, only 47%-60% (Table 3.1) of the variation was explained by mixture models. Similar results were not observed in the Cu-Ni combination. The predictive power of reference models and deviation functions was shown to be similar in predicting the combined effects of Cu-Ni mixtures on root elongation. This indicated that the predictive power of mixture models may be dependent on the specific composition of metal mixtures.

The improvement in fitting by the additional parameters was found to be dependent on the specific subset of metal mixtures and reference model applied. For the Cu-Cd mixtures, a statistically significant better fit was obtained when parameters related to DR or DL dependent deviations were included in the CA- and the IA-based models to describe the mixture toxicity. Although inclusion of the S/A parameter in the CA model showed a statistically significant better fit to the first dataset of Ni-Cd mixtures, adding DR parameters into the IA model improved the data description significantly at the 5% level. For the second dataset of Ni-Cd mixtures, significantly better fits were obtained after extending the CA and the IA models with DR deviation parameters. Extending the IA model with additional parameters did not decrease the residuals significantly for modeling the combined effects of Cu-Ni mixtures in the first experiment, which was different from the results obtained by the CA-based models. For the second dataset of Cu-Ni mixtures, the DR or DL parameters added in the CA- or the IA-based models significantly improved the model fit for mixture toxicity.

3.3.3 Determination of deviation patterns

Based on the MixTox model, the statistically significant deviations from 'additivity' are represented in Table 3.2 and in Figure 3.2 for each dataset of Cu-Cd, Ni-Cd and Cu-Ni mixtures. Deviations from the reference model were generally found in predicting the overall toxicity of Cu-Cd and Ni-Cd mixtures to lettuce apart from the first dataset of Cu-Ni mixtures. The significant deviations for each metal combination investigated in this study were found to be dependent on concentration levels or ratios of metals and not consistent across different reference models. Especially for the 1c dataset of Cu-Ni mixtures, no deviation from 'additivity' was observed using the IA-based functions for toxicity modeling. This was different from the statistically significant dose ratio- or the dose level-dependent deviations found by the CA-based approaches. For the 1b and the 2b datasets of Ni-Cd mixtures, it was demonstrated that patterns shifted between antagonism and dose ratio-dependent deviation in modeling the toxicity of Ni-Cd mixtures. For the 1a and the 2c datasets, dose level-dependent deviations were obtained with CA as the reference model and dose ratio-dependent deviations when IA was used as the reference model.

Moreover, the joint action of metal mixtures (Table 3.2) was determined according to the additional parameters estimated by the MixTox model (Table S3.2). Antagonistic effects were commonly found for the first datasets of Cu-Cd, Ni-Cd and Cu-Ni mixtures at lower dose levels and synergistic effects occurred at higher dose levels. However, for the second datasets of Ni-Cd and Cu-Ni mixtures, the joint action was contradictory to the first datasets or when different reference models were applied. The joint actions of Ni-Cd and Cu-Ni mixtures changed from antagonism in the first experiment to synergism in the second experiment. This indicated that interactions between mixture components were not reproducible. Synergistic effects between Ni^{2+} and Cd^{2+} were found where the mixture toxicity is mainly caused by Ni^{2+} using the IA-based models which was in contrast to the results of the CA-based models. Similar results were also obtained for the 2c dataset that synergism occurred between Cu^{2+} and Ni^{2+} at low dose levels using the CA-based models for assessing the mixture toxicity whereas antagonism found by the IA-based models. Although the experiments of mixture toxicity were repeated, the specific interactions between

mixture components could not be defined as the joint actions found by the MixTox model were different for diverse reference models or datasets selected.

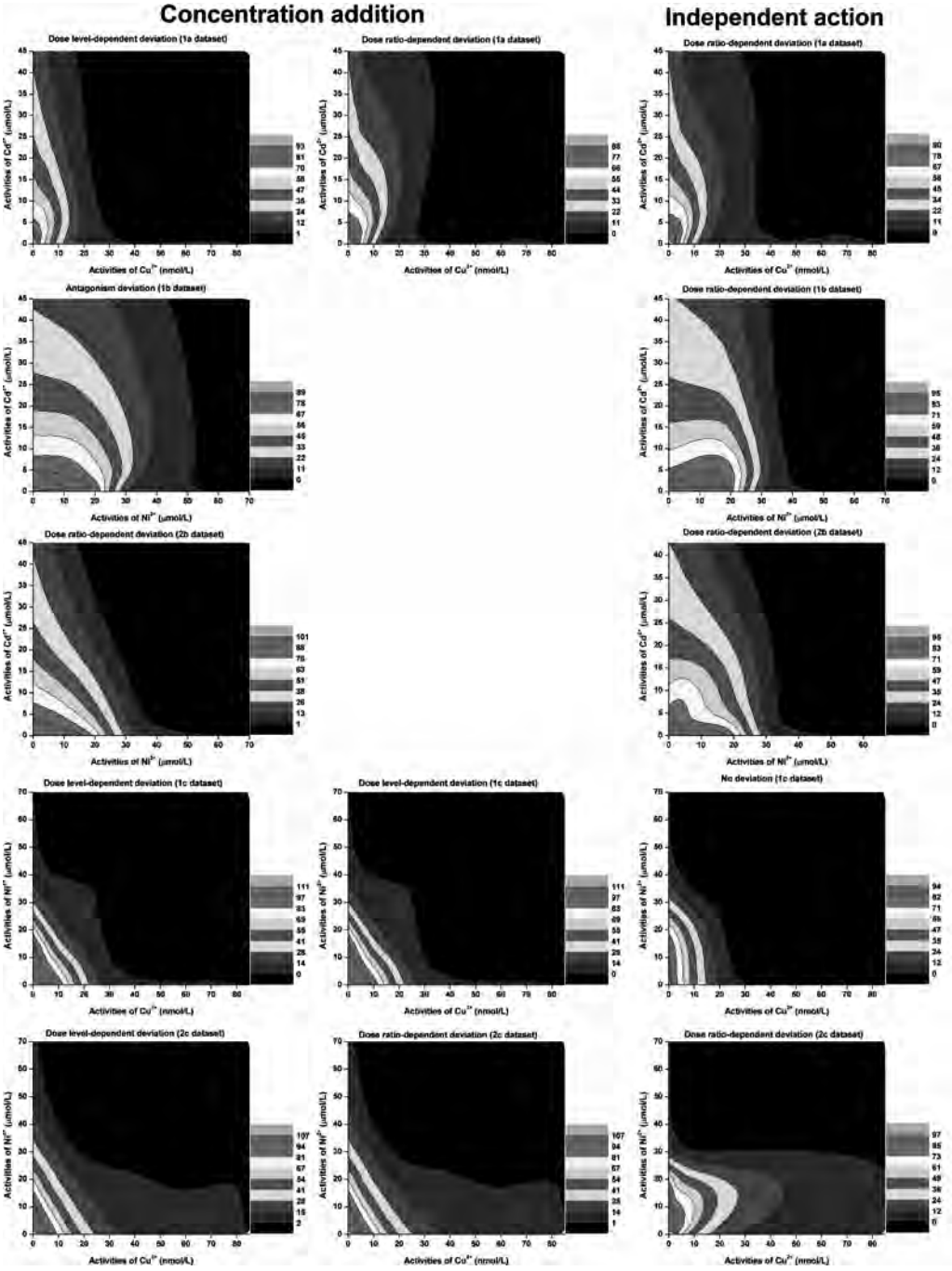

Figure 3.2 2D isobolic representations of the response surfaces fitted by the

statistically significant, most likely deviation models based on concepts of concentration addition (two rows on the left) and independent action (one row on the right) to describe the effects of mixtures of Cu-Cd, Ni-Cd and Cu-Ni on root elongation of *L. sativa*. The different colors indicate diverse response levels. The bigger the number in the addendum, the higher the root elongation rate.

3.4 Discussion

Chemical-chemical interactions occur at various processes which complicates the toxicity assessment for metal mixtures. First, at the environmental level outside the organism, metals can interact with the substances existing in the surrounding media which may affect their bioavailability. Secondly, interactions between metals at the toxicokinetic phase would influence the uptake of mixtures by organisms. Thirdly, interactions that occur at the toxicodynamic phase may influence the accumulation of metals at the biotic ligands, and subsequently affect joint toxicity of metal mixtures (Calamari and Alabaster, 1980). In our study, estimated bioavailable fractions of metals were used in mixture modelling, in which interactions of metals with environmental compartments were preliminarily addressed by the WHAM software. The potential interactive effects found between metal ions more likely occurred at the chemical-organism level.

According to the results shown in this study, deviations from 'additivity' always occurred in assessing the overall toxicity of binary metal mixtures regardless of the reference models applied. However, the statistically significant deviations patterns were found to be not reproducible across the whole dataset for each metal combination which was consistent with the findings of Cedergreen et al. (2007). These inconsistent deviations may be the result of over-simplifications of the model, of the model itself as applied to judge interactions, and of experimental errors. Although the MixTox model was a powerful tool in finding statistically significant deviations, the improvements in fit by adding parameters were rather small in our case. For instance, apart from Cu-Ni mixtures, the goodness of fit in terms of R^2 was increased by less than 10% when the S/A, DR or DL deviation parameters were added into the reference models. On the one hand, as the toxicological response of lettuce in exposure to binary metal mixtures was translated to the integrated

endpoint *RRE* (%), the variations of root growth at a small scale (e.g. mm) which fell well within the range of experimental uncertainty, would lead to the difference between a synergistic effect and an antagonistic effect. On the other hand, the sensitivity of this tool may be improper to distinguish interactions from deviations in our case. Fisher (1957) has suggested that a level of significance (e.g. α=0.05) could be set according to specific circumstances. A more stringent alpha level (e.g. α=0.01) may help avoid testing variability and raise the power of determining interactions in metal mixtures.

Additionally, most of these significant deviations were found to be dependent on dose ratios of Cu-Cd, Ni-Cd and Cu-Ni mixtures, which is similar to the findings of Sharma et al. (1999). This may be the cause of the different and even opposite deviation patterns in the second datasets as compared to the results of the first datasets of Ni-Cd and Cu-Ni mixtures. It is thus good to note that the use of a fixed concentration ratio for experiments may bias the interpretation of interactions between mixture components in assessing the total toxicity (Drescher and Boedeker, 1995). Although a comprehensive series of acute toxicity experiments can have a degree of replication (Tipping and Lofts, 2015), real experimental duplicates are still needed (Cedergreen et al., 2007) to systematically examine the effects of interactions on mixture toxicity assessment. These findings emphasized the importance of intensive and confirmatory experiments in analyzing mixture toxicity, as the 'noise' in the experimental toxicity data can be easily interpreted as interactions by statistical tools such as the MixTox model. However, blindly enlarging the sample size was also not recommended since a statistical test will always demonstrate a significant difference for a huge sample size (Sullivan and Feinn, 2012). Since the patterns of statistically significant deviations were not reproducible, scientists should take care in deriving any conclusions associated with interactions and the strength of interactive effects based on the statistical significance alone.

Some researches already reported that interactions occurred in metal mixtures involving Cu, Zn, and Cd. Versieren et al. (2014) found that 74% of the interactions between Cu^{2+} and Zn^{2+} could be explained by the biotic ligand model based on a partial factorial and ray design (21 points repeated 3 times) for *Hordeum vulgare* L.

and they postulated that synergistic effects would occur for soil grown plants exposed to this mixture. The study of Le (2012) on *Lactuca sativa* indicated that significant alleviative effects of Zn^{2+} were found on the toxicity of Cu^{2+} based on a single dataset with 122 points as input in the extended CA and IA models. Sharma et al. (1999) found complex interactive effects depending on concentrations between components of Cu-Zn, Cu-Cd and Zn-Cd mixtures through more than 10 times repeated root elongation tests and accumulation tests on *Silene vulgaris*. Tipping and Lofts (2015) showed that the toxicity of Cd to *Daphnia magna* (542 data points), *Oncorhynchus clarkia lewisi* (162 data points), and *Oncorhynchus mykiss* (207 data points) could be markedly reduced by Cu and Zn according to the WHAM-F_{TOX} model. Although many replications or near-replications were conducted in the studies listed above, Tipping and Lofts (2015) pointed out the difficulty in obtaining reproducible results of toxicity experiments. Improvements in measurements and modeling are still needed before confidently accepting and applying conclusions concerning toxicologically relevant interactions. Better methods to advance the understanding of mechanism may assist in evaluating non-additive deviations or interactions between metals.

Moreover, it is not possible to make a distinction between the CA- and the IA-based models as both models performed equally well in assessing the overall toxicity of Cu-Cd, Ni-Cd and Cu-Ni mixtures. This finding was in line with the result of Syberg et al. (2008) on dimethoate, pirimicarb and linear alkyl benzene sulfonate. Cedergreen et al. (2008) also proposed that on the basis of predictive accuracy alone, neither of the CA and IA models was significantly better than the other. The similar results of the CA- and the IA-based model predictions are likely to be caused by the slopes of the log-logit response curves being approximately equal to 1.0 (Farley and Meyer, 2015) especially for the single metal exposures of Cu and Cd. As the MixTox model is developed based on the isobologram approach, the CA isoboles are difficult to be distinguished from the corresponding IA isoboles when the slope parameters of log-logit curves are around 1.0 (Drescher and Boedeker, 1995). Until now, the comparison for a superior model in describing the joint effects of a given mixture mostly relies on experience as the knowledge of mechanism is still lacking (Jonker et al., 2005) especially for metals. Unlike organic pollutants,

metals are difficult to be classified based on their mode or mechanism of action due to organism-specific characteristics (Liu et al., 2014b). For example, the effects induced by Cu^{2+} may occur in the form of cellular destabilization via metal substitution reactions within *Patracentrotus lividus* (Manzo et al., 2010). Both Cu and Ni were found to influence the ionic balance of *Gammarus pulex* L. (Charles et al., 2014). In addition, Cu was also reported to interfere with the photosynthesis process in algae (Stauber and Florence, 1987). In contrast, Cd was always found to bind to the apoplastic and the symplastic and to block cell division by disrupting active components in *Triticum aestivum* (Lu et al., 2013). Terrestrial plants are in general complex organisms that may have multiple target sites (Zwart and Posthuma, 2005; Syberg et al., 2008). It is possible that metals within the higher plants like lettuce have primary and secondary modes of action (Manzo et al., 2010) and consequently influence the toxicity of each other in the mixture through distinct subsystems. Thus, without correct assignment, the mode of action may not be used as the sole tool for selecting the likely best model to predict the toxicity of metal mixtures. Similarly, an observed deviation cannot be exclusively assigned to a specific model. We suggested that the CA- and IA-based models can be used just as a representation of mathematical relationships between metal mixtures and their biological responses other than the indication of underlying mechanisms. Although the CA model was found to produce a relatively better prediction of mixture toxicity even for compounds with different modes of action (Faust et al., 2003; Zwart and Posthuma, 2005), it is recommended in the research of Bödeker et al. (1992) and in this study to use the range of expected responses predicted by both the CA and IA models for environmental quality regulations and to use both concepts instead of selecting one of them based on uncertain mechanisms of toxicity to assess the combined effects of metal mixtures.

3.5 Conclusions

The MixTox model was proven to be a very sensitive tool to define statistically significant deviations from ‘additivity’ in assessing the combined effects of binary metal mixtures. However, the replicated mixture experiments showed that the assessment of deviations strongly depended on the fitting of experimental data, the predictive methods applied and the specific range of exposure concentrations.

Therefore, it was concluded that the statistically significant deviations did not directly indicate the biological relevance of interactions. Instead of actually occurring interactions between metals, other factors discussed in this study would also result in statistically significant deviations in modeling mixture toxicity based on the CA and the IA reference models. Unless the underlying mechanism is clearly determined, the two reference models are suggested to be used as mathematical relationships for metal mixtures. To avoid the model development and the interaction investigation for mixture toxicity of metals becoming more like a data-fitting exercise and a consequence of experimental design, further studies should be focused on identifying the underlying mechanisms of metal mixtures. Instead of the endpoint of research, finding a statistically significant deviation can be the starting point of further mechanistic research concerning toxicologically relevant interactions.

Table 3.1 Fitting results of the toxicity of Cu-Cd, Ni-Cd, and Cu-Ni mixtures by the MixTox model.

Dataset	Parameter	The CA-based models				The IA-based models			
		CA	S/A	DR	DL	IA	S/A	DR	DL
1a Cu-Cd	R^2	0.81	0.82	0.84	0.84	0.82	0.82	0.85	0.83
	$p(\chi^2)$ CA or IA vs.	-	0.01*	<0.0001*	0.0003*	-	0.18	0.0003*	0.08
	S/A vs.	-	-	0.0004*	0.002*	-	-	0.0001*	0.07
1b Ni-Cd	R^2	0.72	0.77	0.77	0.78	0.79	0.79	0.80	0.80
	$p(\chi^2)$ CA or IA vs.	-	<0.0001*	<0.0001*	<0.0001*	-	0.30	0.03*	0.11
	S/A vs.	-	-	0.68	0.05	-	-	0.02*	0.07
1c Cu-Ni	R^2	0.49	0.53	0.55	0.55	0.85	0.86	0.86	0.86
	$p(\chi^2)$ CA or IA vs.	-	0.01*	0.004*	0.004*	-	0.07	0.07	0.18
	S/A vs.	-	-	0.03*	0.03*	-	-	0.16	0.68
2b Ni-Cd	R^2	0.47	0.50	0.53	0.51	0.58	0.58	0.60	0.58
	$p(\chi^2)$ CA or IA vs.	-	0.0009*	<0.0001*	0.0007*	-	0.78	0.01*	0.72
	S/A vs.	-	-	0.001*	0.07	-	-	0.003*	0.45
2c Cu-Ni	R^2	0.63	0.64	0.67	0.68	0.83	0.84	0.86	0.84
	$p(\chi^2)$ CA or IA vs.	-	0.09	0.001*	0.0005*	-	0.02*	<0.0001*	0.01*
	S/A vs.	-	-	0.001*	0.0004*	-	-	0.0002*	0.07

1a: the dataset of Cu-Cd mixtures; 1b: the dataset of Ni-Cd mixtures; 1c: the dataset of Cu-Ni mixtures; 2b: the second dataset of Ni-Cd mixtures; 2c: the second dataset of Cu-Ni mixtures; R^2: the coefficient of determination; $p(\chi^2)$: the outcome of the likelihood ratio test; *: significant at the 5% significance level; CA: the concentration addition model; IA: the independent addition model; S/A: the synergism/antagonism model; DR: the dose ratio-dependent model; DL: the dose level-dependent model; vs.: versus, which was used to show comparison between two models; -: not applicable.

Table 3.2 Deviation patterns determined by the significantly most likely deviation models for each dataset of Cu-Cd, Ni-Cd, and Cu-Ni mixtures.

Dataset number	The CA-based models	The IA-based models
1a Cu-Cd	DR (antagonism when the toxicity is mainly caused by Cu) or DL (antagonism at low dose level and synergism at high dose level changed at dose level higher than EC_{50})	DR (antagonism when the toxicity is mainly caused by Cu)
1b Ni-Cd	Antagonism	DR (antagonism when the toxicity is mainly caused by Ni)
1c Cu-Ni	DR (antagonism) or DL (antagonism at low dose level and synergism at high dose level)	IA (No deviation)
2b Ni-Cd	DR (antagonism when the toxicity is mainly caused by Ni)	DR (synergism when the toxicity is mainly caused by Ni)
2c Cu-Ni	DR (synergism when the toxicity is mainly caused by Cu) or DL (synergism at low dose level and antagonism at high dose level changed at dose level higher than EC_{50})	DR (antagonism when the toxicity is mainly caused by Cu)

1a: the dataset of Cu-Cd mixtures; 1b: the dataset of Ni-Cd mixtures; 1c: the dataset of Cu-Ni mixtures; 2b: the second dataset of Ni-Cd mixtures; 2c: the second dataset of Cu-Ni mixtures; CA or IA: concentration addition or independent action; S/A: synergism/antagonism; DR: dose ratio-dependent deviation from additivity; DL: dose level-dependent deviation from additivity.

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Supporting Information

S3.1 Formula of the Steiner solution (see Supplementary Materials 2.1)

S3.2 Additional description for the mixture models

As the statistical software we applied is established by the Centre for Ecology & Hydrology (CEH), we only roughly introduce the theoretical basis and the basic algorithm in the additional description for the mixture models. For more details of this predictive tool, please see the article of Jonker et al. (2005) or visit the following website http://www.ceh.ac.uk/products/stats/mixturetoxicity-analysistools.html. A deviation function (*G*) is added in the equations (3-2) and (3-3) shown in the body text to quantify the degree of deviations from additivity in the supporting information. Where *G*=0 (exp(*G*)=1), the actual effect of the mixture is adequately described by either concentration addition or independent action (CA or IA,) the 2D isobolic representations of which are shown as straight lines (or linear relationships). To quantify the deviations from concentration addition (CA), equation (3-2) in the manuscript can be rewritten as follows

$$\sum_{i=1}^{n} c_i / f_i^{-1}(Y) = \exp(G) \qquad \text{(S3-1)}$$

For independent action (IA), the dose-response relationship can be calculated by multiplying the probabilities of nonresponse or response

$$Y = u_{\max}\Phi\{\Phi^{-1}[\prod_{i=1}^{n} q_i(c_i)] + G\} = u_{\max}\Phi[\Phi^{-1}(P_{1,\ldots,n}) - G] \qquad \text{(S3-2)}$$

where c_i : the concentration of individual chemical *i* in the mixture with *n* chemicals; *Y*: the biological response; $u_{\max}$: the maximum biological response; $q_i(c_i)$: the probability of non-response of individual chemical *i* in the mixture with *n* chemicals; Φ: the standard cumulative normal distribution function; $P_{1,\ldots,n}$: probability of response.

Since the toxicity of each component in a mixture may differ a lot, the deviation functions should depend on each component's relative contribution to the combined toxicity instead of their actual concentrations. The relative amount of toxic units (TU) of each chemical component *i* in a mixture can be defined as follows

$$z_i = \mathrm{TU}x_i / \sum_{j=1}^{n} \mathrm{TU}x_j \quad \text{(S3-3)}$$

where $\mathrm{TU}x_i = c_i / \mathrm{EC}x_i$ (S3-4)

The following deviation functions are substituted in equation (S3-1) or (S3-2) for describing diverse deviation patterns. For synergism or antagonism (S/A), the deviation function can be described as

$$G(z_1,...,z_n) = a\prod_{i=1}^{n} z_i \quad \text{(S3-5)}$$

The deviation function describes antagonism when parameter *a* is positive and synergism when *a* is negative. The lines of 2D isobolic representations would become convex toward the high concentrations for antagonism, and be downward concave for synergism. For the binary mixtures investigated in this study, the equation (S3-5) can be made dose ratio-dependent (DR) by adding another parameter *b*. The overall antagonistic or synergistic deviation changes with chemical 1, where b_1 determines the magnitude of change.

$$G(z_1,z_2) = (a + b_1 z_1) z_1 z_2 \quad \text{(S3-6)}$$

The deviation function describes antagonism when parameter *a* or *b* is positive and synergism when *a* or *b* is negative. Antagonism can be observed where the toxicity of the mixture is caused mainly by chemical 1, whereas synergism can be observed where the toxicity is caused mainly by chemical 2. To describe synergism and antagonism depending on the dose level (DL), the equation (S3-5) is extended by including quantified isoboles. As the 50% effect concentration (EC_{50}) can be estimated with the least amount of variability, the deviation function is defined as follows for concentration addition by incorporating the EC_{50} isobole

$$G(z_1,...,z_n) = a(1- b_{\mathrm{DL}} \sum_{i=1}^{n} \mathrm{TU50}_i)\prod_{i=1}^{n} z_i \quad \text{(S3-7)}$$

For independent action, the EC_{50} isobole is defined by $P_{1,...,n}$=0.5 and the function can be written as

$$G(z_1,...,z_n) = a(1- b_{\mathrm{DL}} P_{1,...,n})\prod_{i=1}^{n} z_i \quad \text{(S3-8)}$$

The function for dose-level dependent deviation describes antagonism when parameter *a* is positive and synergism when *a* is negative. The detailed interpretation of additional parameters can be found in Table 1 of the paper of Jonker et al. (2005). The function mentioned in equation (S3-1) and (S3-2) is the log-logistic dose-response model.

$$h_i(c_i) = 100 / [1 + (c_i / \mathrm{EC50}_i)^{\beta i})] \quad \text{(S3-9)}$$

where $h(c_i)$: a cumulative distribution function, functionally related to concentration *c* of compound *i*; β_i: the slope parameter.

These models are all fitted to the dataset using the method of maximum likelihood or minimizing the sum of squared residuals (*SS*). The parameters that most significantly improve the model fit are then left in the model. The model fit is always improved if a reference model is extended with additional parameters. To test the significance of improvements, the difference in *SS* can be used for a pairwise model comparison through the likelihood ratio test at degrees of freedom (the difference in the number of parameters in two models) which is always referred to a chi-square test or a χ^2 test. Since the equation (S3-6) and the equation (S3-7) or (S3-8) are not nested, the CA or IA model is first compared with the S/A, DR, and DL extended models respectively, and then the S/A model is compared with the DR and the DL models. If the $p\ \chi^2$ value is lower than conventional criteria for statistical significance (0.05), the difference in *SS* between two models is supposed to be significant which also indicates a statistically significant deviation from additivity.

S3.3 Table S3.1 Raw data (not shown in this Ph.D. thesis)

S3.4 Table S3.2 Estimates of additional parameters using the MixTox model for Cu-Cd, Ni-Cd and Cu-Ni mixtures

Dataset number	Additional parameter	The CA-based models			The IA-based models		
		S/A	DR	DL	S/A	DR	DL
1a Cu-Cd	*a*	1.43	-2.61	5.85	0.91	-3.54	5.09
	b_{Cu}	-	7.97	-	-	9.92	-
	b_{DL}	-	-	0.37	-	-	1.19
1b Ni-Cd	*a*	1.63	1.25	3.26	0.59	-2.30	4.13
	b_{Ni}	-	0.59	-	-	6.60	-
	b_{DL}	-	-	0.30	-	-	1.31
2b Ni-Cd	*a*	-0.64	0.13	-1.34	-0.16	2.64	-1.17
	b_{Ni}	-	0.36	-	-	-7.41	-
	b_{DL}	-	-	0.90	-	-	2.61
1c Cu-Ni	*a*	0.36	0.031	0.033	1.28	3.30	1.97
	b_{Cu}	-	-0.00032	-	-	-4.44	-
	b_{DL}	-	-	-0.0002	-	-	0.52
2c Cu-Ni	*a*	-0.28	-0.13	-0.01	1.56	-5.30	0.004
	b_{Cu}	-	-0.05	-	-	11.31	-
	b_{DL}	-	-	0.0004	-	-	-990.05

1a: the dataset of Cu-Cd mixtures; 1b: the dataset of Ni-Cd mixtures; 1c: the dataset of Cu-Ni mixtures; 2b: the second dataset of Ni-Cd mixtures; 2c: the second dataset of Cu-Ni mixtures; CA or IA: concentration addition or independent action; S/A: the synergism/antagonism model; DR: the dose ratio-dependent model; DL: the dose level-dependent model; -: not applicabl.