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

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# Chapter 1

## General Introduction

## 1.1 Metals and their effects

### *1.1.1 Metal resources*

Minerals on the planet are widely present in oceans and in the crust of the earth. Due to their specific properties, minerals bind tightly to the crust, which causes lower concentrations of metals close to the Earth's surface. Although at such lower concentrations, a few metals play a crucial role in the proper functioning of living organisms on earth. These metals are involved in various biological processes that sustain the life of organisms and are therefore called essential elements. For example, calcium, magnesium and potassium are defined as major elements or macronutrients since they are needed in a great amount within most plants and animals. In addition, the growth and the metabolism activities of organisms are inseparable from the presence of trace elements or micronutrients e.g., copper, iron, manganese, molybdenum, nickel and zinc, which are required in a small amount (Yruela I, 2013). Generally, the essential elements can be replenished through uptake from the soil and water by the plant roots. On the one hand, a lack of any one or at a very low supply can lead to nutrient deficiency and subsequently result in early mortality due to the lack of vitality. On the other hand, an excess of trace metals or nonessential metals may result in adverse effects, toxicity or even death of organisms.

### *1.1.2 Metal contamination*

With the progress of human civilization over recent centuries, metals have become concentrated on the Earth's surface mainly by mining, smelting and industrial products. Although several adverse effects of metals have been known for a long time, exposure to heavy metals continues, and is even increasing in particular in less developed countries (Järup, 2003). Organisms on the earth can therefore be exposed to metals at elevated concentrations. Furthermore, metals in soil are difficult to clean up (Tangahu et al., 2011) which makes their threats long-term persisting in the terrestrial ecosystems. Plant growth, ground cover and soil micro-flora have been known to be affected by metal exposure (McLaughlin, 2001; Roy et al., 2005). Crops grown in contaminated land may accumulate a range of metals in their harvestable parts. Ingestion of those contaminated plant- or animal-based foods (Radojevic and Bashkin, 1999) and skin contacts (Qu et al., 2012) are two main possibilities for metals to enter the human body. Metals cannot be degraded or destroyed (Pezzarossa et al., 2011) that once absorbed by organism, remain residents over decades. Their increasing cumulative amounts may therefore cause disorders and diseases to humans. The most typical case was the itai-itai disease that occurred in Japan first starting around 1912. Due to mining, large quantities of cadmium and other metals were discharged into the Jinzū River which was mainly used for irrigation of rice fields and washing. Long-term intake of cadmium-contaminated rice leads to toxic effects on kidney (renal disfunction) and bones (osteomalacia, osteoporosis) among itai-itai victims (Järup, 2003). This horrible event made people aware of the seriousness of metal contamination.

## 1.2 Metal-based nanoparticles and their effects

### *1.2.1 Metal-based nanoparticles*

With the fast growth of the world population and of urbanized societies, how to fulfill the rising demand for metal supplies will become a pressing problem in the future. Engineered metal-based nanoparticles (NPs) with their specific characteristics may provide a solution to raise metal-recycling rates and therefore address resource scarcity and mitigate environment impacts. Nanoparticles are often defined as microscopic particles with at least one dimension between 1 and 100 nanometers in size (Lin and Xing, 2007). Besides size, other physicochemical properties of metal-based NPs such as magnetism, electrical and optical properties (Schrand et al., 2010), surface area, reactivity and sensitivity (Lin and Xing, 2007) can also be altered according to corresponding requirements which make them different from conventional larger sized materials. Due to those unique characteristics, diverse engineered metal-based NPs appear in industrial products, consumer and household commodities (Chang et al., 2012). Zinc oxide (ZnO) and copper oxide (CuO) are two typical representatives of metal-based nanoparticles. ZnO NPs have been manufactured to be a highly reactive catalyst in automobile exhaust treatment (Colvin, 2003). Because of their strong absorption abilities, ZnO NPs are also widely applied as UV-absorbers in cosmetics and modern sunscreens (Chang et al., 2012). As CuO NPs can improve fluid viscosity and thermal conductivity (Chang et al., 2012), they are used amongst others in gas sensors (Chowdhuri et al., 2004), catalysis (Jammi et al., 2009), batteries (Zhang et al., 2005), high temperature superconductors (Dar et al., 2008), solar energy conversion (Yin et al., 2005) and field emission emitters (Dar et al., 2008). In addition, due to the high surface areas and unusual crystal morphologies, CuO NPs were found to inhibit the microbial activity of *Escherichia coli* strains (Pan et al., 2010) that may guide CuO NPs to be for instance specific antibacterial agents in the future (Stoimenov et al., 2002).

### *1.2.2 Contamination due to engineered metal-based nanoparticles*

Large numbers of applications induce direct or indirect environmental release of engineered metal-based NPs from the manufacture and processing industries. The ability of cells and bacteria to absorb nano-sized particles provides the possibility of bio-accumulation of metal-based NPs in the food chain (Biswass and Wu, 2005) and therefore may pose hazards to humans and ecosystems. Recent studies have reported that metal-based NPs can interact with proteins or enzymes of mammalian cells and generate reactive oxygen species (ROS) and oxidative stresses to humans and rodents (Brunner et al., 2006; Soto et al., 2006; Schrand et al., 2010). Engineered nanoparticles can also end up in terrestrial plants through direct application (e.g. fertilizers), accidental release, contaminated soil/sediments, or atmospheric fallouts (Rico et al., 2011). It has been found that many metal-based NPs can exert toxic effects on seedlings and seeds of crops such as rape, radish, lettuce, corn, ryegrass, cucumber, mung bean, and wheat (Lin and Xing, 2007; Lee et al., 2008; Barrena et al., 2009). However, these studies primarily focus on observational toxicity testing with little knowledge or insights in the underlying pathways of toxicity. Innovative methods and technologies are needed to advance the understanding of phytotoxicity and underlying mechanisms of toxicity of metallic NPs to higher plants (Savolainen et al., 2013).

## 1.3 Bioavailability

### *1.3.1 Metal exposure, uptake and effect*

The concept of bioavailability is used to express the fraction of a chemical that can be available for uptake by organisms in specific environmental compartments (Meyer, 2002). Bioavailability of metals has been considered to be a three-step approach (Dickson et al., 1994; Peijnenburg et al., 2007) including exposure, uptake and effect. Interactions between metals and various environmental compartments would affect actual exposure of metals. In aquatic systems, the complexation of metal ions can be highly affected by natural organic matter, hardness and alkalinity (Van Gestel et al., 2010). Exposure and uptake of metals in the soils are influenced by abiotic factors such as metal and water content, pH, and oxidation-reduction potential (Eh) (Yang et al., 2005) and by biotic factors including soil engineering bacteria and mycorrhizal fungi which can interact with plants and excrete enzymes or organic compounds to change the mobility of metals in soil (Salt et al., 1995). In the process of plant growth, protons and organic acids secreted from roots which can also acidify the rhizosphere, increase the metal solubility and finally influence metal absorption of plants (Bernal et al., 1994; Krishnamurti et al., 1997; Yang et al., 2005).

The exposure stage may play an important role in subsequent metal uptake rates on the membrane or cell wall of organisms (Wang and Rainbow, 2005). Mechanisms of metal uptake in plants may involve processes of passive diffusion, facilitated transport, active transport and endocytosis (McLaughlin, 2001; Le, 2012). Apart from fat-soluble metals, most of the hydrophilic metals are absorbed via proton pumps (-ATPases), co- and antitransporters (proteins use the electrochemical gradients), and channels in the plant cell plasma membrane (Tangahu et al., 2011). The factors that modify the fate of metals in the environment as described above can also affect metal uptake through changing membrane fluidity (Norwood, 2007). In addition, competition between multiple metals for transporters on the membrane can lead to the binding sites being blocked and consequently influence the degree of absorption.

Metals absorbed react with the target sites within the organisms and cause physiological effects. Most plants have developed multiple constitutive and adaptive mechanisms to adjust their internal metal concentrations and maintain homeostasis (Yang et al., 2005). Excessive metal exposure within a certain range can be dealt with by plants via sequestration, detoxification and storage (Le, 2012). Metals can be distributed to apoplast tissues in cell walls, can form metal-ligand chelation and then be stored in vacuoles (Yang et al., 2005). The vacuole is known to be the predominant location in cells for storage of citrate and malate (Ryan and

Walker-Simmons, 1983) which can effectively chelate metal at the acidic pH of the vacuole (Dawson et al., 1986). Exposure levels beyond the capacity for metal storage in the cell wall and the vacuoles may cause toxicity and cell death (Ni et al., 2005) as metals can be accumulated in the cytoplasm and may bind to important biomolecules in the cell (e.g. Cys in proteins, glutathione, nucleotides) (Dawson et al., 1986).

### *1.3.2 Fate and behavior of metal-based NPs*

Using a life-cycle model, Mueller and Nowack (2008) found that the predicted concentrations of nano-$TiO_2$ in Swiss surface water were already close to or higher than the No Observed Effect Concentration (NOEC). Metal-based NPs can easily enter the water and soil compartments through application of sewage sludge from wastewater treatment as shown in Figure 1.1 (Batley et al., 2012; Tourinho et al., 2012). In recent years, a number of studies regarding the effects of NPs have been published but specifically for the aquatic environment. Little information is generated for terrestrial ecosystems (Tourinho et al., 2012), especially for higher plants.

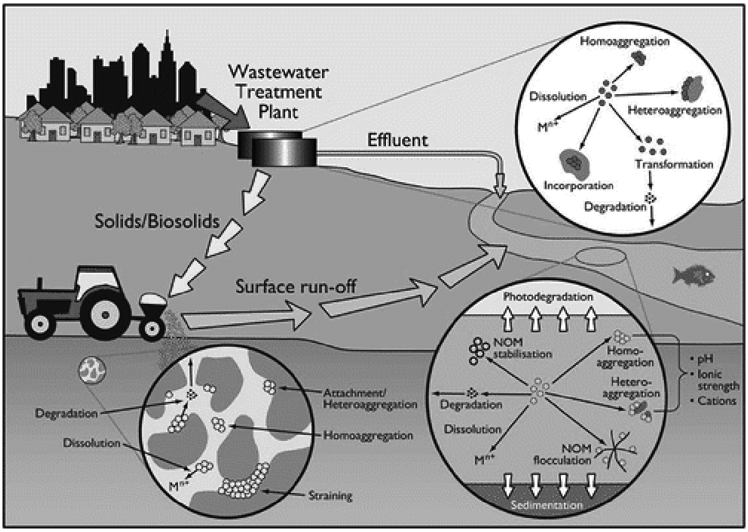

Figure 1.1 Pathways and transformations of nanomaterials in the environment. (Cited from Batley et al., 2012)

As compared to water systems, behavior of metal-based NPs in soils is relatively complex since metals can be found in several pools of the soil (Shuman, 1991). The fate of metal-based NPs in soil varies according to different soil types and diverse physiochemical characteristics of NPs. Ionic strength, zeta potential, organic matter content and pH are found to be highly correlated to the behavior of NPs in the soil. For example, as humic substances are negatively charged in the soil (Ghosh et al., 2008), the negative charges of particle-humic conglomerates would increase the stability of particles in solution (Tourinho et al., 2012). There are two possibilities by which metal-based NPs behave once released into soil: (1) NPs can be strongly adsorbed to soil particles due to their high surface areas and would be immobile in soil; (2) NPs can fit into soil pores because of their small size that allows NPs to travel further before being stabilized in the soil matrix (USEPA, 2007).

Agglomeration/aggregation is a basic characteristic for metal-based NPs and is of crucial importance in predicting the hazards of NPs. In the natural environment, nanoparticles can bind to other nanoparticles (homoaggregation) or to natural mineral and organic colloids (heteraggregation) which may change their fate and toxicity in terrestrial ecosystems (Batley et al., 2012). The homoaggregation rate of NPs was found to depend not only on size, shape and type of particles but also on initial concentration and solution chemistry (Batley et al., 2012; Tourinho et al., 2012). Nowadays, metal-based NPs are frequently manufactured with surface coatings which would extremely affect their surface chemistry and thereby influence agglomeration/aggregation rates or particle stability (Tourinho et al., 2012). Furthermore, dissolution may also play an important role in understanding the potential effects of metal-based NPs on terrestrial organisms. Metal species dissolved in the solution have been proven to be toxic to specific organisms. Therefore, both dissolved parts produced by dissolution and nano-sized particle forms may contribute to the toxicity of metal-based NPs. Considering these factors, it is difficult to quantify the fate and behavior of NPs in soil since general technologies such as dynamic light scattering (DLS) and microscopy-based techniques are still limited to be used for aqueous solution (Tourinho et al., 2012).

## 1.4 Ecological effects assessment

When hazardous chemicals are released into the environment, the response of biota especially plants occupying the lower trophic levels may act as an ‘early warning signal’ for the presence of pollutants. Higher plants tend to retain greater concentrations of metals as compared to free-floating species because of their root tissues (Doust et al., 1994). Their seeds often possess relatively lower sensitivity to pollutants in their ambient environment than seedlings as the germination rate mainly depends on the reserves within the cotyledons (Pfleeger et al., 1991). It is therefore that in this thesis the root growth of lettuce is used as the endpoint in toxicity tests to assess the impacts of metals and metal-based nanoparticles on terrestrial ecosystems.

In order to make sure that the large-scale applications of metallic pollutants in different sizes are safe to the environment, safety criteria are needed based on a comprehensive understanding of their properties and toxicity. Toxicity experiments are a direct method to establish dose-effect relationships that can evaluate to what degree metal pollutants are toxic to environmental receptors. Tests have been conducted with different exposure media e.g., soil, sediment, air, water and food. However, toxicological tests are time-consuming and resource-intensive (Burello and Worth, 2011). With an extensive and complex set of data, mathematical models can be a more straightforward way to describe observed phenomenon in toxicological experiments. Models can assist in setting scenarios for estimating effects of chemicals to the environment under initial conditions and for exploring the underlying mechanisms of chemicals within organisms (Ashford, 1981). Toxicity of metals or metal-based NPs is metal- and species-specific, and is strongly influenced by the environmental chemistry or characteristics of NPs (e.g. size, shape). Current risk assessments focus on individual chemicals. However, it has been recognized that metal pollutants naturally occur in the environment as mixtures (Bongers 2007). Interactions between metal pollutants may also contribute to the toxicokinetics, toxicodynamics and the overall toxicity of metal mixtures (Le, 2012). Noteworthy, the process of ecological effects assessment involves inherent assumptions and limitations which may produce uncertainties. To reflect the actual risks of metal pollutants of different sizes, scientific researchers try to incorporate the relevant parameters above for assessing bioavailability and toxicity.

### *1.4.1 Single toxicity modelling*

#### Speciation

Chemical speciation is vital in determining fate and transport, bioavailability and toxicity of pollutants. Metal speciation indicates the distribution of an element amongst various chemical species in a system. The analytical methods of chemical speciation have been divided into two ways, namely laboratory analysis and overall equilibrium distribution modelling (Van Briesen et al., 2010). Many instruments for measuring metal concentrations in water have been generated in the last decades, e.g. atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) (Paquin et al., 2002). Recently, ion-selective electrodes (ISE) are exploited to directly measure the activity of a specific ion dissolved in a solution. Alternatively, geo-chemical speciation models are applied to compute solution equilibria and the bioavailable fraction of metals with given water chemistry parameters. Chemical speciation modelling programs that are widely used by researchers include MINEQL+4.6 (Environmental Research Software, U.S.), MINTEQA2 (Environmental Protection Agency, U.S.), and WHAM 6/7.0 (Centre for Ecology and Hydrology, U.K.). Often, the chemical speciation is determined by combining direct analytical technology with indirect speciation modelling.

### *Total Metal Model*

At first, total or dissolved metal concentrations were used to establish connections between exposure levels and effects for deriving water quality criteria in the US and Canada (Paquin et al., 2002). The total dissolved metal concentration as a subset of the total metal concentration (0.45-μm membrane filtration) contains the free metal ion, the organic and inorganic metal complexes in the water column. The total metal model (TMM) assumed that the total or dissolved fraction of metals may closely approximate the biologically available fraction that leads to toxicity. For the aim of conservatism, the US EPA still suggests the total metal concentration to be used in specific ecological risk assessments (Suter II et al., 2000).

### *Free Ion Activity Model*

Further studies have shown that total or dissolved metal concentrations are poor in predicting the acute toxicity of metals to aquatic biota (Borgmann, 1983). Highly dynamic factors (e.g. pH, alkalinity, and hardness) of water or of the soil column may affect the actual metal uptake (Meyer, 2002). The free-ion-activity model (FIAM) was first formulated by Morel (1983) and was further improved by Campbell (1995) to model the bio-uptake fluxes outside the cells. The free metal ion and metal complexes with dissolved biotic and abiotic ligands are identified as parts of the bioavailable fraction responsible for toxicity. The plasma membrane is presumed to be the primary site for metal interactions with organisms. Three steps are involved in interactions of metals with organisms in aquatic environment (Campbell, 1995; Qiu, 2014):

(1) Advection or diffusion of metal ions in bulk solution;

(2) Sorption or surface complexation of the metal ions at the active sites of the cell membrane;

(3) Uptake or transport of the metal ion through the cell membrane into the organism. The interaction of free active sites on the cell membrane with different metal species in bulk solution can be described as follows:

$$\{\mathrm{M}^{z+}\} + [\mathrm{L}] \leftrightarrow [\mathrm{ML}]\,;\, K_1 \times \{\mathrm{M}^{z+}\} = \frac{[\mathrm{ML}]}{[\mathrm{L}]} \qquad (1\text{-}1)$$

$$\{\mathrm{M}^{z+}\} + \{\text{-}X_{\mathrm{cell}}\} \leftrightarrow \{\mathrm{M}\text{-}X_{\mathrm{cell}}\}\,;\, \{\mathrm{M}\text{-}X_{\mathrm{cell}}\} = K_2 \times \{\mathrm{M}^{z+}\} \times \{\text{-}X_{\mathrm{cell}}\} \qquad (1\text{-}2)$$

$$[\mathrm{ML}] + \{\text{-}X_{\mathrm{cell}}\} \leftrightarrow \{\mathrm{M}\text{-}X_{\mathrm{cell}}\} + [\mathrm{L}]\,;$$

$$\{\mathrm{M}\text{-}X_{\mathrm{cell}}\} = K_3 \times \frac{\{\text{-}X_{\mathrm{cell}}\} \times [\mathrm{ML}]}{[\mathrm{L}]} = K_1 \times K_3 \times \{\text{-}X_{\mathrm{cell}}\} \times \{\mathrm{M}^{z+}\} \qquad (1\text{-}3)$$

where $\{\mathrm{M}^{z+}\}$ is the free metal ion activity; [L] is the concentration of biotic or abiotic ligands dissolved in solution; [ML] is the concentration of the metal complex bound to a ligand; $\{\text{-}X_{\mathrm{cell}}\}$ is the concentration of free surface sites on the cell membrane; $\{\mathrm{M}\text{-}X_{\mathrm{cell}}\}$ is the activity of the surface complex; $K_1$, $K_2$, and $K_3$ are the conditional stability constants. Since the nature of the biological surface and the concentration of free sites are assumed to be constant, variations in $\{\mathrm{M}\text{-}X_{\mathrm{cell}}\}$ follow the change of $\{\mathrm{M}^{z+}\}$ according to the equations above. Although speciation calculation is incorporated, interactions during uptake at biotic plasma membrane are ignored (Norwood, 2007) which makes the FIAM inaccurate in describing actual effects of metals in certain systems.

*Biotic Ligand Model*

By taking both chemical speciation and biotic binding into account, the FIAM was extended to the biotic ligand model (BLM) (Di Toro et al., 2001). The modern BLMs also contain the theory of the gill surface interaction model (GSIM) that toxicity to fish results from salt and water unbalance within the gill tissue as caused by cationic metals (Pagenkopf, 1983; Niyogi and Wood, 2004). Free metal ions and the corresponding competing cations (e.g. $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $H^{+}$) bind to the fish gill with specific affinities (log $K$) and capacities ($B_{\max}$). These competitions with complexation by abiotic ligands (e.g. NOM, carbonates, chlorides, sulfides) are used to frame a geochemical equilibrium in quantifying the fraction of metal ions accumulated at the biotic ligand (BL) (Niyogi et al., 2008). The BLM was a theoretical framework first developed for single metal species (Paquin et al., 2002). Toxic effects of a metal are determined by the amount of metal ions binding to the specific site of toxic action which is treated as a BL (Van Gestel et al., 2010).

Since soil metal concentration has been regarded not to represent metal bioavailability and toxicity, further methods based on bioavailable fractions are needed to assess the risk levels of metals in soil (Thakali et al., 2006). As general binding sites (e.g. sodium and calcium transporters) are intrinsic in almost every living cell (Niyogi and Wood, 2004), later studies have applied the aquatic BLM to terrestrial ecosystems (tBLMs) by Thakali et al. (2006). The interaction of the cation activities ($\{X^{z+}\}$) with the biological phase (in Figure 1.2) was incorporated into a log-logistic toxicity model expressing the relationship between biological response and fraction of free metal ions ($M^{z+}$) that bind to the BL.

$$R = \frac{100}{1 + \exp[\beta \times (f_{50} - f)]} \tag{1-4}$$

According to the equilibrium relationships described in FIAM, the concentration of total BL sites ([TBL]) is specified as follows

$$[\mathrm{TBL}] = [\mathrm{BL}^-] + [\mathrm{MBL}^+] + \sum[\mathrm{XBL}^+] = [\mathrm{BL}^-] \times (1 + \sum K_{\mathrm{XBL}} \times \{\mathrm{X}^{Z+}\} + K_{\mathrm{MBL}} \times \{\mathrm{M}^{Z+}\}) \tag{1-5}$$

The fraction ($f$) of the total BL sites bound by $M^{z+}$ is defined as

$$f = \frac{[\mathrm{MBL}^+]}{[\mathrm{TBL}]} = \frac{K_{\mathrm{MBL}} \times \{\mathrm{M}^{Z+}\}}{1 + K_{\mathrm{MBL}} \times \{\mathrm{M}^{Z+}\} + \sum K_{\mathrm{XBL}} \times \{\mathrm{X}^{Z+}\}} \tag{1-6}$$

where $R$ is the biological response; $\beta$ is the shape parameter; $f_{50}$ is the fraction of the total BL sites occupied by $M^{z+}$ at which a 50% response is induced; *K* is the conditional binding constant; {} is the activity of the cation and metal ion; $[MBL^{+}]$ is the concentration of metal ion-biotic ligand complexes.

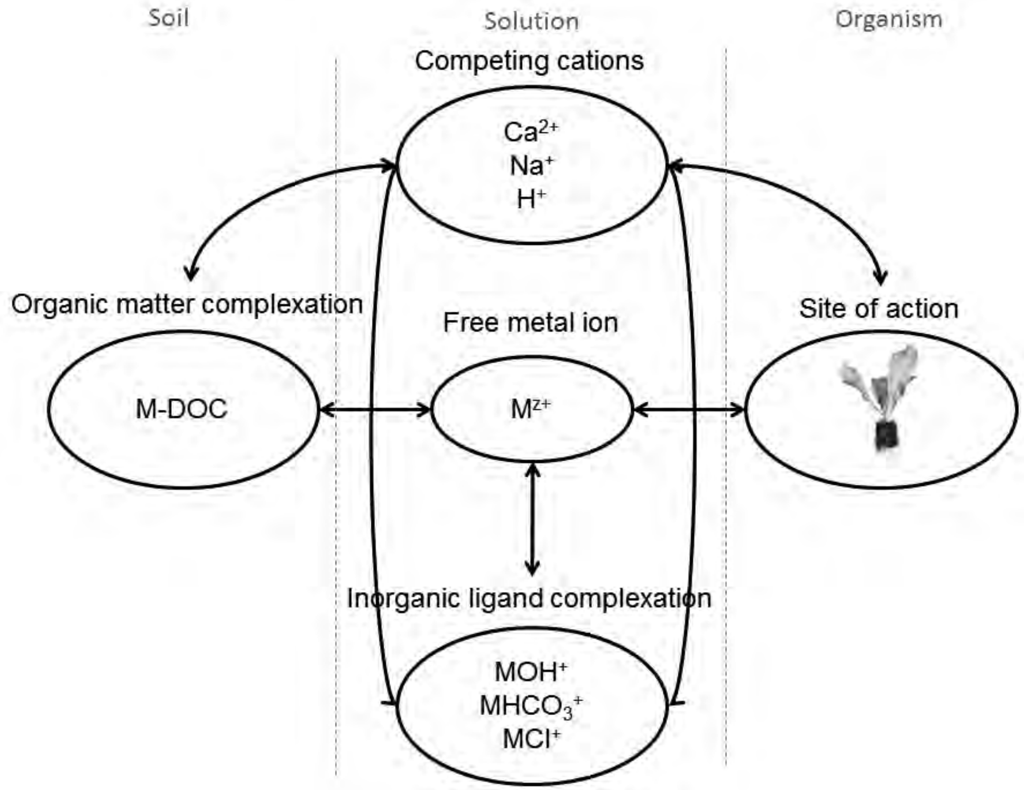

Figure 1.2 Diagram of the biotic ligand model framework in terrestrial ecosystems. (Adapted from Thakali et al., 2006)

*Other toxicity models*

Toxicity models can be altered according to the approaches above and used in different ecosystems. For instance, multicomponent Freundlich models concerning the pH-dependent metal ion binding to BLs were developed instead of BLM to predict Cu-toxicity to maize, fungal, and yeast (Plette et al., 1999; Qiu, 2014).

Furthermore, some researchers suggested that the surface electrical potential of plasma membranes (PMs) seems also important to explain bioavailability of metal ions. The permeability of a membrane is the rate of passive diffusion of molecules through the membrane. Permeability depends mainly on the electric charge and polarity of the molecule and to a lesser extent the molar mass of the molecule. The PM electrical properties therefore play a key role in the distribution of ions at the exterior surface of PMs, ion transport across PMs and ion intoxication (Wang et al., 2011). On the basis of electrical potential at the PM surface ( $\psi_0$), the electrostatic toxicity modeling (ETM) was developed to assess metal bioavailability and toxicity taking into account the plant-ion interactions at the PM surface. The ETM can be applied as a complement for the BLM when observed toxic effects cannot be interpreted in terms of site-specific competition such as in the case of synergistic interactions (Kinraide, 2006; Le, 2012).

### *1.4.2 Mixture toxicity modelling*

Mixtures are defined as any combination of two or more chemicals, regardless of source and spatial or temporal proximity that may act jointly to induce actual or potential effects in a receptor population (US ATSDR, 2004). Since humans and other organisms living in the environment are exposed to a variety of substances, increasing concerns from both scientific and legislative perspectives have shifted from individual chemicals to mixtures. Guidelines for evaluating data on the health risks from exposure to chemical mixtures were first established by the Environmental Protection Agency of the United States in 1986 (US EPA, 2000). Afterwards, the European Commission also set relevant regulations for toxicity assessment of chemical mixtures (European Commission, 2012). Researchers are also constantly improving methods on how to increase the accuracy of toxicity assessments for multiple chemicals.

#### *Additivity models*

Toxic effects of a mixture can be characterized by four possible types of joint action as presented in Table 1.1 (Plackett and Hewlett, 1952). However, only non-interactive or additive mixture effects have been well defined in the form of multicomponent models. These models for assessing mixture toxicity are based on the term 'additivity' that mixture components act together to produce an effect without enhancing or diminishing each other's actions (Van Gestel et al., 2010). The additive effects of mixtures can be predicted by summing the scaled exposure levels (Dose Addition or Concentration Addition) or the responses (Response Action or Independent Action) of mixture components. The concentration addition (CA) approach assumes that components in a mixture act on similar physiological systems within the organism. The independent action (IA) approach on the other hand presumes that each component present in a mixture acts independently but triggers similar effects on the organism (Bliss, 1939). This indicates that the predictive capability of these two additivity models may depend on the similarity of mode of action (MoA) or mechanism of action (MOA) of mixture components. Nevertheless, information on toxicity mechanisms is rarely available which hinders the selection of a most suitable model for risk assessment of metal mixtures. In addition, the majority of metal mixtures do not meet the assumptions of additivity models (Bongers, 2007) e.g. purely independently acting MoA, as organisms are always treated as a coordinated system (Ashford, 1981).

A total concentration of the mixture, at which a certain effect is generated, can be expressed according to the concept of CA as follows (Altenburger et al., 2004)

$$\mathrm{EC}_{x\mathrm{mix}} = (\sum_{i=1}^{n} \frac{p_i}{\mathrm{EC}_{xi}})^{-1} \tag{1-7}$$

where $\mathrm{EC}_{x\mathrm{mix}}$ is the total concentration of the mixtures provoking $x$% effect on the test organism; $\mathrm{EC}_{xi}$ is the concentration of the $i_{\mathrm{th}}$ component provoking $x$% effect solely; $p_i$ is the fraction of component $i$ in the mixture.

The IA model can be defined as

$$E(c_{\mathrm{mix}}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \tag{1-8}$$

where $c_i$ is the concentration of the $i_{\mathrm{th}}$ component in the mixture; $E(c_{\mathrm{mix}})$ is the total effect on the test organism caused by the mixtures; $E(c_i)$ is the toxic effect on the test organism caused by the $i_{\mathrm{th}}$ component in the mixture.

Table 1.1 Four possible joint actions of chemical mixtures (Adapted from Plackett and Hewlett, 1952).

| Types | Similar joint action | Dissimilar joint action |
|---|---|---|
| Non interactive | Simple similar action<br>(DA or CA) | Independent joint action<br>(RA or IA) |
| Interactive | Complex similar action* | Dependent joint action* |

DA or CA: Dose Addition or Concentration Addition interaction pattern; RA or IA: Response Action or Independent Action interaction pattern; *: no mathematical descriptions available.

*Toxicity indices*

To facilitate the calculation of the strength of a given compound, the fraction of component *i* in the mixture shown in equation (1-7) was previous called toxic units (TU) (Sprague and Ramsay, 1965). The sum of TUs can represent the empirical observation of the strength of a mixture since they are expressed in the same units (Sprague, 1970). All components in the mixture can be described as dilutions of each other and their contributions can be scaled relative to their single toxicity (Bongers 2007). In applying the TU approach, concentration addition is the basic assumption regardless of interactions between mixture components (Playle, 2004). As the fraction of the total mixture concentration can be known as the concentration of each component in the mixture, equation (1-7) can be rewritten as (Altenburger et al., 2004)

$$\mathrm{TU_{mix}} = \sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_{xi}} \qquad (1\text{-}9)$$

where $c_i$ is the concentration of the $i_{\mathrm{th}}$ component in the mixture; $\mathrm{EC}_{xi}$ is the standard effect concentration of the $i_{\mathrm{th}}$ component in the mixture, it can be the 50% effective concentration of the organisms ($\mathrm{EC}_{50}$) or even the lowest observed effect concentration (LOEC). If the 50% toxic effects are observed based on $\mathrm{EC}_{50}$ when the sum of TU equals to 1, the mixture toxicity is supposed to be strictly additive. If the 50% toxic effects are observed when the sum of TU is significantly less than one, toxicity is greater than additive (synergistic) and if the sum of TU is significantly greater than one, toxicity is less than additive (antagonistic).

Although the mathematical expression of the TU approach is very simple, this method may be invalid for mixtures containing more than two components or when antagonistic effects occur (Lloyd, 1987). The Mixture Toxicity Index (MTI) was developed as an alternative for the TU concept and was shown to be more appropriate to quantify the extent of the joint action because of the largest fraction of $\mathrm{LC}_{50}$ defined (Könemann, 1981). The different fixed MTIs also have physical meanings such as the tolerance concentrations and relative toxicities of mixtures as compared to their components which may help explain acute toxicity data.

$$\mathrm{MTI} = 1 - \frac{\log M}{\log M_0} \tag{1-10}$$

where $M_o = \sum f_i/f_{max}$; $f_i = c_i/LC_{50i}$; $M = \sum f_i$; $LC_{50}$ is the lethal concentration for 50% of the organisms; $f_{max}$ is the largest $f_i$ value in the mixture. MTI<0, $M>M_o$ ($f_{max}>1$) indicates that the type of joint action is antagonism; MTI=0, $M=M_o$ ($f_{max}=1$) indicates no addition (the expected result for independent action, positive correlation between susceptibilities of the individual organisms to the individual compounds in a mixture); 0<MTI<1, $M_o>M>1$ ($f_{max}<1$) indicates partial addition; MTI=1, $M=1$ ($f_{max}<1$) indicates concentration addition (simple similar action); MTI>1, $M<1$ ($f_{max}<1$) indicates supra addition (potentiation of the toxic actions of one or more of the compounds in a mixture).

Besides the above approaches, some other toxicity indices were generated to facilitate effect and risk assessment of mixtures. For example, the toxic equivalency (TEQ) concept has been utilized to assess cumulative risks related to dioxins and dioxin-like compounds (Ahlborg et al., 1994) and is endorsed by the World Health Organization (Van den Berg et al., 2006). The toxic equivalency factor (TEF) expresses the toxicity of a single pollutant in terms of the most toxic one in chemical groups. With TEFs, TEQs report the toxicity-weighted masses of mixtures of PCDDs, PCDFs and PCBs as a single number.

$$TEQ = \sum_{i=1}^{n} c_i \times TEF_i \tag{1-11}$$

where $TEF$ is the toxic equivalency factor comparing to the index chemical ($TEF_1=1$); $c_i$ is the concentration of the $i_{th}$ component ($c_1$=2,3,7,8-TCDD). However, the TEF approach is still limited to be used for specific organic chemical groups which may be resulted from various degrees of uncertainty under certain assumptions as follows: (1) individual compounds act through the same biological pathway; (2) individual effects are additive; (3) dose-response curves of individual compounds are parallel; (4) individual compounds are similarly distributed in the organism body.

Although there are some literatures focusing on mixture toxicity, it is still largely unknown that how to reduce the 'noise' in modeling the toxicity of metal mixtures. In addition, less information of metal mixtures was gained from terrestrial ecosystems as compared to aquatic systems. Thus, this thesis tried to improve the predictive ability of existing mixture models by extending them in different ways and compare their performance for assessing the toxicity of multiple metals to terrestrial plants.

*Deviations from additivity models*

Ion-ion interactions may occur naturally in terrestrial ecosystems (Påhlsson, 1989) at various levels: (1) during exposure in the environment, (2) uptake at the root surface, (3) at target sites within the plant, (4) in the internal detoxification pathway (Calamari and Alabaster, 1980). Toxicity of metal mixtures may deviate significantly from the addition of biological actions of single metals because of ion-ion interactions. Interaction patterns may be inconsistent depending on the total concentration of mixtures (Figure 1.3 DL) and the relative proportion of component concentrations in the mixture (Figure 1.3 DR) (Bongers, 2007; Qiu, 2014). Therefore, more complex interaction patterns are distinguished to quantify how observed data deviate from additivity models (Jonker et al., 2005):

(1) No deviation: the actual effects of the mixtures are well explained by additivity models (Figure 1.3 Control).

(2) Synergism or antagonism: if the effects of the mixtures are less than that suggested by the toxic effects of individual components present in the mixture, antagonism is observed (Figure 1.3 S/A). If the effects of the mixtures are greater than that suggested by the toxic effects of the individual components, synergism is observed.

(3) Dose level-dependent deviation: the deviation from additivity models at low dose levels is different from the deviation at high dose levels. For example, antagonism can be observed at low dose levels of mixtures and synergism can be observed at high dose levels of mixtures (Figure 1.3 DL).

(4) Dose ratio-dependent deviation: the deviation from additivity models depends on the relative proportion of mixture components. For instance, for binary mixtures, antagonism can be observed when Component 1 dominates the overall toxicity, whereas synergism can be observed when Component 2 dominates the mixture toxicity (Figure 1.3 DR).

*Extended models*

Although the additivity models can provide an approximate estimation for the toxic effects of metal mixtures in ecosystems, these simplified models not only ignore the ion-ion interactions but also the ion-organism interactions. Without considering interactions, the CA and IA models may fail to accurately assess the combined toxicity of multiple metals in specific cases (Spurgeon et al., 2010). Jonker et al. (2005) have already presented the MixTox program to distinguish the statistically significant chemical-chemical interactions. In this thesis, statistically significant deviations found in the plant-bioassays data were incorporated into the mathematical models to describe the dose-response relationships for metal mixtures.

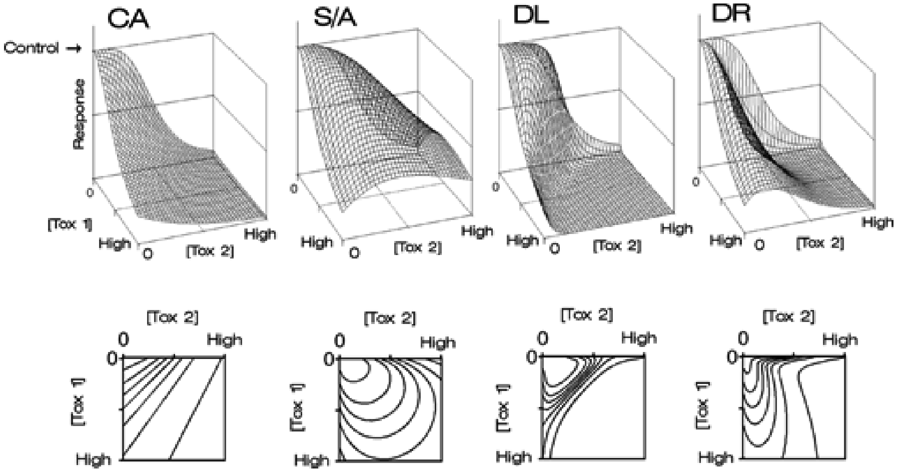

Figure 1.3 Three dimensional dose-response relationships (Top) of binary mixtures and isobologram (Bottom) illustrating interaction patterns from the additivity (CA): antagonistic deviation (S/A), dose level-dependent deviation (DL), and dose-ratio-dependent deviation (DR). (Cited from Jonker et al., 2005)

Some researchers have tried to comprise the parameters that may influence the bioavailability of metals into mixture toxicity modelling, e.g. environmental chemistry. Furthermore, different toxicity descriptors which have been used in single toxicity modelling are substituted into the mixture models in order to deduce the bioavailable metal-related fractions. For example, the BLM and the ETM approaches considering main reactive metal forms (e.g. free metal ions in bulk solution), ion-ion competitions, ion-plant interactions have been extended to predict overall effects of metal mixtures by combining bioavailability or toxicity models with addition models (Hatano and Shoji, 2008; Jho et al., 2011; Le, 2012). However, no universally accepted framework is available to determine an approach to assess the combined toxicity of a given metal mixture across different exposure conditions and different combinations.

### *1.4.3 Toxicity assessment of metal-based NPs*

Compared to the case of dissolved metal ions, the toxicity assessment of metal-based NPs is still at an initial stage. Physicochemical characteristics of nanoparticles (e.g. particle size, shape, surface area, types, activity and concentration), and of specific organism species have been both suggested to be correlated with toxicity of metal-based NPs (Yang and Watts, 2005; Ma et al., 2010). Some researchers tried to use quantitative structure-activity relationship (QSAR) methods to make connections between theoretical descriptors (e.g. physicochemical properties and behavior of NPs) and toxicity testing data (Burello and Worth, 2011). Due to the particular morphology of metal-based NPs, Song et al. (2014) used the response addition model to separate the toxicity contribution of particulate forms of CuNPs and $Cu^{2+}$ to mammalian and piscine cell lines. A similar approach was also used for a whole organism (Hua et al., 2014). The findings of these authors emphasized the contributions of ion release rate of NPs as well as species-specific traits in explaining and extrapolating toxicity testing results of metal-based NPs.

$$E_{CuNPs} = 1 - \frac{(1 - E_{total})}{(1 - E_{Cu2+})} \tag{1-12}$$

where $E_{total}$ is the total cell toxicity caused by the copper suspensions; $E_{CuNPs}$ and $E_{Cu2+}$ are the cell toxicity caused by the particulate form of CuNPs and $Cu^{2+}$, respectively.

Increasing numbers of studies have been published recently concerning the interactions of metal-based NPs with animals, but scant attention has been published so far for plant species. Engineered metal-based NPs can adhere to external root surfaces of plants and thereby reduce the root hydraulic conductivity and plant availability of external water sources (Asli and Neumann, 2009). Both metal ions released from NPs and nano-sized particles can be absorbed and transported within plants and thereupon cause toxicity. Nanoparticles can enter the plant cells through carrier proteins, aquaporins, ion channels, endocytosis, newly created pores (by CNTs), and wrapped by organic chemicals in the media (Rico et al., 2011). However, modes of uptake and accumulation of metal-based NPs are variable for specific plant species and various NPs (e.g. different size, shape, type). To improve the understanding of toxicity mechanisms of metal-based NPs in plants, lettuce was chosen as the test-organism in this thesis and exposed to metal-based NPs, i.e., nano-Cu and nano-ZnO. Moreover, dissolution and aggregation processes of metal-based NPs are found to be highly associated with their toxicity (Franklin et al., 2007). In other words, parameters that affect these two main processes such as characteristics of the surrounding media may also influence toxicological responses of metal-based NPs. However, to our knowledge, research related to impacts of surrounding media on nano-toxicology is sorely lacking. To mimic a more realistic exposure environment, interactions of metal-based NPs with other common pollutants such as metals dissolved in water, or other types of NPs would be discussed in this thesis to improve the understanding of nano-toxicology.

## 1.5 This thesis

### *1.5.1 Objective*

Elevated concentrations of metals have diminished the biodiversity of aquatic and terrestrial ecosystems and endangered the health of human beings. Plants play an important role in the biogeochemical cycling of the elements and can be efficient biomarkers for metal-related chemical stresses. However, ecological effects assessment for metal mixtures sizes of which ranging down to the nanoscale is still in its infancy. Multiple layers of interacting processes increase the difficulty of accurate estimation of bioavailability and toxicity of metals. In addition, simple correlations cannot satisfy the specific physiological processes in higher plants such as metal-specific selectivity. This PhD thesis aims at quantifying mixture toxicity of metals to lettuce (*Lactuca sativa* L.) and the impacts of interacting processes on the actual effects of metals in different sizes. How to choose a suitable approach in mixture toxicity modelling across various exposure situations and across different combinations of metal pollutants is also discussed in this thesis. To achieve this objective, the following sub-questions are addressed:

(1) How does water chemistry affect the toxicity of individual metals (Ni and Cd) to

lettuce and how to quantify the influence of water chemistry?

(2) Can the toxicity-modifying factors of water chemistry be incorporated into toxicity models and will the prediction of acute toxicity of individual metals (Ni and Cd) to lettuce seedlings be improved because of incorporation of these factors in the toxicity models?

(3) What kind of statistically significant deviation patterns from additivity are induced in assessing the combined effects of metal mixtures (Cu-Cd, Ni-Cd and Cu-Ni) to lettuce?

(4) Can the statistically significant deviations from additivity be reproduced and how likely is it that metal ions ($Cd^{2+}$, $Ni^{2+}$ and $Cu^{2+}$) interact with each other?

(5) How to incorporate the impacts of environmental chemistry in assessing the toxicity of metal mixtures (Cu-Ni, Cu-Zn and Cu-Ag) to lettuce?

(6) Will the estimation of mixture toxicity be improved considering ion-ion interactions?

(7) Will the dissolved metal species and the particulate fractions of each type of metal-based NP act jointly according to the rules of additivity?

(8) Will Cu NPs interact with ZnO NPs and influence the toxicity of each other to lettuce?

### *1.5.2 Outline*

**Chapter 1** provides an overview of the PhD thesis describing the state-of-the art of the science on issues involving effects of metals and metal-based NPs. The research objectives and the fundamental principles for different modelling approaches in terrestrial ecosystems are outlined.

In **Chapter 2**, the impacts of $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Na^{+}$ and pH were investigated on the acute toxicity of Ni and Cd to butter-head lettuce seedlings (*Lactuca sativa* L.). The total metal model (TMM), the free ion activity model (FIAM) and the biotic ligand model (BLM) were all used to quantify the 4-day root elongation inhibition. The predictive power of TMM, FIAM and BLM was compared for determining the toxicity of Ni and Cd.

In **Chapter 3**, using root elongation of lettuce (*Lactuca sativa* L.) as a toxicity endpoint, the combined effects of Cu, Cd, and Ni were studied. The joint actions of binary metal mixtures were investigated using statistical software i.e., the MixTox model. The reproducibility of deviations from the reference models i.e., Concentration Addition (CA) and Independent action (IA) in assessing the mixture toxicity was tested based on a comprehensive experiment design.

In **Chapter 4**, the biotic ligand model was extended to predict the overall toxicity of Cu-Ni, Cu-Zn, and Cu-Ag mixtures to lettuce (*Lactuca sativa L.*) in three approaches based on the concept of additivity, i.e. the toxic unit approach, the toxic equivalency factor approach and the approach by determining fraction of total number of biotic ligand sites bound by metal ions of mixtures. The predictive capabilities of these different BLM-based approaches for each combination were compared by the bootstrapping method.

In **Chapter 5**, the combined toxicity of copper nanoparticles (50 nm) and zinc oxide nanoparticles (150 nm) to *Lactuca sativa* L. was assessed by the IA model to check whether mixtures of metal-based NPs would also act jointly following the rules of ‘additivity’. To systematically examine whether chemical-chemical interactions would affect their joint toxicity, a step by step experiment was designed with six nested combinations of Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO. The suspension of each type of metal-based NP was presumed to be a mixture including a soluble part and an undissolved particulate part. The $EC_{50}$ values of one compound were plotted as a function of increasing concentrations of other compounds in the mixture to assign where and how chemical-chemical interactions occurred.

In **Chapter 6**, the results obtained in Chapters 2-5 are synthesized in order to answer the research questions proposed in **Chapter 1**. Based on the synthesis, the choice of a suitable model for predicting mixture toxicity across different combinations of metal pollutants in different sizes is discussed by considering the observed chemical-chemical interactions and comparing the predictive power of the different approaches applied in this thesis. This chapter also gives recommendations for potential applications of the modelling approaches developed and brief outlooks for further research.

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# Chapter 2

## Impacts of major cations ($K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$) and protons on toxicity predictions of nickel and cadmium to lettuce (*Lactuca sativa* L.) using exposure models.

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## Abstract

Biotic Ligand Models (BLM) explicitly accounting for hypothetical interactions with biotic ligands and bioavailability as dictated by water chemistry have been developed for various metals and different organisms. It is only recently that BLMs for plants have received increasing attention. Lettuce is one of the most important vegetable crops in the world. This study investigated the impacts of $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Na^{+}$ and pH, on acute toxicity of Ni and Cd to butter-head lettuce seedlings (*Lactuca sativa* L.). Four day assays with the root elongation inhibition as the endpoint were performed in hydroponic solutions. Magnesium was found to be the sole cation significantly enhancing the median inhibition concentration ($IC_{50}$) of $Ni^{2+}$ with increasing concentration. By incorporating the competitive effects of $Mg^{2+}$, the Ni-toxicity prediction was improved significantly as compared to the total metal model (TMM) and the free ion activity model (FIAM). The conditional stability constants derived from the Ni-BLM were $\log K_{\mathrm{MgBL}}$=2.86, $\log K_{\mathrm{NiBL}}$=5.1, and $f_{\mathrm{NiBL}}^{50\%}$ =0.57. A slight downtrend was observed in the 4-d $IC_{50}$ of $Cd^{2+}$ at increasing $H^{+}$ concentrations, but this tendency was not consistent and statistically significant ($p$ = 0.07) over the whole range. The overall variations of $Cd^{2+}$-toxicity within the tested $Na^{+}$, $K^{+}$, $Ca^{2+}$ and $Mg^{2+}$ concentration ranges were relatively small and not statistically significant. Eighty percent of lettuce root elongation inhibition (*REI*) by Cd could be explained using both TMM and FIAM instead of BLM in the present study. Thus, the mechanistically underpinned models for soil quality guidelines should be developed on a metal-specific basis across different exposure conditions.

*Keywords*: Root elongation; nickel; cadmium; biotic ligand model; toxicity

## 2.1 Introduction

Elevated concentrations of metals in soil are a worldwide problem (Gupta et al., 2013), the adverse effects of which could threaten the environment (Thakali et al., 2006a). In order to minimize the entry of metals into the environment, standards continue to be developed for underpinning the risk assessment procedures (Wang et al., 2010a). Many models have been developed to take into account the factors which may affect metal toxicity to organisms (Hatano and Shoji, 2010).

At first, metal toxicity is correlated with the total or dissolved metal concentrations as used by U.S. Environmental Protection Agency (EPA, 1985) which include not only the free metal ion, but also the organic and inorganic metal complexes (Paquin et al., 2002). Afterward, some researchers pointed out that the link between effects and metal exposure levels can be improved by considering the underlying processes of intricate chemical and physiological details about what was occurring in nature. The free metal ion has been generally accepted as one of the dominant reactive species that could be taken up by organisms directly (Lexmond and van der Vorm, 1981; Gopalapillai et al., 2013) and can impose adverse effects. With this theoretical basis, the free ion activity model (FIAM) was proposed and has been used to predict toxicity of metals to organisms (Morel and Hering, 1993; Li et al., 2009). However, besides the metal speciation in the water phase, $H^+$ and common cations (e.g. $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$) were found to form complexes with biotic ligands (BL) which may also have impacts on the metal bioavailability and toxicity (Di Toro et al., 2001). Thus, by taking solution chemistry and competing components into consideration, the Biotic Ligand Model (BLM) was originally synthesized and conceptualized by Di Toro et al. (2001) and used extensively for acute metal toxicity prediction in aquatic organisms (Clifford and McGeer, 2010). However, toxicity of metals is complex and affected by various factors, such as exposure conditions (Hatano and Shoji, 2010), and organism species (Le, 2012).

The excessive level of metals is one of the principal abiotic pressures leading to the potential hazardous effects to the flora (Hossain et al., 2012) as plants can be more sensitive to the ambient stresses (Valerio et al., 2007) compared to animals. Thus, it needs to be investigated whether metal toxicity to plants could be explained by variations of metal species in the solution and competitive effects of major cations or protons as in aquatic animals. Until recently, BLMs have been developed to estimate metal toxicity in terrestrial systems (Thakali et al., 2006 a, b).

Cd and Ni have been considered as being toxic to plants and may induce respiratory, photo-synthetic and structural disorders at relatively low concentrations (Dixon, 1988). Researches concerning the ameliorative effects of cations and protons on $Ni^{2+}$ and $Cd^{2+}$ toxicity to plants have been better conducted on aquatic plants, such as green algae (François et al., 2007; Deleebeeck et al., 2009) and duckweed species (Gopalapillai et al., 2013). By contrast, metal toxicity to terrestrial plants is less understood and highly variable (Läuchi and Epstein, 1984; Dixon, 1988; Das et al., 1997). Even within one plant species, the ameliorative effects of cations and protons are found to be different in soils and in soil solutions. When barley seedlings were exposed in nutrient solution, higher activities of $Ca^{2+}$ (Li et al., 2009) and $Mg^{2+}$ increased the $EC_{50}$ of $Ni^{2+}$ significantly (Lock et al., 2007; Antunes and Kreager, 2009). In soils, the protective effect of protons was observed (Rooney et al., 2007) and the effect of $Ca^{2+}$ was found to be not simply competitive as proposed in the equilibrium BLM (Thakali et al., 2006a). Hence, the development of BLMs for terrestrial plants to predict metal toxicity needs to be further explored.

Butter-head lettuce (*Lactuca sativa* L.) was selected as a monitoring tool in assessing environmental contamination of Ni and Cd since it is an important crop plant and is recommended by EPA (1988) and Organization for Economic Cooperation and Development (OECD, 2006) as a key species. The total global commercial production of lettuce was up to 23.6 million metric tons in 2010 according to the Food and Agriculture Organization of the United Nations (van Wyk, 2005). The metals accumulated in the lettuce may cause damage to human health through the food-chain. On the other hand, *L. sativa* has been found to hyper-accumulate metals such as Cd and Zn (Garate et al., 1993) and to be sensitive to Ni and Cu (Charles et al., 2011). Thus, in order to determine the potential risk of Ni and Cd for ecosystems, the present study aimed at predicting Ni and Cd toxicity to lettuce (*L. sativa*) in hydroponic exposure setting, taking into account the effects of $H^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$. This was achieved by developing mathematical relationships between the main reactive form of metals in the solution and plant responses to metals using total metal model (TMM), FIAM and BLM.

## 2.2 Methodology

### *2.2.1 Plant culture and endpoint*

Seeds of *L. sativa* (Milan) were bought from a Dutch commercial seed company. Lettuce was cultured in the Steiner solution which has been shown to be a good plant growth and rooting media (Steiner, 1961; Peijnenburg et al., 2000). The composition of Steiner solution was given in the Supplementary material S2.1. The relative root elongation inhibition (*REI*, %) was used to determine the toxicity of $Ni^{2+}$ and $Cd^{2+}$ (details in 2.4) as the higher accumulated amounts of metals were found in roots of lettuce with the increased exposure concentrations (Garate et al., 1993).

### *2.2.2 Experimental design*

Effects of pH and cations on Cd- and Ni- toxicity were determined by systematically varying either pH or the concentrations of one of the cations, while keeping all other conditions constant. The designed levels of cation concentrations were supposed to be metal-specific and species-specific, and therefore were set up based on previous studies (Le, 2012) under the same experimental conditions. The concentration ranges of the four sets of major cations added in the solution and one set of proton experiments for each Ni and Cd toxicity assessment were fixed as follows: Na (from 0 to 20 mM), K (from 0 to 20 mM), Ca (from 0 to 10 mM), Mg (from 0 to 16 mM) and pH (from 5 to 8). Some of the tests were duplicated to check experimental uncertainty. In the solution, the Ni concentrations ranged from 0 to 4.5 mg/L and the Cd concentrations covered ranges from 0 to 10 mg/L, except the Mg-set, in which the Ni concentration was increased to 10 mg/L and the Cd concentration was increased to 12 mg/L. Because after the preliminary experiments, the protective effect of $Mg^{2+}$ was found to be stronger as compared to other major cations.

Apart from the experiments in which effects of varying pH were assessed, all test solutions were kept at a pH value of 7.0 during exposure by adding either $HNO_3$ or NaOH. The pH value of the test solutions was measured using a 691 pH meter (Metrohm, Herisau, Switzerland). The pH was determined every day in the experiments in which the effect of pH on toxicity was tested, while it was measured every other day for the other experiments. Cd, Ni and the cations ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$) were added as nitrate salts.

### *2.2.3 Toxicity assays*

Root growth is recommended as a suitable endpoint linked to the toxic effects of metals since it is relatively simple to assess (Fodor, 2002; Hagemeyer, 2004), and accurately to quantify (Clemens, 2006). Before the toxicity test, lettuce seeds were germinated on sterilized expanded perlite in Steiner solution for 4 days in a climate room at a temperature of 15˚C, 80% humidity, and a 16:8 hours light: dark cycle. The 4 d-old seedlings were fixed in parafilm sheets floating on the surface of glass beakers (100 ml volume) with their roots fully immersed in the metal-spiked hydroponic medium. In each exposure, four plant seedlings were used. To prevent excess evaporation and subsequent lack of exposure of the roots, the beakers were placed in a container with a small layer of water inside. The intuitive diagram of the experimental process has been shown in the Supplementary material S2.2. The relative increase in root length was measured after 4 days and was assessed as the distance from the transition point between the hypocotyl and the root, to the root tip. The lengths of all roots were measured to the nearest millimeter (EPA, 1988). Five ml of the exposure medium was reserved for chemical analysis before or after the exposure in vials and stored at 4°C in the refrigerator. The concentrations of Cd and Ni in solution were analyzed by flame atomic absorption spectroscopy (FAAS, Perkin Elmer AAnalyst 100, Waltham, U.S.). Electrical conductivity (EC) was checked after exposure by means of a Microprocessor EC/TDS Meter HI 98360 (Hanna instruments, Woonsocket, U.S.). Besides root elongation, visual inspection on the appearance of the seedlings was done on leaves (color and size), hypocotyls (length and thickness), lateral roots (amount, length and color) and taproot (thickness and color).

### 2.2.4 Data analysis and speciation calculation

Root elongation inhibition (*REI*) was calculated by comparing the measured root elongation in the solution containing Cd/Ni after 4 days of growth with the root elongation in the controls according to the following equation:

$$REI = (1 - \frac{RG_S}{RG_c}) \times 100\% \tag{2-1}$$

where $RG_s$: the average root growth of plants in the sample solution with added Cd/Ni; $RG_c$: the average root growth of plants in the control solution, the controls for the cation/hydrogen -sets were the ones with only cation/hydrogen inside.

The speciation of Ni, Cd and major cations (i.e. K, Na, Ca, and Mg) was calculated using the Windermere Humic Aqueous Model 7.0.1 (Centre for Ecology & Hydrology, Wallingford, UK). The activities of the metal ions and major cations in solution ($Cu^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $Na^{+}$, $Zn^{2+}$) were calculated based on the measured concentrations using FAAS, whereas the entered concentrations of the other cations or anions (e.g. $NO_3^{-}$, $SO_4^{2-}$, and $PO_4^{3-}$) were determined in terms of total concentrations which were calculated from the known formula of the Steiner medium (S2.1). Because the hydroponic system was open to the ambient air, the $pCO_2$ was set at $10^{-3.5}$ atm.

### *2.2.5 Estimation of the BLM parameters*

The BLM-methodology is based on the assumption that stability constants remain similar under various physico-chemical conditions (Heijerick et al., 2002). Stability constants, for example of $Ni^{2+}$, $K_{NiBL}$ (L/mol) were defined as follows:

$$K_{NiBL} = \frac{[NiBL^{+}]}{\{Ni^{2+}\} \times [BL^{-}]} \tag{2-2}$$

where $\{Ni^{2+}\}$: the activity of the free nickel ion (mol/L); $[NiBL^{+}]$: the concentration of Ni bound to the biotic ligand (mol/L); $[BL^{-}]$: the concentration of free biotic ligand, not occupied by any cation (mol/L). Similar equations could be written for $Cd^{2+}$.

The toxic effect is determined by the fraction of the total number of metal binding sites occupied by $Ni^{2+}$ or $Cd^{2+}$ ($f_{NiBL}$, $f_{CdBL}$) (De Schamphelaere and Janssen, 2002; Lock et al., 2007). The formula for calculating $f_{NiBL}$ was derived as follows:

$$f_{NiBL} = \frac{[NiBL^{+}]}{[BL]_T} =$$

$$\frac{K_{NiBL} \times \{Ni^{2+}\}}{1 + K_{NiBL} \times \{Ni^{2+}\} + K_{HBL} \times \{H^{+}\} + K_{CaBL} \times \{Ca^{2+}\} + K_{MgBL} \times \{Mg^{2+}\} + K_{NaBL} \times \{Na^{+}\} + K_{KBL} \times \{K^{+}\}}$$
(2-3)

In the BLM concept, $f_{NiBL}$ is constant at the 50% effect level for each species regardless of water chemistry (Meyer et al., 1999). When inhibition of lettuce root elongation is up to 50%, the above equation can be organized as:

$$\mathrm{IC}_{50\{\mathrm{Ni}^{2+}\}} = \frac{f^{50\%}_{\mathrm{NiBL}}}{(1-f^{50\%}_{\mathrm{NiBL}})\times K_{\mathrm{NiBL}}} \times \quad (2\text{-}4)$$
$$(1+K_{\mathrm{CaBL}}\times\{\mathrm{Ca}^{2+}\}+K_{\mathrm{MgBL}}\times\{\mathrm{Mg}^{2+}\}+K_{\mathrm{NaBL}}\times\{\mathrm{Na}^{+}\}+K_{\mathrm{KBL}}\times\{\mathrm{K}^{+}\}+K_{\mathrm{HBL}}\times\{\mathrm{H}^{+}\})$$

Similar formulas can be derived for $Cd^{2+}$ if needed. According to the assumptions of the BLM, if there is a linear relationship between $IC_{50}$ of $Ni^{2+}$ or $Cd^{2+}$ and the activity of one cation including hydrogen, when the others are constant, the toxicity of $Ni^{2+}$ or $Cd^{2+}$ could be considered to be significantly influenced by this cation/hydrogen. Then the values of $K_{HBL}$, $K_{NaBL}$, $K_{CaBL}$, $K_{MgBL}$ and $K_{KBL}$ may be calculated from the slope and intercept of the linear regression line between $IC_{50}$ of $Ni^{2+}$ or $Cd^{2+}$ and the activities of either $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ or $H^+$ (Le, 2012). Linear regression analysis of cations effects on $IC_{50}$ of $Ni^{2+}$ or $Cd^{2+}$ was performed using Graphpad Prism 5.00 (GraphPad Software Inc., San Diego, U.S.).

The median inhibition concentrations ($IC_{50}$ of $Ni^{2+}$ or $IC_{50}$ of $Cd^{2+}$) were calculated by fitting the root growth response (*REI*) to a symmetrical sigmoidal curve using the non-linear regression method in GraphPad Prism 5.00. Duplicate data points were expressed as the average value.

$$REI = \frac{100}{1+10^{((\log \mathrm{IC}_{50}-x)\times Hillslope)}} \quad (2\text{-}5)$$

where *Hillslope*: the steepness of the curve; *x*: the corresponding activities of Ni or Cd inducing the toxic response.

The values of $K_{NiBL}$ and $f^{50\%}_{NiBL}$ were derived using the data of pH-set or cation-sets in which statistical and constant impacts on the $IC_{50}$ of $Ni^{2+}$ were observed. The detailed procedure followed the methodology presented by De Schamphelaere and Janssen (2002): the optimum $K_{NiBL}$ was selected as the value which resulted in the best fit between the logit of the 4-d *REI* and $f_{NiBL}$; the associated constants ( $f^{50\%}_{NiBL}$ and $\beta$) were obtained based on the logistic relationship (Thakali et al., 2006a) of $f_{NiBL}$ versus the *REI* using the software Origin 8.0725 (Origin Lab, Northampton, UK) as follows:

$$REI = \frac{100}{1+(\frac{f_{\mathrm{NiBL}}}{f_{\mathrm{NiBL}}^{50\%}})^{\beta}} \qquad (2\text{-}6)$$

where $\beta$: the shape parameter that determines the steepness of the response curves.

### 2.2.6 Mathematical description of model comparison

Additionally, the TMM and the FIAM were also fitted to the same dataset to compare the model performance with BLM. An additional description of the models developed in this study is presented in the Supplementary material S2.3. The following equation was set up for Ni as an example:

$$REI = \frac{100}{1+(\frac{\mathrm{Total\,Ni}}{\mathrm{IC50}})^{\beta}} \qquad (2\text{-}7)$$

$$REI = \frac{100}{1+(\frac{\{\mathrm{Ni}^{2+}\}}{\mathrm{IC50}_{\{\mathrm{Ni}^{2+}\}}})^{\beta}} \qquad (2\text{-}8)$$

The measured concentrations of Ni and Cd in solution were used as the dose in the TMM (equation 2-7). The FIAM used the calculated metal ion activities of Ni and Cd as the dose (equation 2-8). The fitting parameters, namely $\beta$ and the concentration ($IC_{50}$) or the free metal ion activity ($IC_{50}$ of $Ni^{2+}$) inducing 50% inhibition of root elongation were estimated by minimizing the root-mean-squared error (*RMSE*) of the predicted *REI*s using Origin 8.0725. The lower value of *RMSE* was used as an indicator of a better model among the TMM, the FIAM and the BLM:

$$RMSE = \sqrt{\frac{SS}{n}} \qquad (2\text{-}9)$$

where *SS* = residual sum of squares; n = number of points.

## 2.3 Results

### 2.3.1 Impacts of Ni and Cd on lettuce morphology

In addition to inhibition effects on the root elongation, leaves of lettuce seedlings exposed to medium concentrations of Ni and Cd were smaller and lighter green than leaves in the control groups. The hypocotyls of exposed plants became swollen and lateral roots were shortened or even absent. At higher concentrations of Ni and Cd, both tops of lateral roots and taproots turned brown and became thinner. Leaves of seedlings exposed to Cd were influenced to a higher extent when compared with plants exposed to the same concentration of Ni (data not shown in this study).

### *2.3.2 Impacts of pH and cations on the toxicity of $Ni^{2+}$ and $Cd^{2+}$*

The initial concentrations of $Cu^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $Na^+$, and $Zn^{2+}$ in the Steiner solution were measured to be 0.003, 1.674, 2.103, 5.662, 1.251, and 0.002 mM, respectively. The individual effects of $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, and $H^+$ on the toxicity of $Ni^{2+}$ and $Cd^{2+}$ are presented in Figure 2.1 a-e and Figure 2.2 a-e. The pH value was the average of each adjustment during the 4-d exposure period.

The $IC_{50}$ of $Ni^{2+}$ for lettuce was found to be in the range of 15 to 56 μM with varying pH and concentrations of $Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$. An increase in $Mg^{2+}$ activity from 0.85 to 5.90 mM (Figure 2.1 d) resulted in a significant increase of the $IC_{50}$ of $Ni^{2+}$ ($r^2$=0.90, $p$=0.0001) i.e. the $Ni^{2+}$ toxicity was reduced by a factor of 2.4. No consistent or statistically significant ($p$>0.05) linear correlations were found between the activity of $H^+$, $Na^+$, $K^+$, $Ca^{2+}$ and the $IC_{50}$ of $Ni^{2+}$ in the concentration range investigated in this study (Figure 2.1 a, b, c, e).

The $IC_{50}$ of $Cd^{2+}$ to lettuce ranged from 5 to 22 μM. An inconsistent reduction in $Cd^{2+}$ toxicity was observed with decreasing $H^+$ activity (Figure 2.2 e) which was opposite to the BLM assumption. When pH ≤ 7 this inverse relationship became significant ($r^2$=0.90, $p$=0.013). No significant changes in the 4-d $IC_{50}$ of $Cd^{2+}$ were found when the activities of either $Na^+$, $K^+$, $Ca^{2+}$ or $Mg^{2+}$ were varied (Figure 2.2 a, b, c, d).

### *2.3.3 Model development*

Based on the above findings, only the ameliorative influence of $Mg^{2+}$ on Ni-toxicity was statistically significant in this study across the concentration ranges tested. This implies that $Mg^{2+}$ should be taken into account when assessing Ni toxicity to lettuce instead of only using total Ni concentration or the activity of $Ni^{2+}$.

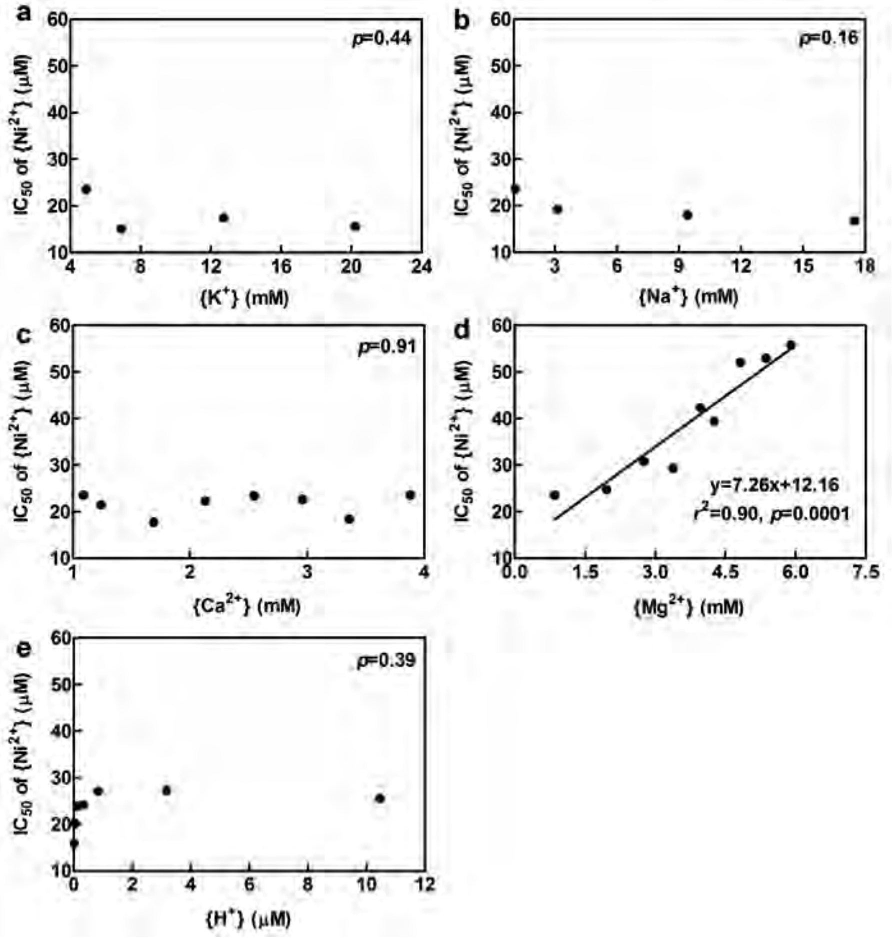

Figure 2.1 Relationships between the median inhibition activity of $Ni^{2+}$ expressed as $IC_{50}$ values and the activities of $K^+$ (a), Na+ (b), $Ca^{2+}$ (c), $Mg^{2+}$ (d) and $H^+$ (e) in the exposure medium for lettuce root elongation inhibition. The solid line represents the statistically significant linear correlation. The activities of hydrogen ions, i.e. 10.47, 3.16, 0.83, 0.33, 0.11, 0.04, and 0.01 (μM) correspond to the pH values as following: 4.98, 5.5, 6.08, 6.48, 6.94, 7.45, and 7.84.

The Mg-set (the toxic effects of Ni in the presence of different concentrations of Mg), totaling 51 treatments, was used for modeling and associated constants estimation. When the $IC_{50}$ of $Ni^{2+}$ (M: mol/L) was considered as a function of $Mg^{2+}$ (M), linear regression gave the following expression to predict the $Mg^{2+}$ dependence of $Ni^{2+}$

toxicity to root elongation of lettuce:

$$\mathrm{IC}_{50\{\mathrm{Ni}^{2+}\}} = 0.0073 \times \{\mathrm{Mg}^{2+}\} + 1 \times 10^{-5} \quad (r^2=0.90,\ p=0.0001) \qquad (2\text{-}10)$$

The $Mg^{2+}$ stability constant was calculated from the slope of the relationship between $IC_{50}$ of $Ni^{2+}$ and the activity of $Mg^{2+}$, divided by the intercept (Figure 2.1 d), as $\log K_{MgBL}$=2.86. The optimal values of $\log K_{NiBL}$ and $f_{NiBL}^{50\%}$ were calculated to be 5.1 and 0.57, respectively.

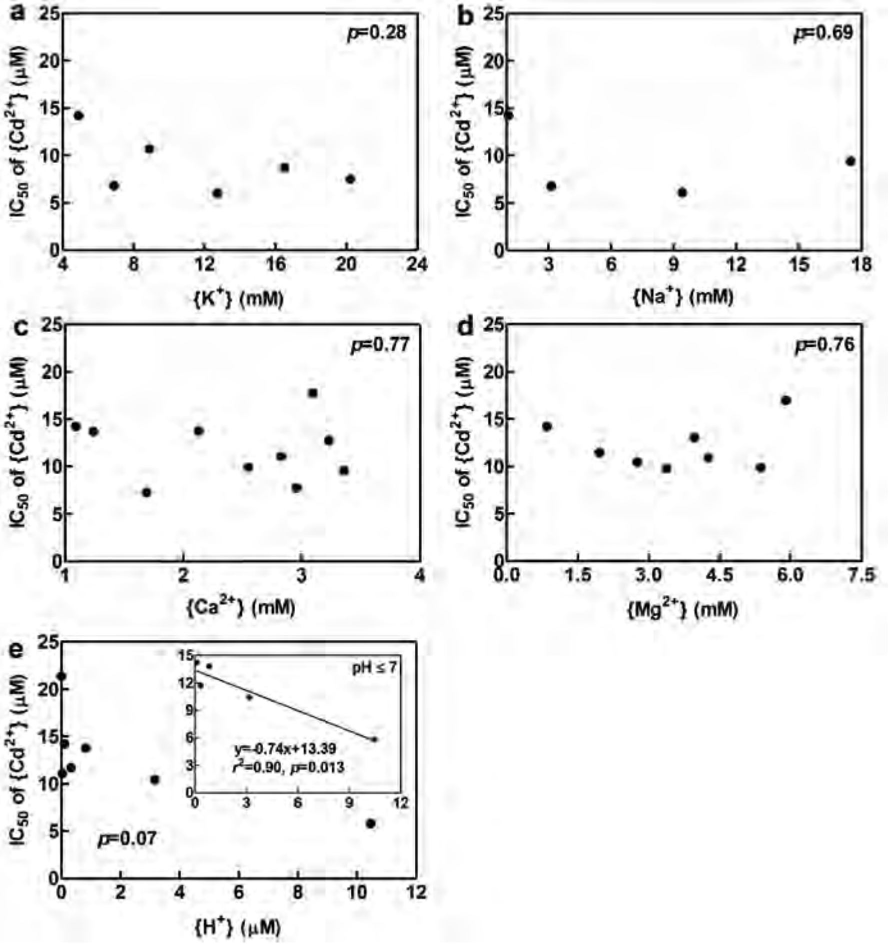

Figure 2.2 Relationships between the median inhibition activity of $Cd^{2+}$ expressed as $IC_{50}$ values and the activities of $K^+$ (a), $Na^+$ (b), $Ca^{2+}$ (c), $Mg^{2+}$ (d) and $H^+$ (e) in the exposure medium for lettuce root elongation inhibition. The activities of hydrogen

ions, i.e. 10.47, 3.16, 0.83, 0.33, 0.11, 0.04, and 0.01 (μM) correspond to the pH values as following: 4.98, 5.5, 6.08, 6.48, 6.94, 7.45, and 7.84. The solid line in the small graph represents the statistically significant linear correlation when pH ≤ 7.

Using the TMM, the FIAM and the BLM for toxicity assessment of Ni and Cd, the dose-response curves obtained are shown in Figure 2.3 and Figure 2.4. Compared to the TMM (*Adj.* $R^2$=0.49) and the FIAM (*Adj.* $R^2$=0.60), the Ni-BLM (*Adj.* $R^2$=0.80) with the lowest *RMSE* (14.07) which was derived using the Mg-set in the present study significantly improved the predictive capability for the 4-d *REI* of lettuce (*L. sativa*). Moreover, 90% of the $IC_{50}$ of $Ni^{2+}$ can be explained by considering competitive effects of $Mg^{2+}$. However, since non-significant and constant competitive effects of cations or pH were observed in the Cd-toxicity experiments, a Cd-BLM could not be established in the present study. Thus, the TMM and the FIAM were used in predicting toxicity of Cd to lettuce based on the dataset without adding cations or varying pH values. Eighty percent of the variance in Cd toxicity to lettuce root elongation can be explained by using both the TMM and the FIAM. The detailed model fits and estimated parameters are all presented in Table 2.1.

Table 2.1 Model fit and the estimated parameters of the TMM, FIAM and BLM for Ni and Cd toxicity to lettuce root elongation inhibition.

| Metal | Model | *Adj.* $R^2$ | *RMSE* | $f_{NiBL}^{50\%}$ or $IC_{50}$ (M) | $\beta$ |
|---|---|---|---|---|---|
| Ni | TMM | 0.49 | 22.02 | 6.60e-05 | -2.18 |
| | FIAM | 0.60 | 19.61 | 3.15e-05 | -2.47 |
| | BLM | 0.80 | 14.07 | 0.57 | -5.42 |
| Cd | TMM | 0.80 | 8.92 | 2.85e-05 | -0.78 |
| | FIAM | 0.80 | 8.92 | 1.50e-05 | -0.79 |

*Adj.* $R^2$ indicates the adjusted determination coefficient of the models between the observed and the predicted *REI*; $\beta$ indicates the fitting parameter determining the slope of the dose-response curve; *RMSE* indicates root-mean-squared error of the predicted *REI*; $f_{NiBL}^{50\%}$ indicates the fraction of the total number of binding sites occupied by toxic ions when inhibition of lettuce root elongation is up to 50%; $IC_{50}$ indicates the concentrations or free ion activities of toxic metals inducing 50% inhibition of root elongation.

## 2.4 Discussion

### *2.4.1 Cations and protons effects on Ni toxicity*

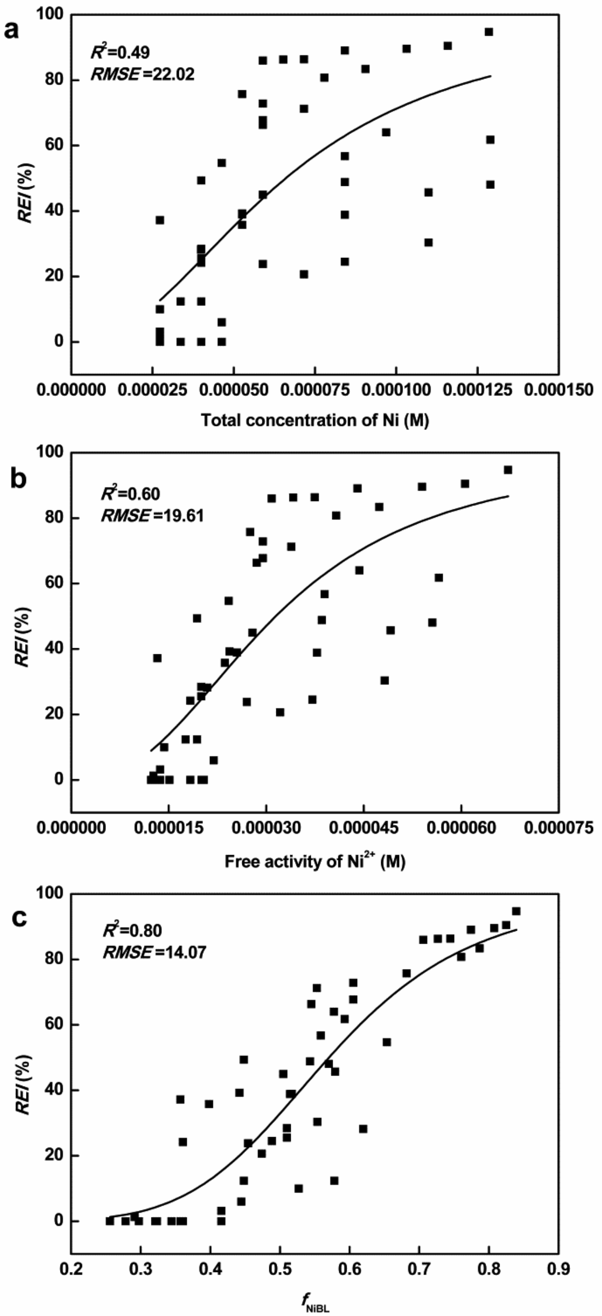

Figure 2.3 Dose-response relationships between 4 d root elongation inhibition (*REI*) and total Ni concentration (a), free $Ni^{2+}$ activity (b), fraction of the total biotic ligand sites occupied by toxic $Ni^{2+}$ ($f_{NiBL}$) (c). The solid lines represent the logistic model fits

(equation 2-6, 2-7, and 2-8). $R^2$ indicates the adjusted determination coefficient of the models between the observed and the predicted *REI*. *RMSE* indicates root-mean-squared error of the predicted *REI.*

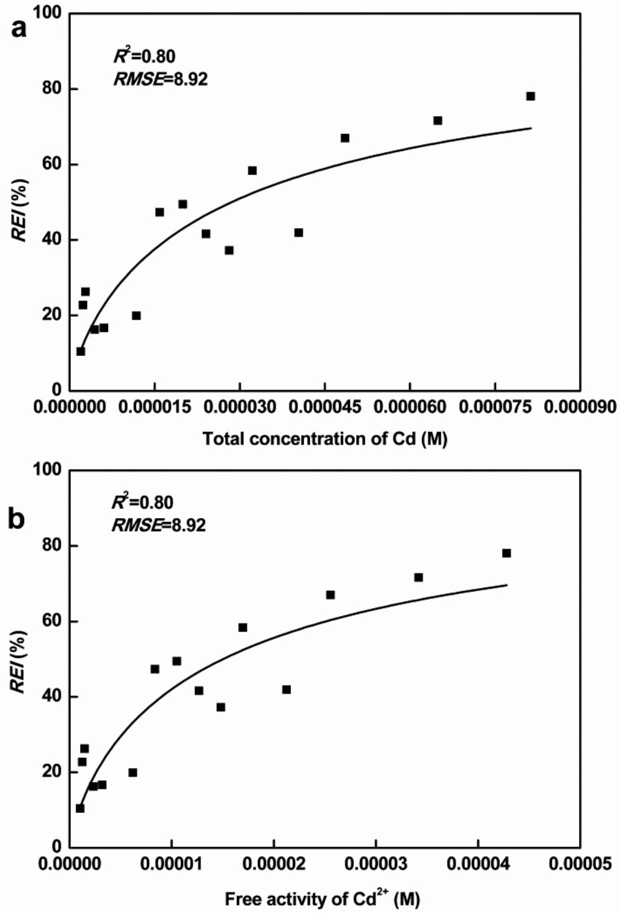

Figure 2.4 Dose-response relationships between 4 d root elongation inhibition (*REI*) and total Cd concentration (a), free $Cd^{2+}$ activity (b). The solid lines represent the logistic model fits (equation 2-7 and 2-8). $R^2$ indicates the adjusted determination coefficient of the models between the observed and the predicted *REI*. *RMSE* indicates root-mean-squared error of the predicted *REI*.

Based on the data obtained from the present study, the TMM and the FIAM were used to quantitatively explain the toxicity of Ni to root elongation of lettuce (*L. sativa*). By considering the mitigating effect of $Mg^{2+}$ in the solution, a Ni-BLM was developed which significantly improved the toxicity estimation of Ni. These findings for Ni supported one of the key assumptions of the BLM that cations compete with metal ions for transport sites (De Schamphelaere and Janssen, 2002). The number of the binding sites occupied by metal ions which was integrated in the BLM seemed to be a better indicator for Ni-toxicity assessment to lettuce as compared with total concentration and free ion activity.

The statistically significant competitive effect of $Mg^{2+}$ on Ni-toxicity observed in this study suggested that $Mg^{2+}$ and $Ni^{2+}$ shared the same uptake pathway. This finding also confirmed the hypothesis that $Mg^{2+}$ provided protection to the biotic ligands of lettuce in case of exposure to $Ni^{2+}$ based on the previous studies (Lock et al., 2007; Li et al., 2009). The response mechanism of terrestrial plants to Ni may differ from aquatic plants as no significant interactive effects of Mg could be detected on the toxicity of Ni in aquatic plants (Deleebeeck et al., 2009; Gopalapillai et al., 2013). Our observation can be explained by the fact that Ni and Mg have a similar ionic radius (Snavely et al., 1991). As no other significant impacts from divalent cations (such as $Ca^{2+}$) were observed in our case, reduced Ni toxicity could not be explained only by the change in the electrical negative potential at the plant membrane as suggested by Gopalapillai et al. (2013). Moreover, $Ni^{2+}$ was found to displace $Mg^{2+}$ in ribulose-1, 5-bisphosphate-carboxylase/oxygenase (RuBisCO) and lead to the loss of enzyme activities (Wildner and Henkel, 1979) which illustrated the occurrence of interactions between $Ni^{2+}$ and $Mg^{2+}$ in the organisms. The BLM as a better method for Ni-toxicity prediction comparing with the TMM and the FIAM (Table 2.1) suggested that not only metal species in the solution but also water chemistry did influence Ni-toxicity.

Values of binding constants as found in our study were compared with constants reported in other studies for plants in Table 2.2. The log $K_{MgBL}$ value obtained in our study was similar to the value reported by Deleebeeck et al. for algae (2009) but was lower than the values reported by Lock et al. (2007), Li et al. (2009), Antunes and Kreager (2009) for barley, Thakali et al. (2006b) for barley and tomato. The value of log $K_{NiBL}$ found for lettuce in our study was relatively close to the former results for barley listed in Table 2.2, but lower than the value calculated for tomato.

The optimized value of $f_{\mathrm{NiBL}}^{50\%}$ in this study implied that 57% of the BL sites had to be occupied by $Ni^{2+}$ to cause 50% inhibition, which was similar to the value reported by Lock et al. (2007). The significance of $f_{50}$ depends not only on the test situations but also on the theoretical basis for building the BLM (Antunes et al., 2007) which may also result in the differences of $K_{\mathrm{NiBL}}$ values even for the same test organism, such as barley.

$Ca^{2+}$ was not observed to inhibit $Ni^{2+}$ toxicity on the site of action in lettuce which was in line with the researches of Lock et al. (2007) and Thakali et al. (2006b). Unlike $Mg^{2+}$ in chlorophyll associated with photosynthesis, $Ca^{2+}$ as a cofactor of calmodulin in cellular signaling may explain the observation that no interactions occurred between $Ca^{2+}$ and $Ni^{2+}$ (Hossain et al., 2012).The modification of either the $K^+$ or the $Na^+$-concentration exerted non-significant effects on Ni-toxicity, which was consistent with previous studies presented in Table 2.2. Unlike the researches on barley and algae exposed also in solutions (Deleebeeck et al., 2009; Li et al., 2009), insignificant competitive effects of $H^+$ to lettuce were found in the present study. Lettuce (*L. sativa*) grows better at a soil pH ranging from slightly acid to neutral. When the pH value was below 6, $H^+$ may have adverse impacts on lettuce seedlings which offset the protective effects on plants. This may be the reason that pH was not observed to be significantly involved in Ni-toxicity. In summary, the estimated parameters and the effects of cations and protons found in the present study can serve as a basis for further research on mechanisms that induce differences in bioavailability and toxicity of Ni and for actual risk assessments of Ni in soil.

### *2.4.2 Cations and protons effects on Cd toxicity*

Competitive effects of major cations ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$) in the solution on Cd-toxicity to lettuce were not observed at a physiological level using *REI* as the endpoint in this study. Only a slight but non-significant negative trend of $IC_{50}$ of $Cd^{2+}$ was observed across the whole range of pH levels in the present study (Figure 2.2). Our findings demonstrated that the resistance mechanism of lettuce to diverse metals was metal-specific since no apparent interaction between $Cd^{2+}$ and cations was observed at the water-organism interface. Instead of BLM, TMM or FIAM was found to be a reasonable indicator of Cd-toxicity to lettuce root growth.

The 4-d $IC_{50}$ of $Cd^{2+}$ increased as pH increased to 8 which indicated that there was no competitive interaction between $Cd^{2+}$ and $H^+$ for lettuce. François et al. (2007) also found a non-competitive inhibition by $H^+$ on the green algae *Chlamydomonas reinhardtii*. They suggested that the BLM was not capable of describing the true impact of pH on Cd uptake by algae. Instead of increased unoccupied sites of action, the metal fluxes were increased by conformational changes of the transport proteins when pH values were raised. When $pH \leq 7$ the toxicity of $Cd^{2+}$ was enhanced obviously by the increased hydrogen concentration in the present study which was contrary to the BLM theory. Lei et al. (2012) pointed out that a combination of morphological changes may be the fundamental reason why toxicity of $Cd^{2+}$ on tobacco decreased as the pH gradually increased. At higher pH levels, the activities of other cadmium species increase in the solution, such as $CdCO_3$ and $CdOH^+$. Otherwise, at lower pH values, the dominantly bioavailable Cd species would be $Cd^{2+}$ which concentration would be increased with increased $H^+$. This may also lead to the result that most of the toxic effects of Cd to lettuce can be explained by both TMM and FIAM when pH was maintained around 7 (Fig. 2.4). Another explanation of our finding may be related to electrostatic effects of the cell membrane, as the surface negativity of the membranes was reduced when the proton activity was increased. This reduction of surface negativity will increase the surface-to-surface trans-membrane potential difference (Wang et al., 2010b) which will in turn increase the electrical driving force of toxic metallic ions across the cell membrane (Gopalapillai et al., 2013). Therefore, the mitigating effect of higher pH on Cd-toxicity may be due to the change of the electrostatic nature of the plant cell wall. On the other hand, according to the theory of Lexmond and van der Vorm (1981), the toxicity of metal ions often decreases with increasing pH for plants when grown in nutrient solution. This may result from changes of the morphology and physiology of plant roots in solution cultures that differed from those in the soil environment because of the aeration and microbial status (Voigt et al., 2006). Thus, it seemed to be theoretically possible to reduce the bioavailability of Cd for plants when alkaline fertilizers were applied to increase pH values. Emphasis can be laid on whether moderately contaminated soils are suitable for specific crop plants culture by increasing pH levels in future work.

Besides pH, changes in $Na^+$, $K^+$, and $Mg^{2+}$ concentrations also had no apparent effects on Cd-toxicity, which was consistent with the previous findings in plant system (Das et al., 1997) and indicated that $Na^+$, $K^+$, and $Mg^{2+}$ did not interfere with $Cd^{2+}$ at the site of action or vice versa. Although the toxic effects of Cd to lettuce grown in a soil matrix have been shown to be ameliorated by $Ca^{2+}$ (Voigt et al., 2006), the antagonistic effect between $Cd^{2+}$ and $Ca^{2+}$ was not observed in the present study. The higher concentration of dissolved organic matter (DOM) in the forest soil extracts may account for the differences of results by influencing the metal speciation (Haghiri, 1974). The uptake of Cd in the nitrate test solutions was predominantly controlled by $Cd^{2+}$ (Cabrera et al., 1988). Metal toxicity of Cd decreased with increasing DOM concentration, which may be caused by the formation of less toxic organically complexed metals. In other words, metals may be sequestered by chelating with specific organic acid and the toxic effects of which would be reduced (Dong et al., 2007). On the other hand, root damage was assumed to cause the reduced uptake of Ca in barley shoot rather than the specific ion antagonism between $Cd^{2+}$ and $Ca^{2+}$ (Cabrera et al., 1988). Based on the findings above, the traditional BLM-type as single-site competition suggested was not applicable in assessing the toxicity of Cd to lettuce exposed in hydroponic solution.

### *2.4.3 Effects of solution salinity and counter-ion on metal toxicity predictions*

In our toxicity experiments, Steiner solution prepared by adding inorganic salts was used as the hydroponic nutrient solution to prevent reduction in growth rate due to low availability of mineral nutrients. Some researchers declared that additions of large amounts of soluble metal salts may confound toxicity by increasing the ionic strength or salinity in the soil solution (Stevens et al., 2003; Rooney et al., 2007). Besides coexistent major cations discussed in 2.4.1 and 2.4.2, metal counter-ions (e.g. $NO_3^-$) were also found to lead to overestimation of $EC_{50}$s of Zn and Pb in soil samples (Stevens et al., 2003). However, no apparent osmotic change was observed due to the increased salinity in irrigation water (Shannon and Grieve, 1999). Lettuce was chosen as a moderately salt sensitive crop (Andriolo et al., 2005) and *L. sativa* showed a higher tolerance for salt than other lettuce species (Shannon and Grieve, 1999). In this study, no negative effects were observed on lettuce growth using Steiner solution. Conversely, the calcium salts added, may first alleviate the nutrients deficiency (Wang et al., 2010b) if malnutrition occurred. The electrical conductivity (EC) that reduces germination by 50% of lettuce was found to be 8 dS/m by adding NaCl (Shannon and Grieve, 1999). The EC values of the solutions used in the present study were all below this threshold. The Steiner solution was used as both plant culture and test medium for all the treatments, it is therefore that the effects of the background ionic environment could be excluded. Although the hydroponic technique is convenient nowadays, the more effective use of the technique in the field of toxicology is worth pondering. Instead of influence on plant responses to Ni, added anions such as sulfate, nitrate, or chloride may affect metal speciation in solution (Gopalapillai et al., 2013). The speciation equilibrium in aqueous solution is the basis for toxicity modeling of metals by FIAM or BLM. The metal species in solution and those binding to the biological surface are expected at a thermodynamic equilibrium as well in the BLM (Worms and Wilkinson, 2007). According to the formula of Steiner solution, the major anions, such as $NO_3^-$, $SO_4^{2-}$ and $PO_4^{3-}$ at relatively higher concentrations were all included in the speciation calculation. Therefore, the effect of anions cannot be ignored in metal risk assessment for terrestrial plants when free metal ion is supposed to be one of the most important species to induce toxicity.

## 2.5 Conclusions

In this study, it was found that the mechanism of lettuce resistance to excessive metals differed for Cd and Ni. By considering the competitive effect of $Mg^{2+}$, the prediction for Ni-toxicity to lettuce (*L. sativa*) was significantly improved as compared to FIAM and TMM. The protective effect of $Mg^{2+}$ was incorporated into the BLM model as represented by the value of log $K_{MgBL}$=2.86. The conditional stability constants were calculated to be log $K_{NiBL}$=5.1, and $f_{NiBL}^{50\%}$=0.57. Impacts of other cations ($Na^+$, $K^+$, and $Ca^{2+}$) and pH were excluded because of lack of significant competitive effects on the toxicity variation. According to observations in Cd-toxicity experiments, major cations or protons did not inhibit the toxicity of $Cd^{2+}$ to lettuce via competition as expected in the BLM concept. Instead, the total concentration or free ion activity succeeded in predicting toxicity of Cd. It was therefore represented that in hydroponic solution, no ionic brakes apparently inhibited the movements of $Cd^{2+}$ entering vegetable crops which may explain that the toxic effects of Cd to lettuce seedlings showed at the lower concentration as compared to Ni. Besides a better understanding of toxic mechanisms of metals, the models developed in the present study could be used to predict toxicity of Cd and Ni to lettuce and 50% inhibition concentration of $Ni^{2+}$ to cut down the costs for regulatory testing of chemicals in soil.

Table 2.2 Comparison of binding constants for the Ni-BLM with literature data.

| Studied Organisms | $\log K_{NiBL}$ | $\log K_{MgBL}$ | $\log K_{CaBL}$ | $\log K_{NaBL}$ | $\log K_{HBL}$ | $f^{50\%}_{NiBL}$ | Reference |
|---|---|---|---|---|---|---|---|
| Barley (*Hordeum vulgare*) | 3.10 | 4.60 | 3.30 | -[c] | 5.40 | -[c] | Autunes and Kreager, 2009 |
| Barley (*Hordeum vulgare*) | 5.27 | 3.47 | -[b] | -[b] | -[b] | 0.57 | Lock et al., 2007 |
| Barley (*Hordeum vulgare*) | 3.60±0.53 | 3.81±0.6 | 1.50[a] | -[c] | 4.53±0.62[a] | 0.05 | Thakali et al., 2006b |
| Barley (*Hordeum vulgare*) | 4.83±0.17 | 4.01±0.03 | 1.60±0.15 | -[b] | 4.29±0.12 | 0.36±0.08 | Li et al., 2009 |
| Tomato (Lycopersicon esculentum cv. Moneymaker) | 6.05±0.15 | 5.23±0.47 | 5[a] | -[c] | 6.52±0.18 | 0.05 | Thakali et al., 2006b |
| Algae (*Pseudokirch-neriella subcapitata*) | -[c] | 3.3 (3.0-3.7) | -[b] | -[c] | 6.5 (6.4-6.6) | -[c] | Deleebeeck et al., 2009 |
| Lettuce (*Lactuca sativa*) | 5.10 | 2.86 | -[b] | -[b] | -[b] | 0.57 | Present study |

[a] The parameter is not significant but still included in the BLM by the authors.

[b] The parameter is not significant and therefore not included in the BLM by the authors.

[c] The parameter is not presented by the authors.

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## Supplementary materials

### S2.1 Formula of the Steiner solution

The Steiner nutrient solution is composed by adding 10 ml of solution A and B and 1 ml of solution C and D to one liter of tap water and then aerating the solution during 16 hours. Afterwards the pH is measured and adjusted at pH 6.5±0.5 using no more than 158 µl concentrated sulphuric acid per liter solution. The precipitation that might have been produced during the aeration will disappear while adjusting the pH.

*Solution A*: 68 g $Ca(NO_3)_2 * 4H_2O$, 62 g $KNO_3$ per 1liter water.

*Solution B*: 46 g $MgSO_4 * 7H_2O$, 13.6 g $KH_2PO_4$ per 1 liter water.

*Solution C*: 2.69 g $H_3BO_3$, 2.0 g $MnSO_4 * H_2O$, 0.506 g $ZnSO_4 * 7H_2O$, 0.126 g $Na_2MoO_4 * 2H_2O$, 0.078 g $CuSO_4 * 5H_2O$ per 1 liter water.

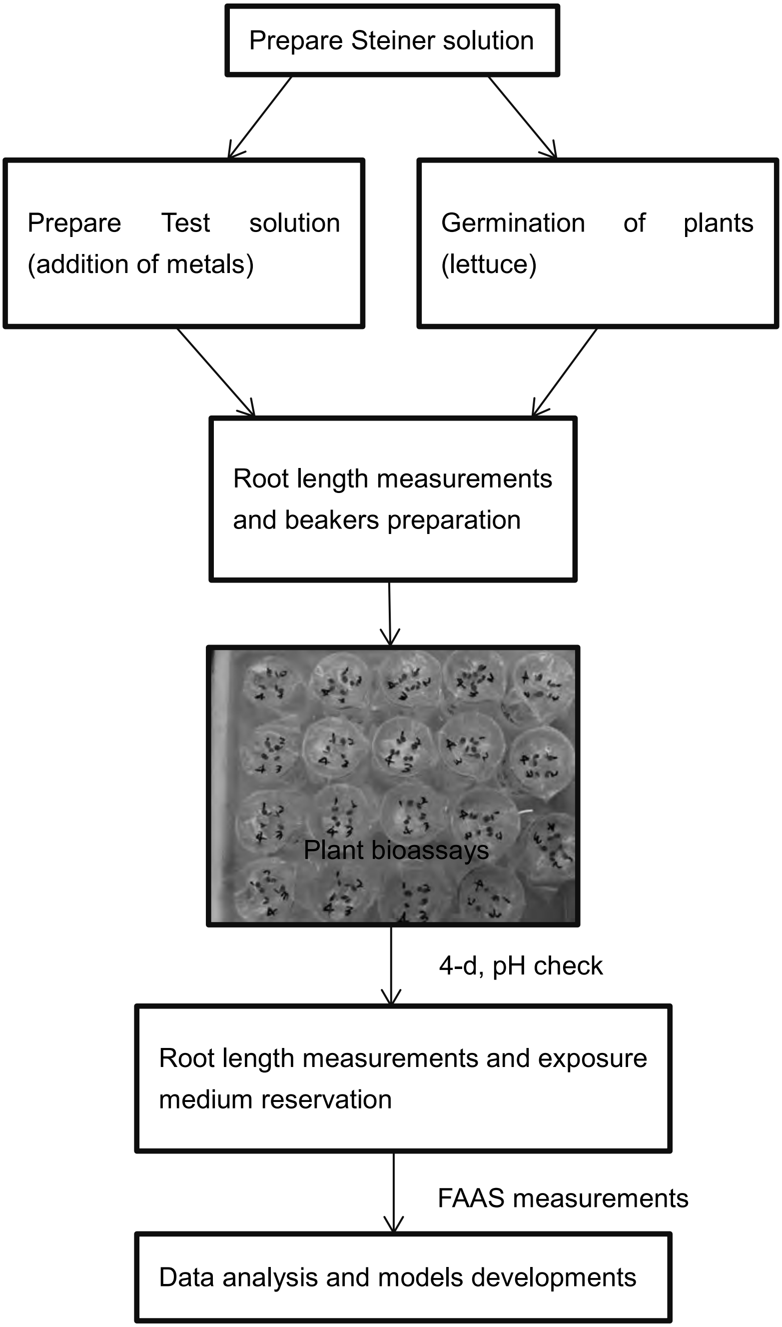
First dissolve these chemicals separately in 100 ml of tap water, where $H_3BO_3$ is dissolved in hot water (±70 °C). Then join the solutions of the first 4 chemicals, supplement with water to ± 850 ml, add the solution of $CuSO_4 * 5H_2O$ and supplement again until 1 liter. If there is a precipitation in the $CuSO_4 * 5H_2O$-solution or in solution C after adding of the $CuSO_4 * 5H_2O$-solution, then add 0.5 or1 M $H_2SO_4$ until the precipitation disappears.

*Solution D1*: 16.659 g Titriplex III ($C_{10}H_{14}O_8N_2Na_2 * 2H_2O$), 2.91 g KOH per 500 ml distilled water, dissolve the Titriplex in hot distilled water (± 70 °C).

*Solution D2*: 12.44 g $FeSO_4 *7H_2O$, 2 ml 0.5 M $H_2SO_4$ per 200 ml distilled water

*Solution D*: Join solutions D1 and D2, supplement until 900 ml with distilled water, aerate the solution during ca. 16 hours and supplement until 1litre with distilled water. After aeration the solution is coloured reddish brown.

## S2.2 Diagram of the experimental process



### S2.3 Additional description of the mechanistically based models

The cations (i.e. $H^+$, $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$) and toxic metal ions ($Cd^{2+}$ and $Ni^{2+}$) investigated in this study were assumed to form complexes with BL at a ratio which expressed as the stability constants ($K$, equation 2-2). The traditional BLM used in this study was based on the binding sites on a single biotic ligand (BL) that would influence the root elongation of lettuce. The total BL site concentration [TBL] was described as

[TBL] = [BL]+[HBL]+[NaBL]+[KBL]+[CaBL]+[MgBL]+[MBL]

When the equilibrium relationships (equation 2-2) were integrated, the above equation could be rewritten as follows

[TBL] = [BL]

$(1+K_{HBL} \times \{H^+\}+K_{NaBL} \times \{Na^+\}+K_{KBL} \times \{K^+\}+K_{CaBL} \times \{Ca^{2+}\}+K_{MgBL} \times \{Mg^{2+}\}+K_{MBL} \times \{M^{2+}\})$

Then according to the BLM concept, toxicity of Ni/Cd was determined by the fraction of the total number of BL occupied by Ni/Cd (equation 2-3). In the BLM, the fraction of metals for each species was assumed to be constant at 50% biological effect. Then equations (2-3) and (2-4) were derived to show relationships between $IC_{50Ni/Cd}$ and cations. The effect experiments were conducted to find out whether $IC_{50Ni/Cd}$ could be influenced by the cations in the solution as suggested by the BLM concept. As shown in Figure 2.1, there was only a linear relationship between $IC_{50Ni/Cd}$ and the activity of $Mg^{2+}$ (equation 2-10) when activities of other cations were constant. Thus, $K_{MBL}$ can be calculted as follows

$$K_{MgBL} = \frac{\text{slope}_{Mg^{2+}}}{\text{intercept}_{Mg^{2+}}} = \frac{\dfrac{f_{NiBL}^{50\%}}{(1-f_{NiBL}^{50\%}) \times K_{NiBL}} \times K_{MgBL}}{\dfrac{f_{NiBL}^{50\%}}{(1-f_{NiBL}^{50\%}) \times K_{NiBL}}} = \frac{0.0073}{1 \times 10^{-5}} = 730$$

$$\text{Log}\, K_{MgBL} \approx 2.86$$

With the fixed $K_{NiBL}$ value, the data of Mg-sets was fitted to equation (2-6) to obtain $f_{NiBL}^{50\%}$=0.57 and $\beta$= -5.42. Then the same dataset was fitted to TMM and FIAM (equation 2-7, 2-8) formulas and the related estimated parameters were shown in Table 2-1. Apart from BLM which cannot be built for Cd due to no competitive effects of cations or protons found in the present study, similar methods were used to develop TMM and FIAM for Cd-toxicity prediction.

# 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.

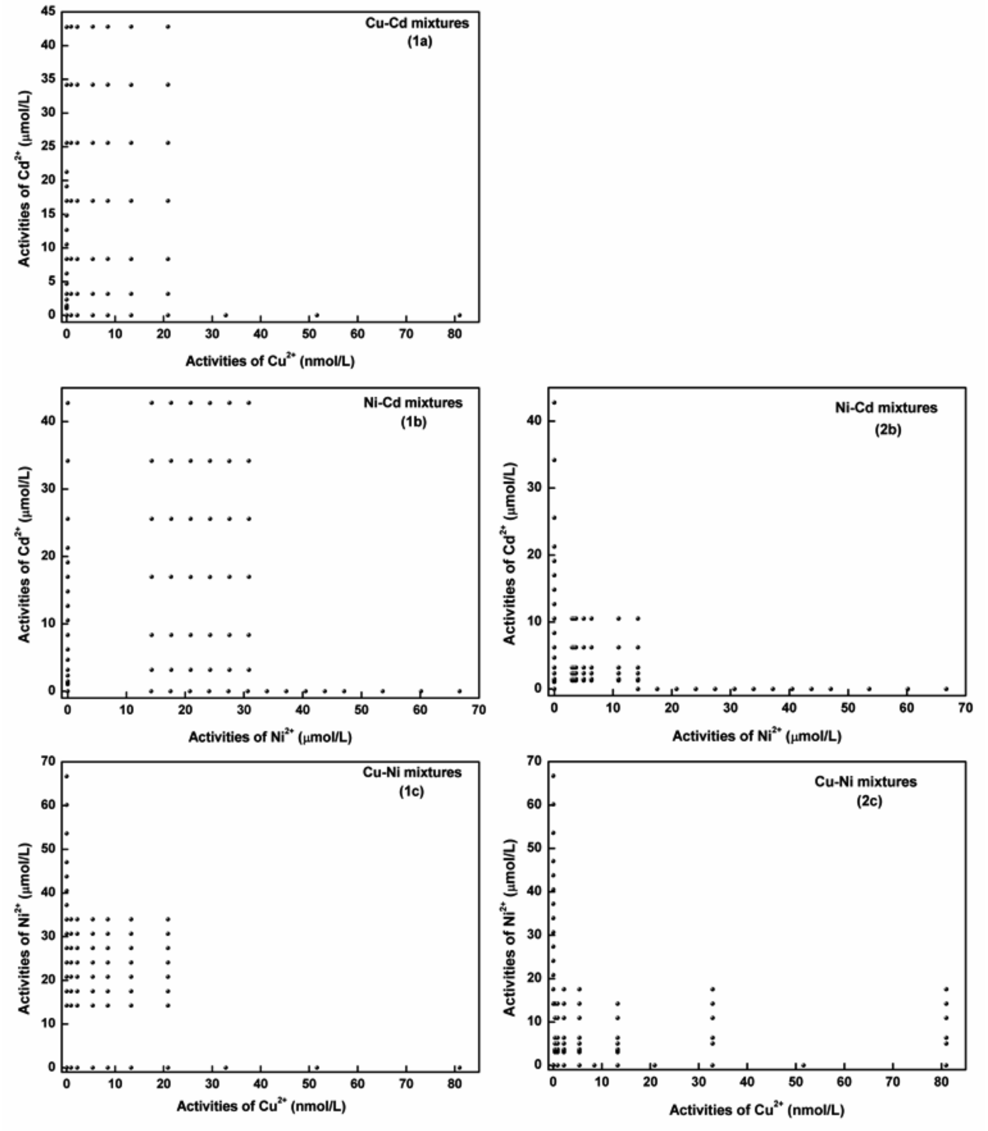


Figure 3.1 Set up of experiments for Cu-Cd, Ni-Cd, Cu-Ni mixtures expressed as free ion activities.

### *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

$$RRE = RG_{\mathrm{S}} / RG_{\mathrm{C}} \times 100\% \tag{3-1}$$

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 ($\mathrm{TU_{mix}}$) equals one.

$$\mathrm{TU_{mix}} = \sum_{i=1}^{n} c_i / \mathrm{EC}x_i \tag{3-2}$$

where $c_i$: the concentration of individual chemical *i* in the mixture with *n* chemicals, free-ion activity was used to express $c_i$; $\mathrm{EC}x_i$: the effect concentration of individual chemical *i* that results in the same effect (*x*%) as the mixture, free-ion activity was used to express $\mathrm{EC}x_i$; $\mathrm{TU_{mix}}$: 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:

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

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 ($\beta$) 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 ($\chi^2$) 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 ($\chi^2$) 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 $p$ ($\chi^2$) are shown to indicate the statistically significant level.

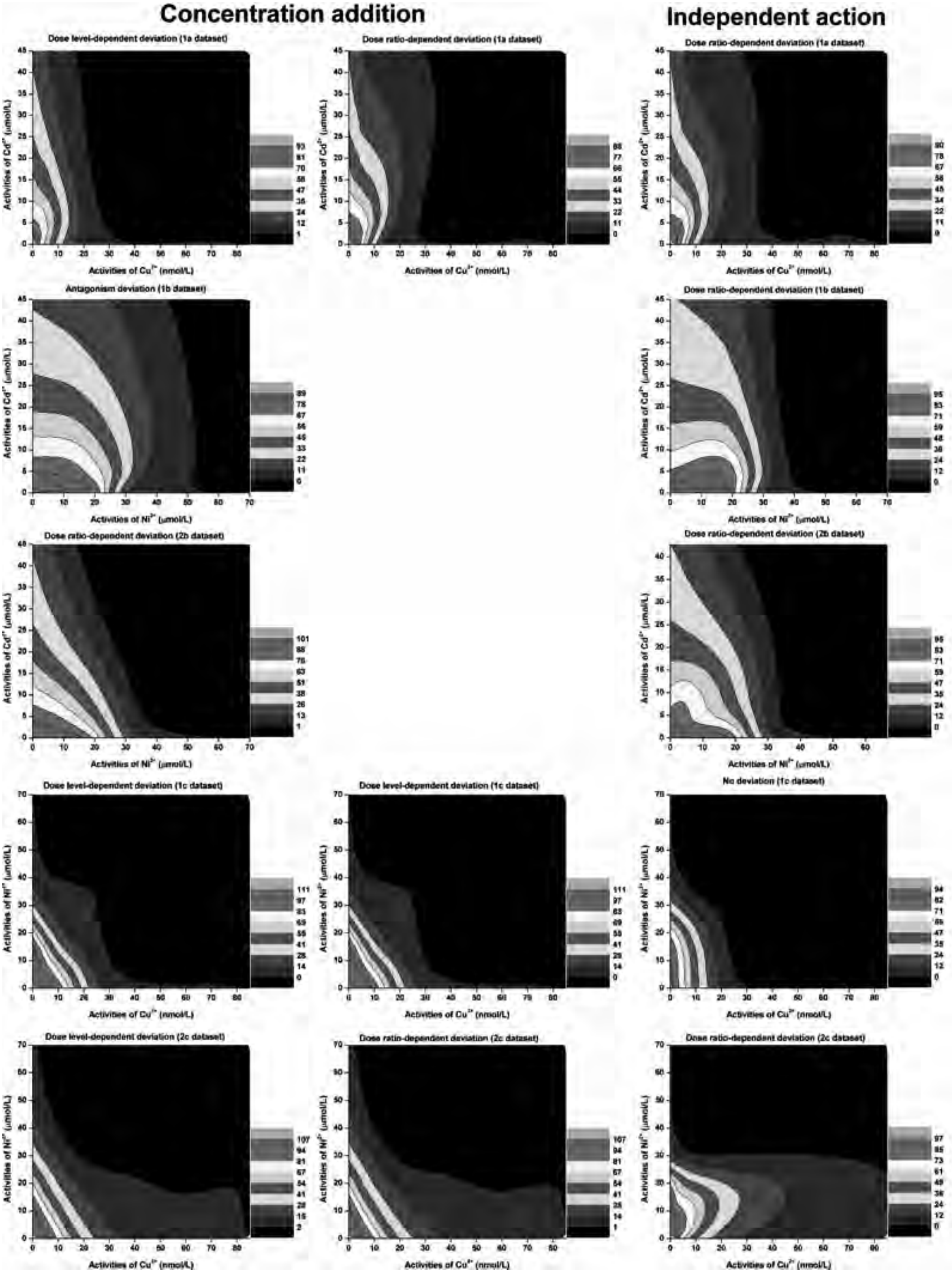
Generally, the combined effects of Cu-Cd, Ni-Cd and Cu-Ni mixtures to *L. sativa* were equally well explained by the CA- and the IA-based mixture models. The predictive ability of the mixture models differed when assessing the toxicity of different datasets of Ni-Cd mixtures. Fitting of the CA- or the 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.



Concentration addition

Independent action

Dose level-dependent deviation (1a dataset)

Dose ratio-dependent deviation (1a dataset)

Dose ratio-dependent deviation (1a dataset)

Antagonism deviation (1b dataset)

Dose ratio-dependent deviation (1b dataset)

Dose ratio-dependent deviation (2b dataset)

Dose ratio-dependent deviation (2b dataset)

Dose level-dependent deviation (1c dataset)

Dose level-dependent deviation (1c dataset)

No deviation (1c dataset)

Dose level-dependent deviation (2c dataset)

Dose ratio-dependent deviation (2c dataset)

Dose ratio-dependent deviation (2c dataset)

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-$F_{\mathrm{TOX}}$ 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<br>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<br>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<br>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<br>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<br>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

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

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

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

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.

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

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 ($\mathrm{EC}_{50}$) can be estimated with the least amount of variability, the deviation function is defined as follows for concentration addition by incorporating the $\mathrm{EC}_{50}$ isobole

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

For independent action, the $\mathrm{EC}_{50}$ isobole is defined by $P_{1,\ldots,n}$=0.5 and the function can be written as

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

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.

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

where $h(c_i)$: a cumulative distribution function, functionally related to concentration $c$ of compound $i$; $\beta_i$: 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 $\chi^2$ 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<br>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<br>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<br>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<br>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<br>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.

# Chapter 4

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## 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 $f_{\mathrm{mix}}$ 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:

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

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, $f_{\text{mix}}$ 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).

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

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

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

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

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

$$\text{TU} = \sum \text{TU}_i = \frac{f_{\text{Cu}}}{f_{50,\text{Cu}}} + \frac{f_{\text{Ni}}}{f_{50,\text{Ni}}} \tag{4-5}$$

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, $f_{\mathrm{mix}}$ and TEQ as follows:

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

In equation (4-11): $\beta$ = the fitted parameter determining the slope of the dose response curve; $x$ = the value of the toxicity index, i.e. TU, $f_{\mathrm{mix}}$ and TEQ at a given mixture concentration; $x_{50}$ = the value of TU/$f_{\mathrm{mix}}$/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 $\beta$ 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 ($3.03\times10^{-5}$ 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 $3.4\times10^{-5}$ 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 $f_{\mathrm{mix}}$ 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 $f_{\mathrm{mix50}}$ 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 $Cu^{2+}$ (< $3.38\times10^{-8}$ M), the $IC_{50}$ of $Zn^{2+}$ was increased as compared to the value when $Zn^{2+}$ operated alone ($1.06\times10^{-4}$ 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 ($2.60\times10^{-8}$ M) except at higher activities of $Zn^{2+}$ ($>1.23\times10^{-4}$ 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.

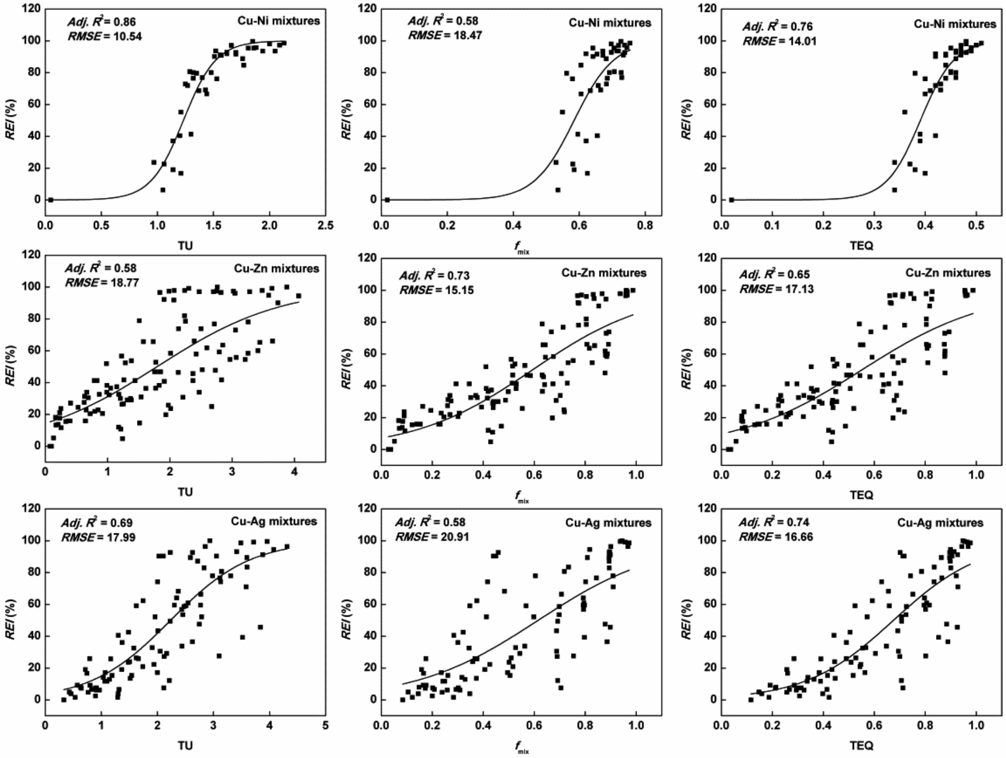

Figure 4.1 Dose-response relationships between root elongation inhibition (*REI*, %) to lettuce *L. sativa* and toxic indexes i.e. TU (first column), $f_{mix}$ (second column) and TEQ (third column) for the mixture combinations Cu-Ni (first row), Cu-Zn (second row) and Cu-Ag (third row). The solid lines 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*.

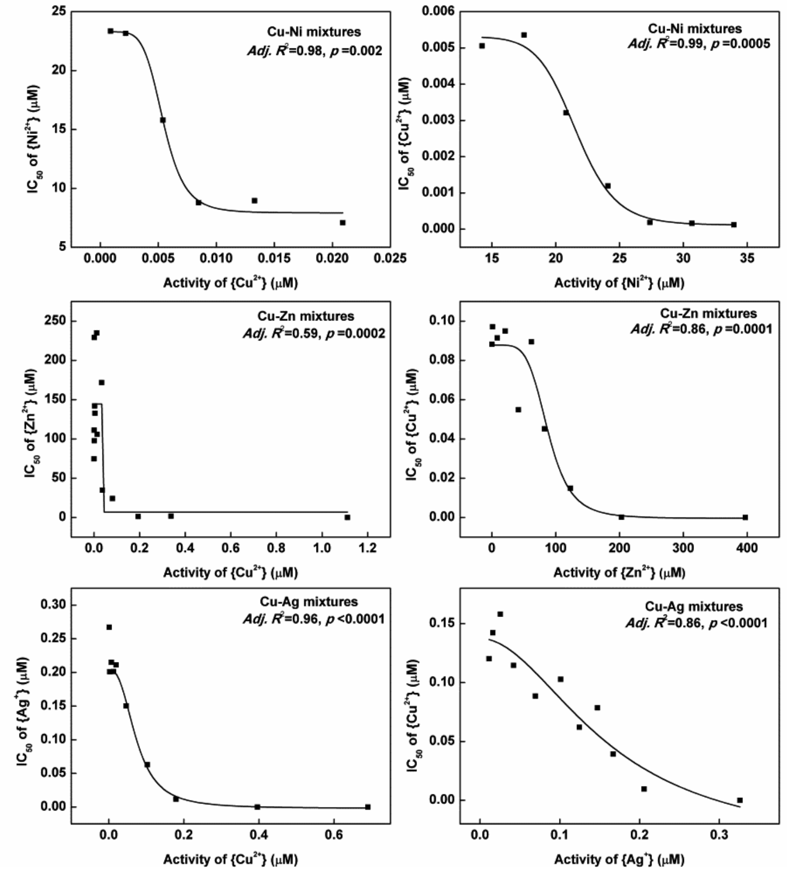

Figure 4.2 Relationships between the median inhibition concentrations ($IC_{50}$s) of $Zn^{2+}/Cu^{2+}/Ag^{2+}/Ni^{2+}$ for *L. sativa* after 4 d exposures and the activities of $Cu^{2+}/Zn^{2+}/Ag^{2+}/Ni^{2+}$ in the mixture. The first row shows impacts in Cu-Ni mixtures, the second row shows impacts 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 $\beta$ 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 ($1.34\times10^{-7}$ M). Up to $1.03\times10^{-7}$ 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) | $\beta$ (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; $\beta$: 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 $\beta$ 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/$f_{\mathrm{mix}}$/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.

# Chapter 5

## Evaluating the combined toxicity of Cu and ZnO nanoparticles: utility of the concept of additivity and a nested experimental design.

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Submitted

## Abstract

We evaluated the combined toxicity of Cu and ZnO nanoparticles (NPs) using six nested combinations, $Cu(NO_3)_2$- $Zn(NO_3)_2$, $Cu(NO_3)_2$-CuNPs, $Zn(NO_3)_2$-ZnONPs, $Cu(NO_3)_2$-ZnONPs, $Zn(NO_3)_2$-CuNPs, CuNPs-ZnONPs. Each type of metal-based NPs was presumed to be a mixture containing soluble metal species and undissolved solid metal particles. A $Zn(NO_3)_2$ or $Cu(NO_3)_2$ solution was used as a reference to assess the toxicity of the dissolved fraction of ZnO NPs or Cu NPs. Effect measurements were performed using root elongation of *Lactuca sativa* L. Results were interpreted with the independent action (IA) model. This showed a good predictive power in estimating mixture toxicity of $Zn(NO_3)_2$-ZnONPs ($R^2$=0.84), $Cu(NO_3)_2$-CuNPs ($R^2$=0.94) and CuNPs-ZnONPs ($R^2$=0.82). The variations left in toxicity modeling of $Zn(NO_3)_2$-ZnONPs mixtures were explained by small antagonistic effects found between particulate ZnO and dissolved Zn which were not observed for $Cu(NO_3)_2$-CuNPs mixtures. Besides antagonistic effects between dissolved Cu and Zn, statistically significant relationships were also observed between increased concentrations of particulate Cu or Zn and increased median effective concentrations of $Zn(NO_3)_2$ or $Cu(NO_3)_2$. Results illustrated that ‘interaction’ between dissolved and particulate fractions of metal-based NPs affected the combined toxicity of Cu NPs and ZnO NPs, which complicated their observed effects as compared to mixtures of Cu and Zn nitrates.

*Keywords*: Cu; Zn; nanoparticles; mixture; toxicity

## 5.1 Introduction

Nanotechnology has been applied to create novel materials with unique characteristics in a large variety of consumer and household products. For example, engineered zinc oxide nanoparticles (NPs) are added into personal care products and coatings, which benefits from their ability to efficiently absorb UV-light and their increased transparency to visible light (Rousk et al., 2012). Nano-Cu powders can be dispersed into catalysts, conductive pastes, sintering additives, anti-bacteria products, and lubricant additives owing to their potential catalytic, dielectric, and biomedical properties (Mortimer et al., 2010). Increasing numbers of applications may lead to direct or indirect releases of engineered metal-based NPs into the environmental. This may pose effects on a variety of organisms in aquatic and terrestrial eco-systems, and in turn requires more attention on their eco-toxicological effects.

Dissolution and aggregation/agglomeration are the two main processes that can strongly influence the state of metal-based NPs present in suspensions, and consequently impact the bioavailability, uptake and toxicity of NPs (Misra et al., 2012). It has been reported that various characteristics of the exposure media can affect dissolution and aggregation of metal-based NPs, e.g. pH, ionic strength and the presence of naturally occurring organic matter (Franklin et al., 2007). Dissolution of NPs is a dynamic process in which constituent molecules of the dissolving solid migrate from the surface to the bulk solution through a diffusion layer (Borm et al., 2006). The adsorption of molecules and ions from solution can promote or delay the dissolution process by modifying the diffusion layer characteristics (Adamson and Gast, 1997). Apart from heteroaggregation, particles can also be bound together (homoaggregation) when their equilibrium solubility is above saturation concentrations (Holsapple et al., 2005), which can increase the overall diffusion layer thickness and hinder dissolution of NPs.

Metal-based NPs are always an intermediate state of bulk and molecular materials. Metal ions or small inorganic complexes produced by engineered metal-based NPs consisting of highly toxic elements inevitably drive the partial toxicity of metal-based NPs to organisms (Misra et al., 2012). However, it is still a challenge to clarify which metal species contribute most to the nano-toxicity. Some studies suggested that the toxic effects of ZnO NPs and Cu NPs on environmentally relevant organisms were most likely due to the dissolved metal species rather than being particle-dependent (Blinova et al., 2010; Bondarenko et al., 2012; Ivask et al., 2013). Other researchers argued that the particulate forms of ZnO NPs and Cu NPs contributed substantially to the cytotoxic effects on mammalian and piscine cell lines (Fernández-Cruz et al., 2013; Song et al., 2014). The translation from an effect on a cell line to a whole organism is not straightforward and depends on numerous factors such as the types of cell lines and metal-based nanoparticles. Karlsson et al. (2014) found that the oxidative stress of mouse embryonic stem (mEs) cells was induced by the released

Cu ions of CuO NPs whereas the stress was particle related for NiO NPs.

Nano-ZnO has been classified as ‘extremely toxic’ to aquatic organisms, followed by ‘very toxic’ nano-Cu (Kahru and Dubourguier, 2010). They were already found to be simultaneously present in wastewater effluents (Bystrzejewska-Piotrowska et al., 2009; Bolyard et al., 2013; Li et al., 2015). These nanoparticles can enter the terrestrial system by the application of bio-solids from sewage systems as a fertilizer (Batley et al., 2012). Higher plants have been reported to be able to ingest and store metal-based NPs in tissues (Rico et al., 2011). To date, the knowledge of the eco-toxicity of Cu NPs and ZnO NPs is far from being adequate as compared to their large-scale application (Hu et al., 2010; Song et al., 2010), especially under conditions of their co-exposure.

This study aims at improving the understanding of effects of Cu NPs, ZnO NPs and their mixtures on *L. sativa* L. and unravelling two questions as follows: (1) Will the dissolved metals and the particulate metals of each type of metal-based NPs act jointly following the common rules of additivity? (2) Will Cu NPs interact with ZnO NPs and influence the toxicity of each other? Theoretically, if Cu NPs and ZnO NPs would act comparable to metal salts, e.g. $Cu(NO_3)_2$ and $Zn(NO_3)_2$, then the existing models for general eco-toxicology of metals such as the free ion activity model (FIAM) and the biotic ligand model (BLM) can be applied to predict the toxicity of metal-based NPs. As shown in our previous studies (Le et al., 2013; Liu et al., 2014), $Cu^{2+}$ competed with $Zn^{2+}$ for binding to the biotic ligand of lettuce. What makes this research more difficult than the case of mixtures of metal salts is that the suspensions of each type of metal-based NPs are a mixture mainly containing dissolved metal species and undissolved particles. Suspensions of Cu NPs and ZnO NPs were therefore assumed to contain four metal species i.e. dissolved Cu, dissolved Zn, particulate Cu and particulate ZnO. Cedergreen et al. (2012) have shown that the joint effect of ternary mixtures can be predicted from binary mixture toxicity results. To trace down the potential ‘interactions’ between Cu NPs and ZnO NPs and where these ‘interactions’ (if any) take place, an elaborate nested experiment was designed including all possible combinations:

- $Cu(NO_3)_2$- $Zn(NO_3)_2$ (dissolved Cu and dissolved Zn, Cu-Zn)

·$Cu(NO_3)_2$ and Cu NPs (dissolved Cu and particulate Cu, Cu-nanoCu)
·$Zn(NO_3)_2$ and ZnO NPs (dissolved Zn and particulate ZnO, Zn-nanoZnO)
·$Zn(NO_3)_2$ and Cu NPs (dissolved Zn and particulate Cu, Zn-nanoCu)
·$Cu(NO_3)_2$ and ZnO NPs (dissolved Cu and particulate ZnO, Cu-nanoZnO)
·Cu NPs and ZnO NPs (particulate Cu and particulate ZnO, nanoCu-nanoZnO)

A $Zn(NO_3)_2$ or $Cu(NO_3)_2$ solution was used as a reference to assess the single toxicity of the dissolved fraction of ZnO NPs or Cu NPs. The combined effects caused by nanoCu-nanoZnO mixtures were then compared with the overall effects of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ the data of which have been reported in the study of Le et al. (2013).

## 5.2 Methods

### *5.2.1 Test compounds and nutrient solution*

The engineered uncoated Cu NPs (nano-spheres, nominal particle size 50 nm, NM-0014, purity 99.8%) and the engineered uncoated ZnO NPs (nano-sticks, nominal particle size 150 nm, NM-110) were purchased from the io-li-tec company (Heilbronn, Germany). $Cu(NO_3)_2•3H_2O$ (purity 99.5%), $Zn(NO_3)_2•6H_2O$ (purity 99.5%) and other salts used in preparing the nutrient solution were all purchased from the Merck KGaA company (Darmstadt, Germany). The nutrient solution was composed of $Ca(NO_3)_2•4H_2O$ (236.1 mg/L), $MgSO_4•7H_2O$ (60 mg/L), $NaHCO_3$ (50 mg/L), and $KHCO_3$ (10 mg/L) totally dissolved in demi-water (pH 7.8) and was applied for culturing plants and preparing exposure medium.

### *5.2.2 Experimental design*

A full factorial experimental design included all the six possible combinations of particles and dissolved metal species. A detailed description of the experimental setup after pre-screening tests is represented in Figure S5.1. Negative controls (nutrient solution) and positive controls (single compounds, i.e. $Cu(NO_3)_2$, $Zn(NO_3)_2$, Cu NPs, ZnO NPs individually) were both conducted every week with mixture treatments and were repeated twice. Hydroponic exposure was used to avoid complex interactions of particles and ions in the soil compartment. To keep the concentrations of compounds in solution constant, the test media were replaced every day. Stock suspensions of Cu NPs and ZnO NPs were daily prepared in nutrient solution and sonicated in an S 40 H Elmasonic water bath sonicator (Elma, Germany) for 10 min. All the stock solutions including the nitrate salts were further diluted 10 times with nutrient solution to obtain the nominal concentrations for each treatment.

### *5.2.3 Exposure of lettuce and toxicity determination*

*Lactuca sativa* L. was selected as the test organism because this species can be manipulated relatively easily and it is sensitive to environmental contaminants (OECD, 2006). The toxicity tests were operated according to guidelines of the US Environmental Protection Agency (EPA, 1988). As compared to the germination rate of seeds, the relative root elongation rate (*RRE*, %) of lettuce seedlings was more realistic in reflecting external stressors (Pfleeger et al., 1991) and therefore was employed as the toxicological endpoint in this study. Lettuce seeds were purchased from the Horti Tops company (Amsterdam, the Netherlands) and germinated on expanded perlite in a climate room (18°C, 80% humidity, and a 16:8 h light: dark cycle) for 96 h. After germination, seedlings with taproot lengths more than 3 cm were chosen to be fixed on parafilm strips floating on the surface of glass petri dishes containing 30 ml test medium. In each petri dish, 4 seedlings were suspended. Before and after 96 h exposure, the length of lettuce taproot was measured from the transition point between the hypocotyls and the root to the root tip. The root growth of each treatment was defined as the mean value of differences in root length of 4 seedlings before and after exposure. Then *RRE* was determined as follows

$$RRE = \frac{RG_\mathrm{S}}{RG_\mathrm{C}} \times 100\% \tag{5-1}$$

where $RG_\mathrm{s}$: the root growth of plants in the sample solution, cm; $RG_\mathrm{c}$: the root growth of plants in the control solution, cm.

### *5.2.4 Characterization of nanoparticles*

The morphology and particle size of metal-based NPs were characterized using a JEOL 1010 Transmission Electron Microscope (JEOL, Japan). The particle size of Cu NPs and ZnO NPs was analyzed using a Nano Measurer 1.2 (Fudan University, China). The distribution of the hydrodynamic diameter and the zeta-potential of NPs in different spiked test media were measured after 1 h and 24 h of preparation by Dynamic Light Scattering (DLS) on a Zetasizer Nano-ZS instrument (Malvern, United Kingdom).

### *5.2.5 Chemical analysis*

A Cu-ion selective electrode (Cu-ISE, Metrohm, Switzerland) was used as a direct way to measure the free Cu-ion activity in solution after 1 h and 24 h. The Zn-ion selective electrode was not used in this study because the detection limit was not sufficient for the test. To check whether particles will reduce the sensitivity of the Cu-electrode membrane, plain polystyrene fluorescent microspheres # 103125-05 (nominal particle size 70 nm, Microspheres-Nanospheres, American) were added to compare the activities of $Cu^{2+}$ with those in solutions of $Cu(NO_3)_2$ alone. The actual total concentrations of Ca, Mg, Na, K, Cu, Zn, and the dissolved concentrations of Cu and ZnO NPs after 1 h and 24 h of equilibration were analyzed using Flame Atomic Absorption Spectroscopy (Perkin Elmer AAnalyst 100, American). Centrifugation of samples removed Cu NPs, ZnO NPs and ions which may be adsorbed to particle surfaces. The supernatants were obtained after 20 min of centrifugation in a Centrifuge 5415D (Eppendorf, Germany) at 13 300 *g* (Fernández-Cruz et al., 2013). The particle suspensions, the supernatants and the liquids with nitrate salts were digested using $HNO_3$ and sampled for FAAS analysis.

### *5.2.6 Data analysis*

To check the potential chemical-chemical interactions before entering the organism, relationships between the free $Cu^{2+}$ activities in the solution (or the dissolved metal species of Cu NPs or ZnO NPs) and the added amount of one compound in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO after 1 h and 24 h of exposure were all analyzed using the linear regression method in the GraphPad Prism 5 software (GraphPad, American). For the Cu-nanoCu mixtures, the activities of $Cu^{2+}$ released from Cu NPs were calculated by subtracting the $Cu^{2+}$ activities of $Cu(NO_3)_2$ from the totally measured activities of $Cu^{2+}$ in mixture solutions. The actually total or dissolved concentrations of Cu NPs or ZnO NPs in Cu-nanoCu and Zn-nanoZnO mixtures were calculated in a similar way.

To check the potential interactions after compounds entered the organism, the independent action (IA) model based on the rules of ‘additivity’ (Bliss, 1939) was initially used to predict the combined toxicity of mixtures of Cu-nanoCu, Zn-nanoZnO and nanoCu-nanoZnO since the effect of each compound in a mixture can be directly measured from positive controls.

$$E(C_{\text{mix}}) = 1 - \prod_{i=1}^{n}[1 - E(C_i)] \tag{5-2}$$

where $E(C_{\text{mix}})$: the estimated effect of an n-compound mixture; $E(C_i)$: the effect of the $i$th compound applied singly at a fixed concentration.

If compounds in a mixture do not act following the rules of ‘additivity’, ‘interactions’ between these compounds may play an important role in their combined toxicity. Finding interactions in mixtures is always a challenge especially when a mixture contains more than two components. Since the suspensions of Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO mixtures involve more than two metal species, searching interactions between these different metal species cannot be done using the existing models for binary mixtures. Therefore, a different approach was used in this study, which will be explained by the following example of Cu-nanoZnO mixtures.

To examine the influence of $Cu(NO_3)_2$ on the toxicity of ZnO NPs, the root growth inhibition caused by $Cu(NO_3)_2$ should be subtracted from the total effects of Cu-nanoZnO mixtures. The *RRE*s induced by ZnO NPs in co-exposure of $Cu(NO_3)_2$ and ZnO NPs can be calculated by substituting the $RG_c$ of positive controls (with $Cu(NO_3)_2$ alone) in equation (1). The *RRE*s induced by ZnO NPs alone can be calculated by substituting the $RG_c$ of negative controls (nutrient solution only) in equation (5-1). The median effective concentrations ($EC_{50}$s) of ZnO NPs in single-exposure or co-exposure with $Cu(NO_3)_2$ were all calculated using the log-logit function in GraphPad Prism 5. The $EC_{50}$ values of ZnO NPs in the co-exposure with $Cu(NO_3)_2$ were then compared with the $EC_{50}$ value of ZnO NPs in single exposure. It was assumed that if the $EC_{50}$s of ZnO NPs in the co-exposure with $Cu(NO_3)_2$ were significantly different from the value of ZnO NPs in single exposure, then the influence of $Cu(NO_3)$ on the toxicity of ZnO NPs was statistically significant. The $EC_{50}$ values of ZnO NPs were plotted as a function of increasing concentrations of

$Cu(NO_3)_2$ by the linear regression in OriginPro 8 (Origin Lab, United Kingdom). As an initial attempt, the slope of straight lines was compared with zero to indicate the overall antagonism or synergism. A non-significant slope is indicative of no substantial effects of $Cu(NO_3)_2$ on the toxicity of ZnO NPs, a significant positive ($p < 0.05$) slope is indicative of antagonistic effects, and a significant negative slope indicates synergistic effects. Similar methods can be used to find out the influence of ZnO NPs (in both dissolved and particulate forms) on the toxicity of $Cu(NO_3)_2$ and for other mixtures investigated in this study.

To determine whether Cu NPs and ZnO NPs have similar effects as $Cu(NO_3)_2$ and $Zn(NO_3)_2$, the biological responses (*RRE*, %) caused by mixtures of nanoCu-nanoZnO and of $Cu^{2+}$-$Zn^{2+}$ were plotted as the 2D isobolic representations by the software of OriginPro 8. Data for the toxicity of $Cu^{2+}$ and $Zn^{2+}$ to *L. sativa* L. were obtained under similar environmental environment, and are published by Le et al. (2013). The x-axis or y-axis was presented as toxic unit (TU) for each compound (1TU=the median effective concentration). The theoretical line of additivity is the straight line that connects the individual doses of each compound in a mixture to produce a fixed equal effect alone. In general terms, isoboles represent an upward curve (round) when the combined effects are less than addition and isoboles become hollow when mixture effects are more toxic than addition (Bongers, 2007).

## 5.3 Results

### *5.3.1 Characterization of nanoparticles*

The TEM images of Cu NPs, ZnO NPs and their mixtures are shown in Figure 5.1. The primary sizes and shapes of the particles were estimated based on the TEM images. The Cu NPs were shown to be of spherical shape, 127 nm in size (size variation of 119-137 nm). The ZnO NPs crystals were approximatively displayed tetragonal morphology (width: 55 nm, size variation of 24-110 nm; length: 144 nm, size variation of 95-224 nm). The size distribution of the hydrodynamic diameter of NPs in lettuce culture solution and in solution of five combinations was determined using DLS and shown in Table S5.1. Initial particle sizes changed quickly after the NPs were submerged in lettuce culture solution. Both NPs were present as aggregates (370 nm - 1531 nm) in lettuce culture solution and in mixture solutions.

The hydrodynamic particle sizes of Cu NPs and ZnO NPs increased by a factor of 1.5 to 2 after 24 h submerged in lettuce culture solution and in solution of mixtures of Zn-nanoCu, nanoCu-nanoZnO, Zn-nanoZnO. Besides, the absolute values of zeta-potential of NPs were < 14 mV. This indicated that the suspensions of NPs were relatively less stable which resulted in the aggregates by Van Der Waals inter-particle force.

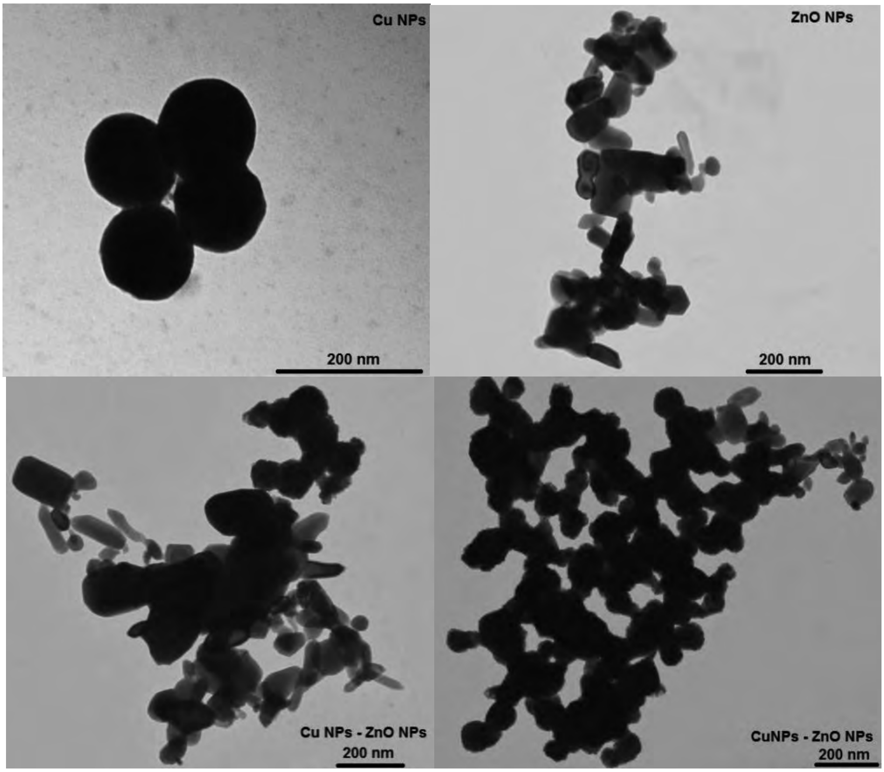

Figure 5.1 The TEM images of Cu NPs, ZnO NPs and their mixtures. Scale bars indicate size (nm).

### *5.3.2 Fate analysis*

Relationships between the free activities of $Cu^{2+}$ in solution and the added ZnO NPs, $Zn(NO_3)_2$, Cu NPs, $Cu(NO_3)_2$ after 1 h and 24 h are plotted in Figure S5.2. After 24 h, the activities of $Cu^{2+}$ were generally increased in mixtures of Cu-nanoCu, Zn-nanoCu, Cu-nanoZnO, and nanoCu-nanoZnO as compared to the values after 1 h. However, no consistently significant effects of increasing concentrations of

$Cu(NO_3)_2$, Cu NPs, $Zn(NO_3)_2$, ZnO NPs were observed on the activities of $Cu^{2+}$ in solution after 1h and 24 h of equilibration using the Cu-ISE. This indicated that the amount of free $Cu^{2+}$ released from either Cu NPs or $Cu(NO_3)_2$ was not substantially affected by other compounds of Cu or Zn added to the solution. It is shown in Figure S5.3 that the growing trend of $Cu^{2+}$ activities in solution with polystyrene fluorescent microspheres remained constant when more $Cu(NO_3)_2$ was added and the slope of linear curves remained positive. This finding showed that polystyrene fluorescent microspheres did not reduce the sensitivity of Cu-ISE.

The background concentrations of Na, K, Ca, Mg in nutrient solution were respectively measured to be 11.9 ± 0.3 mg/L, 3.68 ± 0.07 mg/L, 31.24 ± 0.5 mg/L, 5.49 ± 0.08 mg/L. The impacts of addition of a compound to solution, on the dissolution of Cu NPs and ZnO NPs after 1h and 24 h of equilibration in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO are represented in Figure S5.4. Generally, the more NPs were added to the solution, the lower proportion of dissolved fraction of the same type of metal-based NPs was measured after 1 h and 24 h. The dissolved concentrations of Cu NPs at the same dose levels were found to be higher after 24 h in all combinations which coincided with the increased values of free $Cu^{2+}$ activities. No statistically significant impacts were observed from addition of $Zn(NO_3)_2$, $Cu(NO_3)_2$, and Cu NPs on the dissolution of ZnO NPs. Although the dissolved concentrations of Cu NPs at lower doses were significantly increased by the added $Cu(NO_3)_2$ after 1 h, the influence was not constant across the whole range of concentrations. Only for Zn-nanoCu mixtures, it was found that the dissolved concentrations of Cu NPs were significantly reduced by the added $Zn(NO_3)_2$ after 24 h.

### *5.3.3 Toxicity of individual compounds*

Following the full factorial experimental design, a complete dose-response curve was obtained for each compound investigated in this study, which was used to calculate the $EC_{50}$s to *L. sativa* L. The averaged $EC_{50}$ values of Cu NPs, ZnO NPs, $Cu(NO_3)_2$ and $Zn(NO_3)_2$ are provided in Table 5.1. Nano-ZnO had the minimum acute toxicity to lettuce as shown by its highest $EC_{50}$ value and $Cu(NO_3)_2$ with the lowest $EC_{50}$ resulted in the highest toxic effects on root growth. The $EC_{50}$ values of nano-Cu and $Cu(NO_3)_2$ were similar, and the $EC_{50}$ of nano-ZnO was twice as big as for $Zn(NO_3)_2$.

Table 5.1 The median effective concentrations ($EC_{50}$, mg/L) with the 95% confidence interval (CI) of nano-Cu, nano-ZnO, $Cu(NO_3)_2$ and $Zn(NO_3)_2$ individually on root elongation of lettuce (*L. sativa* L.) in the present study.

| Compounds | Forms | $EC_{50}$ (mg/L) |
|---|---|---|
| Cu NPs | Total Cu | 0.10 (0.08-0.11) |
| ZnO NPs | Total Zn | 4.47 (3.39-5.85) |
| $Cu(NO_3)_2$ | Dissolved Cu | 0.07 (0.06-0.07) |
| $Zn(NO_3)_2$ | Dissolved Zn | 2.08 (1.75-2.39) |

### *5.3.4 Toxicity of mixtures*

In the present study, 82-94% of the variability in the toxicity of nanoCu-nanoZnO, Zn-nanoZnO and Cu-nanoCu mixtures could be explained by the independent action (IA) model (Figure 5.2). To examine whether potential ‘interactions’ were the cause of remaining deviations from the model, the effective concentrations causing a 50% reduction in root elongation of Cu NPs, ZnO NPs, $Cu(NO_3)_2$, $Zn(NO_3)_2$ in single-exposure and in co-exposure of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO mixtures were plotted in Figure 5.3 as a function of the various dose levels of Cu or Zn in solution. The dissolved concentrations of metal-based NPs were expressed as the average values after 1 h and 24 h of equilibration. As shown in Figure 5.3 A-D, the $EC_{50}$s of nano-Cu were not statistically significantly increased with increasing concentrations of $Cu(NO_3)_2$ and significant impacts of Cu NPs were neither observed on the $EC_{50}$s of $Cu(NO_3)_2$. This implied that the dissolved Cu and the particulate Cu did not affect the toxicity of each other for lettuce (Table 5.2). For the combination of Cu-nanoZnO, the $EC_{50}$s of nano-Zn cannot be calculated in the second replicates when concentrations of $Cu(NO_3)_2$ were beyond 0.06 mg/L, which were thus not used in the data analysis. The $EC_{50}$ of nano-Zn was still significantly increased, up to a factor of 5.5 at 0.05 mg/L of $Cu(NO_3)_2$. In Figure 5.3 F-H, the $EC_{50}$ of $Cu(NO_3)_2$ at the highest concentration of ZnO NPs (42.2. mg/L) was not calculated due to the small difference in root length as compared to the positive controls. Without this data point, the $EC_{50}$ values of $Cu(NO_3)_2$ were significantly increased upon increasing amounts of ZnO NPs in the solution. For the combination of Zn-nanoCu, the $EC_{50}$s of $Zn(NO_3)_2$ were found to be sharply increased by the added Cu NPs regardless of the metal species in solution. However, a similar result was not observed in turn. The $EC_{50}$ values of nano-ZnO significantly increased upon increasing concentrations of $Zn(NO_3)_2$ in solution. At lower concentrations of ZnO NPs, the $EC_{50}$s of $Zn(NO_3)_2$ were also increased with increasing concentrations of Zn NPs (b, N-P, Figure 5.3). This finding indicated that the dissolved Zn may compete against the particulate Zn for inducing toxicity to lettuce at lower concentrations of Zn NPs (< 20 mg/L). For the complex nanoCu-nanoZnO mixtures, the $EC_{50}$s of ZnO NPs cannot be calculated in the second replicates when concentrations of Cu NPs were higher than 0.05 mg/L and non-significant impacts of Cu NPs at lower concentrations were observed on the toxicity of ZnO NPs. The $EC_{50}$s of Cu NPs were observed to significantly increase with an increased amount of ZnO NPs in the solution (< 5mg/L).

The biological responses caused by mixtures of nanoCu-nanoZnO and mixtures of $Cu^{2+}$-$Zn^{2+}$ are plotted in Figure 5.4. For the combination of $Cu^{2+}$-$Zn^{2+}$, the toxicity was dominated by $Cu^{2+}$ at lower concentrations of $Zn^{2+}$ and antagonistic effects occurred. At higher concentrations of $Zn^{2+}$, the toxicity was relatively dominated by $Zn^{2+}$ and synergistic effects occurred. A similar dose ratio-dependent deviation pattern was not observed for the combination of nanoCu-nanoZnO. Antagonism is observed at lower effect levels, whereas deviation patterns were found to be dependent on the concentration ratios of Cu NPs and ZnO NPs at high effect levels. Synergistic effects occurred if the combined toxicity was dominated by Cu NPs and a mixture acted antagonistic if ZnO NPs relatively dominated.

## 5.4 Discussion

### *5.4.1 Fate of nanoparticles*

The size of Cu NPs and ZnO NPs in culture media of lettuce was not observed to be strongly affected by the added amount of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ in solution after 1 h and 24 h of equilibration. This may be attributed to the high tendency of both NPs to aggregate after submersion in nutrient solution. The high concentrations of ingredients in nutrient solution can be a reason for rapid and dramatic aggregation (Franklin et al., 2007). The increasing concentrations of Cu or Zn in the exposure media were shown not to hinder or stimulate the dissolution of Cu NPs or ZnO NPs and therefore the ion release of Cu NPs except in the co-exposure of $Zn(NO_3)_2$ and Cu NPs after 24 h. This may be caused by incompletely separating the dissolved metal species from nanoparticles, since there is still no definitely effective technique for assessing dissolution of bulk materials to NPs (Misra et al., 2012). Alternatively, unlike the other properties of water chemistry such as pH, $HPO_4^{2-}$ and DOM (Li et al., 2013), the dissolved Cu and Zn in solution cannot strongly influence the dissolution of Cu NPs and ZnO NPs.

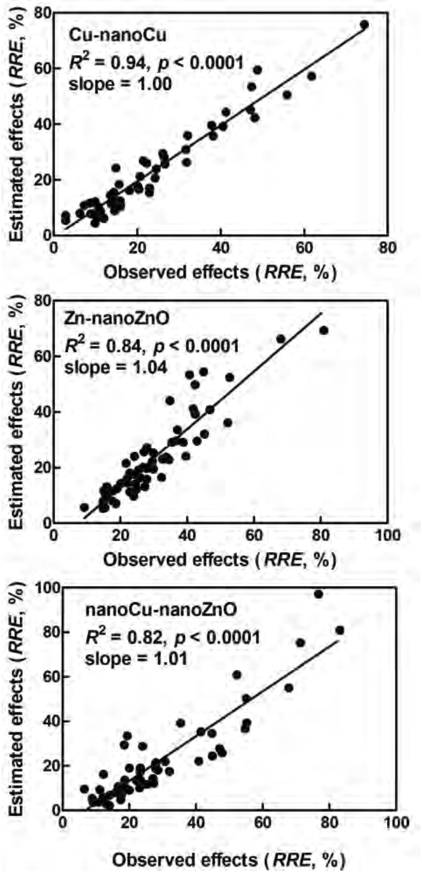

Figure 5.2 Relationships between the estimated and the observed effects of mixtures of Cu-nanoCu, Zn-nanoZnO and nanoCu-nanoZnO on relative root

elongation (*RRE*, %) of lettuce *Lactuca sativa* L. using the independent action (IA) model. $R^2$ indicates the determination coefficient. *p* indicates the statistical significance level.

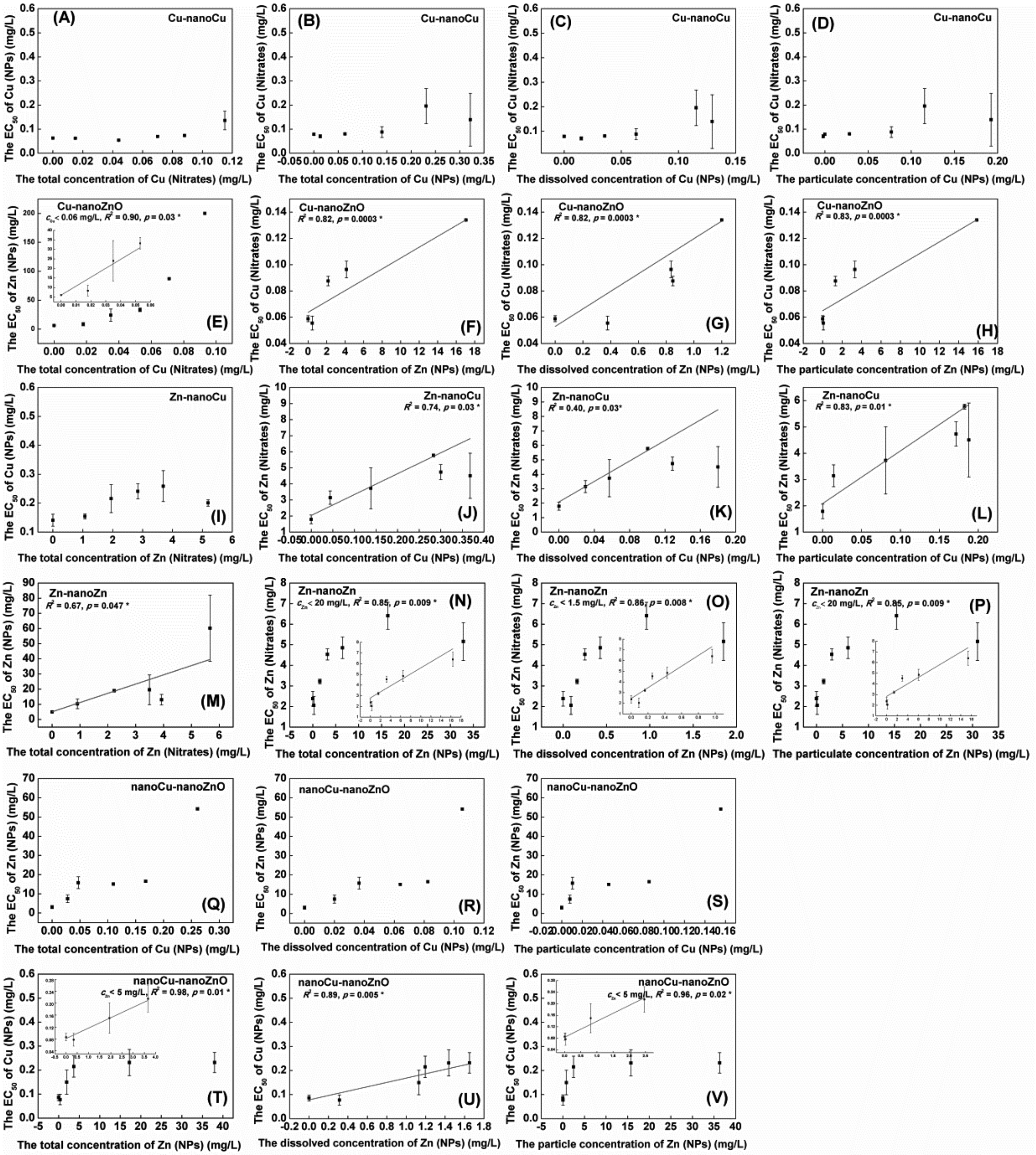

Figure 5.3 Relationships between the median effective concentrations ($EC_{50}$s) of $Cu(NO_3)_2$, $Zn(NO_3)_2$, Cu NPs, ZnO NPs for *L. sativa* L. after 4 d of exposure and the total concentration (or dissolved, or particulate concentration) of Cu NPs, ZnO NPs,

$Zn(NO_3)_2$, or $Cu(NO_3)_2$. Data are presented as mean ± standard error of the mean. Solid lines represent the statistically significant linear regression fits to figure out the overall synergistic or antagonistic effects. $R^2$ indicates the determination coefficient adjusted for the degrees of freedom. *p* indicates the statistical significance level. * indicates that the slope of linear curve is significantly different from zero at the 5% significance level.

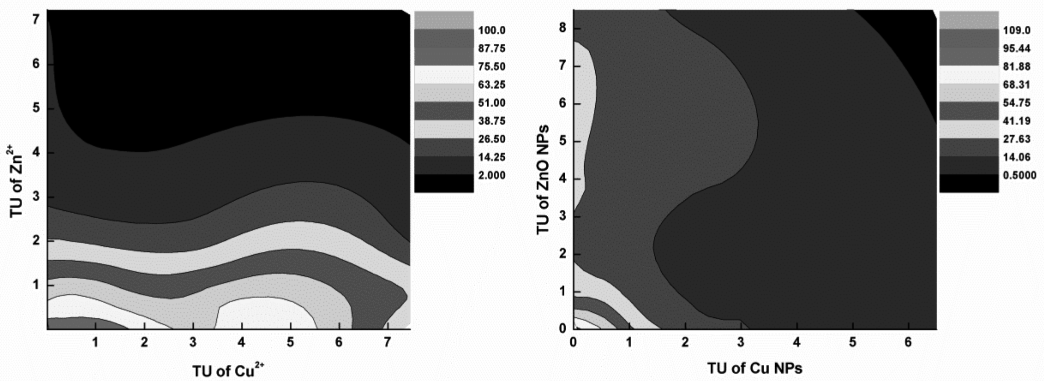

Figure 5.4 2D isobolic representation of the biological responses (*RRE*, %) for mixtures of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ and mixtures of Cu NPs and ZnO NPs. The values of *RRE* are high in the negative control groups and decrease as doses of compounds increase. The x-axis or y-axis is presented as toxic unit (TU) for each compound (1TU=the value of median effective concentration which was shown in Table 5.1).

### *5.4.2 Toxicity of individual compounds*

Our results showed that Cu is more toxic to lettuce seedlings than Zn regardless of the metal being in the form of a cation or a nanoparticle. This may be attributed to the different demands of plant cells for Cu and Zn in the growth and development. To our knowledge, similar studies were not conducted before, which made it difficult to compare the $EC_{50}$ values of Cu NPs and ZnO NPs calculated in this study with those in other studies. Substantial differences in $EC_{50}$s can be caused by diverse properties of metal-based NPs e.g. size and shape, by different sensitivities of plant, and by different periods of plant growth.

### *5.4.3 Combined toxicity of Cu NPs and ZnO NPs*

The good fitting provided by the standard IA model and the non-significant ‘interactions’ observed for Cu-nanoCu mixtures simultaneously verified the assumption of Song et al. (2014) that the addition model can be used to estimate the relative contribution of ionic and particulate forms to the cytotoxicity of Cu NPs. The IA model also showed a reasonable predictive power in estimating toxicity of Zn-nanoZnO mixtures and the rest of variations in modelling were explained by small antagonistic effects found in mixtures of Zn-nanoZnO. Since significant interactions were not observed on the dissolution and aggregation of ZnO NPs, the dissolved Zn may compete with the particulate Zn at the organism level, which influenced the toxicity of each other. Based on the antagonistic effects observed in mixtures of Cu-nanoZnO and Zn-nanoCu, it was suggested that the dissolved Zn may interact with the dissolved Cu which was consistent with the competition between $Cu^{2+}$ and $Zn^{2+}$ reported in previous studies of Le et al. (2013) and Liu et al. (2014). The feed-back mechanism (Qiu and Hogstrand, 2005) may be an explanation that an increase of copper in plant cell decreases the quantity of zinc importer proteins and blocks channels for zinc. In turn, the presence of low amounts of zinc may exert a positive effect on cell homeostasis and on the tolerance of cells to copper (Li et al., 2015). Until now, only Li et al. (2015) reported the potentiation effects on the human hepatoma cell line HepG2 co-exposed to Cu NPs and ZnO NPs and suggested that the nano-particulate fractions of ZnO NPs were attributable to the enhancement of Cu NPs toxicity. In contrast to the first finding of Li et al. (2015), antagonistic effects were observed in this study between Cu NPs and ZnO NPs on the toxicity of each other to lettuce. This may be caused by different features between animal cells and plant cells which lead to a diverse bioavailability or toxicity across species. In compliance with the second finding of Li et al. (2015), the particulate fractions of NPs were also observed to correlate with the ‘interactions’ and the overall toxicity of Cu NPs and ZnO NPs. This can be a reason that lead to a small deviation from ‘additivity’ in IA modeling and a difference in 2D isobolic representations between nanoCu-nanoZnO mixtures and mixtures of $Cu(NO_3)_2$ and $Zn(NO_3)_2$. The results of Zn-nanoCu, Cu-nanoZnO, Cu-nanoCu, and Zn-nanoZnO mixtures showed that the observed antagonistic effects between Cu NPs and ZnO NPs may be attributed to ‘interactions’ between dissolved Cu and dissolved Zn, between particulate Zn and dissolved Zn, of particulate Cu on dissolved Zn, and of particulate Zn on dissolved Cu at the organism level.

## 5.5 Conclusions

Our study first showed the commonly known independent action model can be used as a starting point to predict mixture effects of metal-based NPs. By dividing each type of metal-based NPs into a part of highly soluble metal species and a part of undissolved particles, we did record small antagonistic effects between these two parts of ZnO NPs which resulted in small deviations (16%) from ‘additivity’ in toxicity modelling. Similar effects were not observed for mixtures of Cu-nanoCu and therefore 94% variations in root growth could be explained by the IA model. The toxicity of ZnO NPs was found to be significantly decreased upon increasing concentrations of $Cu(NO_3)_2$ in solution, and vice versa. The $EC_{50}$s of $Zn(NO_3)_2$ were also highly correlated to the total (or dissolved, or particulate) concentrations of Cu NPs. Based on the above results, the small antagonistic effects observed between Cu NPs and ZnO NPs can be attributed to ‘interactions’ found among dissolved metal species as well as particulate fractions and lead to small deviations from ‘additivity’ ($R^2$=0.82), which cannot be easily explained by a simple combination of $Cu(NO_3)_2$ and $Zn(NO_3)_2$. To our knowledge, this is an innovative research in which data were generated on physic-chemical behavior as well as on biological effects of ZnO NPs, Cu NPs and their mixtures. Although the mechanism of interactions remains to be determined, there is no doubt that our research will enrich the rapid evolving field of nano-toxicology and help scientists develop approaches to evaluate the potential impacts of metal-based NPs and their mixtures on eco-systems.

Table 5.2 Interactions found between compounds in mixtures of Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO in the exposure of 4 d lettuce seedlings.

| Compound 1<br>Compound 2 | Zn $(NO_3)_2$ | Cu $(NO_3)_2$ | Cu NPs | Dissolved Cu | Particulate Cu |
|---|---|---|---|---|---|
| Cu $(NO_3)_2$ | Anta\Anta (Le et al., 2013; Liu et al., 2014) | n.d. | No\No | No\n.d. | No\n.d. |
| Zn $(NO_3)_2$ | n.d. | Anta\Anta (Le et al., 2013; Liu et al., 2014) | Anta\No | Anta\n.d. | Anta\n.d. |
| ZnO NPs | Anta\Anta | Anta\Anta | No\Anta | No\n.d. | No\n.d. |
| Dissolved Zn | n.d.\Anta | n.d.\Anta | n.d.\Anta | n.d. | n.d. |
| Particulate ZnO | n.d.\Anta | n.d.\Anta | n.d.\Anta | n.d. | n.d. |

No significant effect (No): the slope of linear fits in Figure 5.3 is not significantly different from zero which indicates that no significant effects of one component observed on the $EC_{50}$s of other components in the mixture; Antagonism (Anta): the slope of linear fits in Figure 5.3 significantly deviates from zero with increasing concentrations of one mixture component which indicates an antagonistic effect of one compound on another compound in the mixture; n.d.: not determined in this study; \: the left side indicates the impacts of Compound 1 on Compound 2 and the right side indicates the impacts of Compound 2 on Compound 1.

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## Supplementary Materials

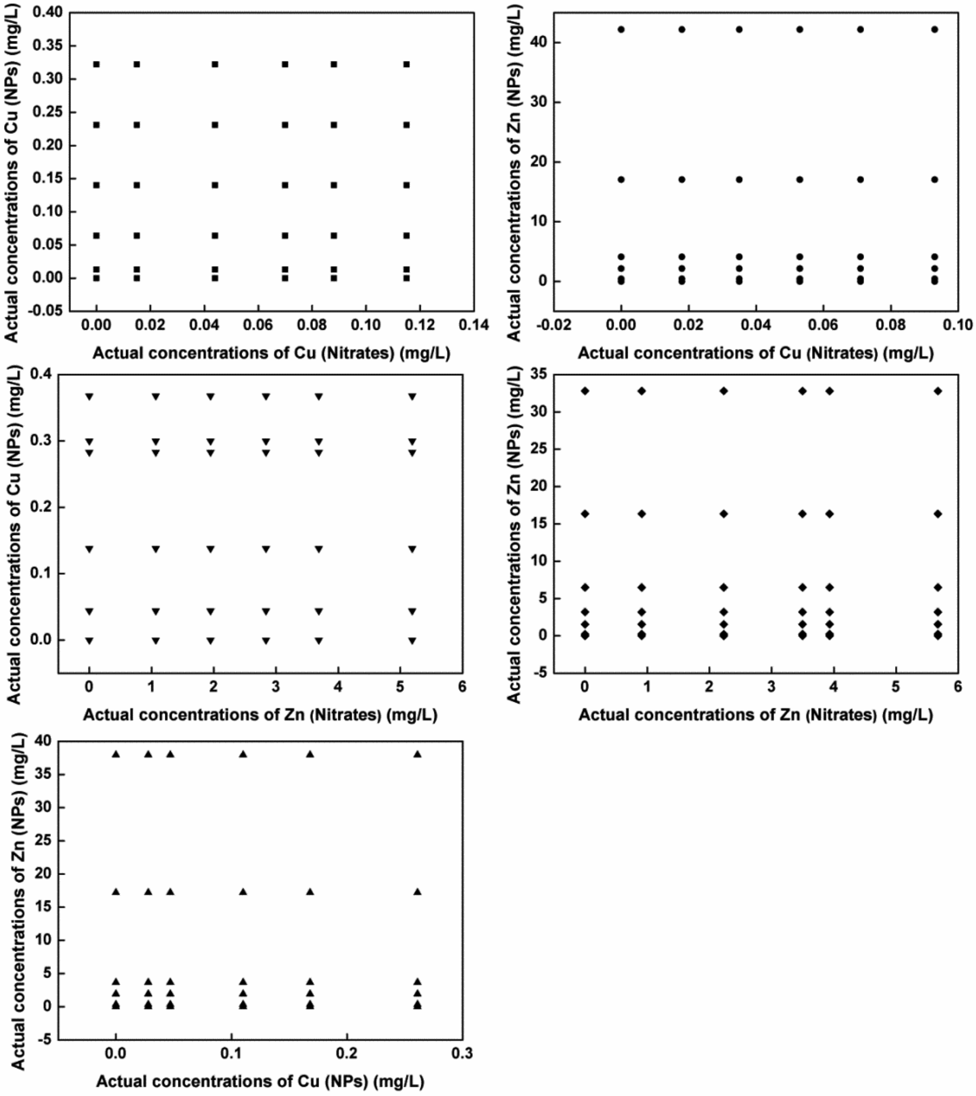

Figure S5.1 The set-up for Cu-nanoCu, Cu-nanoZnO, Zn-nanoCu, Zn-nanoZnO, and nanoCu-nanoZnO mixtures expressed as actual concentrations tested by the Flame AAS.

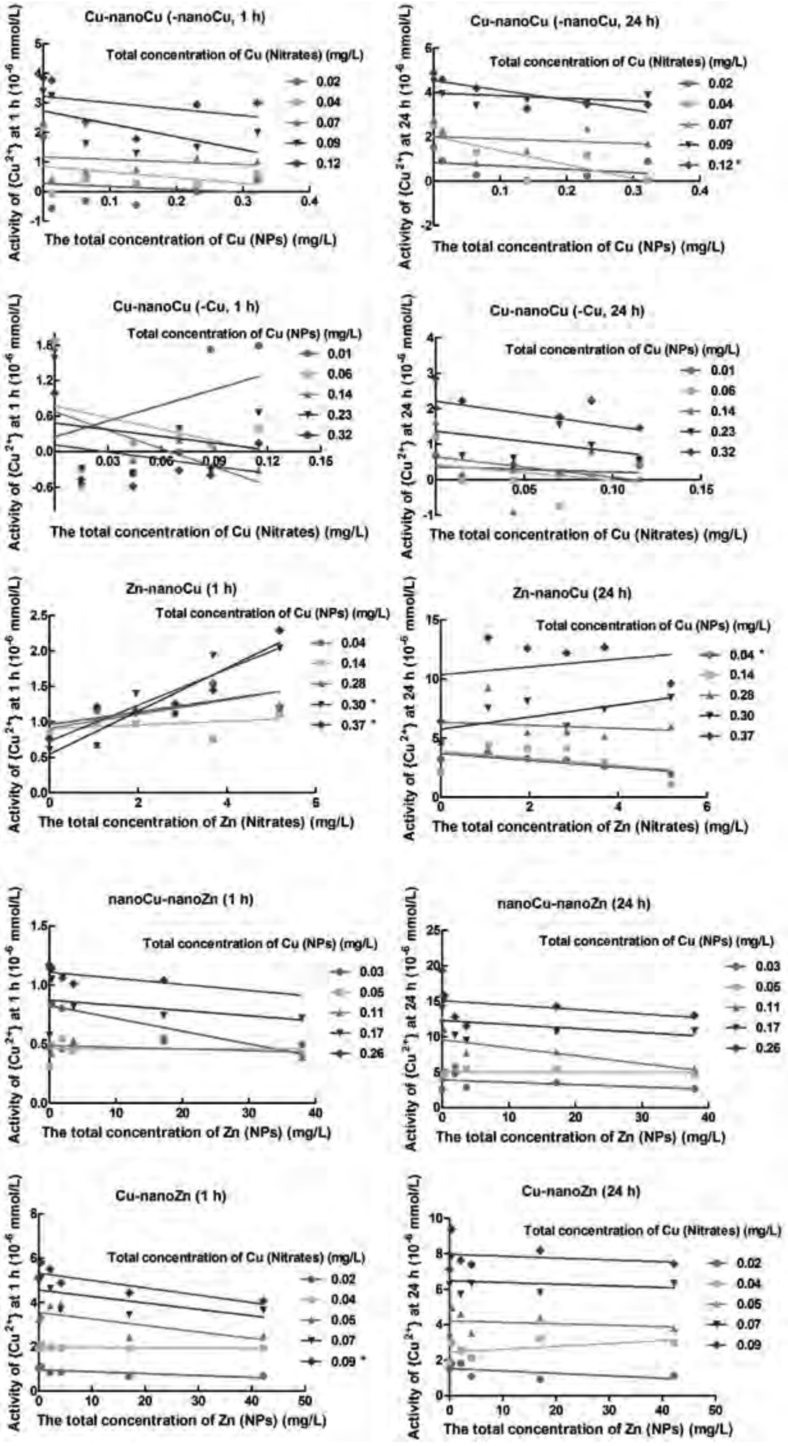

Figure S5.2 Relationships between free $Cu^{2+}$ activities in the solution and the added amount of ZnO NPs, $Zn(NO_3)_2$, Cu NPs, $Cu(NO_3)_2$ after 1 h and 24 h of equilibration. For the combination of Cu-nanoCu, the activities of $Cu^{2+}$ released from Cu NPs equal to the total $Cu^{2+}$ activities minus the activities of $Cu^{2+}$ released from $Cu(NO_3)_2$, and vice versa. The solid lines represent linear relationships. * indicates that the slope of linear curve is significantly different from zero at the 5% significance level. {} indicates the free ion activities.

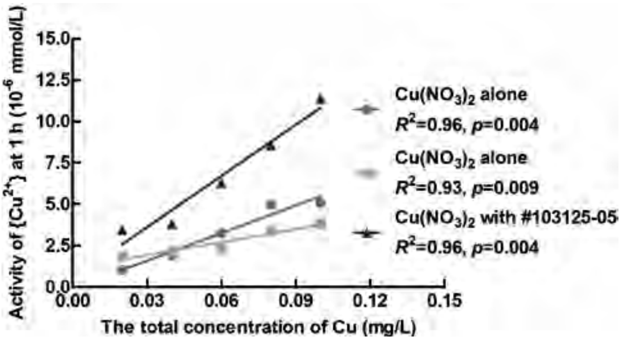

Figure S5.3 Relationships between free $Cu^{2+}$ activities in solution and the added amount of $Cu(NO_3)_2$ in the presence of plain polystyrene fluorescent microspheres after 1 h of equilibration. The total concentration of Cu is the mean value (n=2) of actual concentration of Cu in nitrate salts measured by FAAS. #103125-05 indicates the plain polystyrene fluorescent microspheres. The solid lines represent linear relationships. $R^2$ indicates coefficient of determination. $p$ indicates the statistical significance level.

Table S5.1 Particle characterization of nano-Cu, nano-ZnO and five mixtures expressed as mean ± standard error of the mean (SEM) by dynamic light scattering.

| Compounds | Size distribution (nm) | | Zeta-potential (mV) | |
|---|---|---|---|---|
| | 1 h | 24 h | 1 h | 24 h |
| Nano-Cu | 370 ± 36 | 786 ± 107 | -12.7 ± 0.4 | -5.5 ± 0.4 |
| Nano-ZnO | 1016 ± 28 | 1487 ± 33 | 0.9 ± 0.1 | -6.5 ± 0.3 |
| Cu-nanoCu | 851 ± 26 | 815 ± 26 | -13.6 ± 0.7 | -9.2 ± 0.6 |
| Zn-nanoCu | 418 ± 32 | 631 ± 58 | -11.4 ± 0.6 | -4.4 ± 0.4 |
| NanoCu-nanoZnO | 644 ± 37 | 1531 ± 101 | -8.5 ± 0.5 | -10.8 ± 0.4 |
| Cu-nanoZnO | 1060 ± 90 | 1010 ± 144 | -5.6 ± 0.3 | -4.1 ± 0.3 |
| Zn-nanoZnO | 1222 ± 166 | 1365 ± 93 | 8.1 ± 0.9 | -7.3 ± 0.6 |

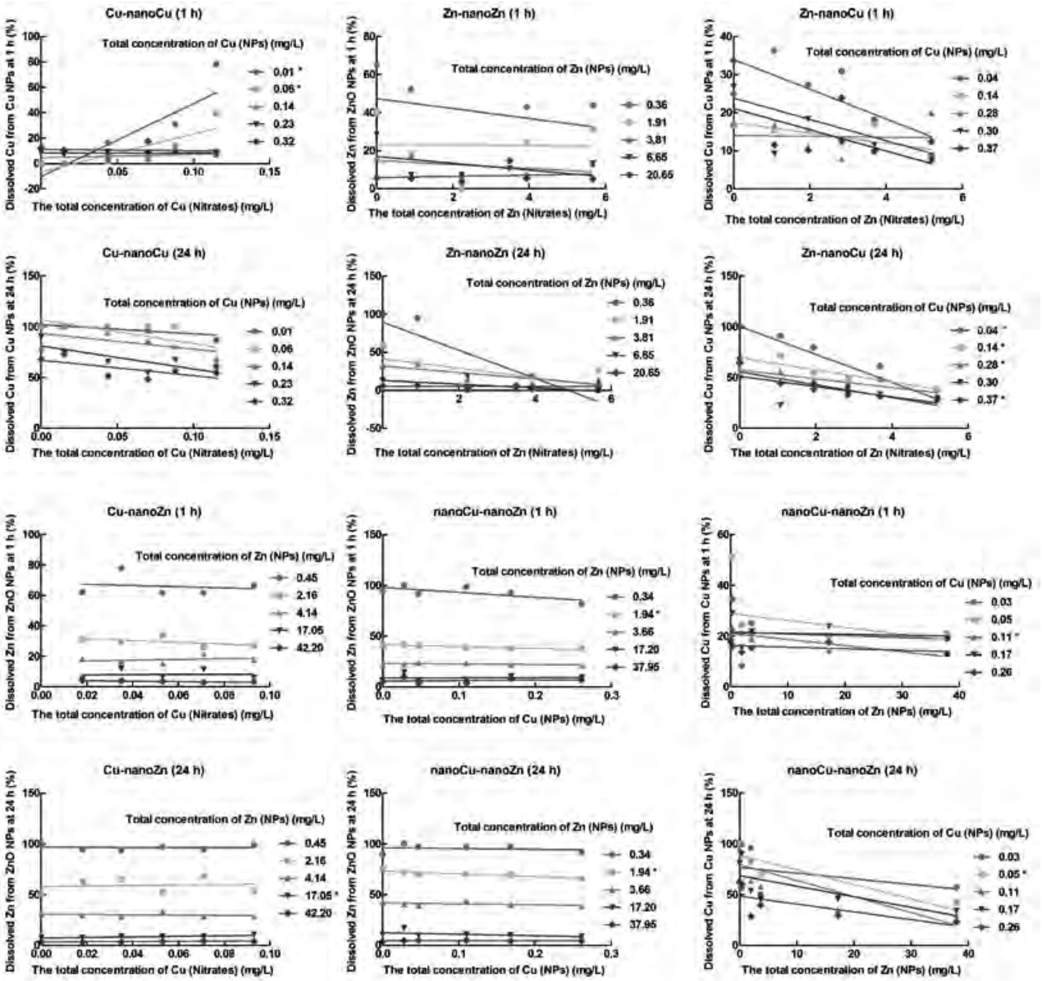

Figure S5.4 Relationships between the percentage of dissolved concentrations divided by the total concentrations of one compound and the added amount of other compounds in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO after 1 h and 24 h of equilibration. The solid lines represent linear relationships. * indicates that the slope of linear curve is significantly different from zero at the 5% significance level.

# Chapter 6

## General Discussion

Organisms are regularly and unavoidably exposed to mixtures of metals which are released into the ecosystems as a result of natural and anthropogenic activities via air, water, food and dermal contacts. However, the majority of published data concerning toxicity testing of metals is focused on single metal effects (Ince et al., 1999). Similar problems occur in the currently accelerating research topic of hazard assessment of synthesized metal-based nanoparticles (NPs) which may subsequently enter the natural environment by for instance the use of bio-solids from sewage systems for fertilizing agricultural soils. It then becomes the challenge of accurately determining interactions of metals and metal-based NPs with biological systems. In this PhD thesis, the influence of the surrounding environment ($H^+$, $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$) was incorporated in the quantification of the adverse effects of metals (Ni and Cd) on root elongation of *Lactuca sativa* L. Besides the interactions within the exposure media, ion-ion interactions were also included in estimating the combined effects of metal mixtures (Cu-Zn, Cu-Ag, Cu-Ni, Cu-Cd, and Ni-Cd) and the relative contributions of each metal to the overall toxicity. Deviations towards overestimated effects (antagonism) or underestimated effects (synergism) using the ‘additivity’ principle were also discussed to search a biologically relevant link. To improve the understanding of the behavior and effects of metal-based NPs on terrestrial plants, lettuce seedlings were respectively exposed to $Cu(NO_3)_2$, $Zn(NO_3)_2$, Cu NPs, ZnO NPs and their five combinations i.e. mixtures of $Cu(NO_3)_2$ and Cu NPs (Cu-nanoCu), mixtures of $Zn(NO_3)_2$ and ZnO NPs (Zn-nanoZnO), mixtures of $Cu(NO_3)_2$ and ZnO NPs (Cu-nanoZnO), mixtures of $Zn(NO_3)_2$ and Cu NPs (Zn-nanoCu), and mixtures of Cu NPs and ZnO NPs (nanoCu-nanoZnO). This PhD thesis is primarily focused on the metals and metal-based NPs mentioned above since they were always found present together at elevated concentrations in contaminated fields (Han et al., 2002; Bystrzejewska-Piotrowska et al., 2009). The aim of this research is translated into a number of research questions as follows:

(1) How does water chemistry affect the toxicity of individual metals (Ni and Cd) to lettuce and how to quantify the influence of water chemistry?
(2) Can the toxicity-modifying factors of water chemistry be incorporated into toxicity models and will the prediction of acute toxicity of individual metals (Ni and Cd) to

lettuce seedlings be improved because of incorporation of these factors in the toxicity models?

(3) What kind of statistically significant deviation patterns from additivity are induced in assessing the combined effects of metal mixtures (Cu-Cd, Ni-Cd and Cu-Ni) to lettuce?

(4) Can the statistically significant deviations from additivity be reproduced and how likely is it that metal ions ($Cd^{2+}$, $Ni^{2+}$ and $Cu^{2+}$) interact with each other?

(5) How to incorporate the impacts of environmental chemistry in assessing the toxicity of metal mixtures (Cu-Ni, Cu-Zn and Cu-Ag) to lettuce?

(6) Will the estimation of mixture toxicity be improved considering ion-ion interactions?

(7) Will the dissolved metal species and the particulate fractions of each type of metal-based NP act jointly according to the rules of additivity?

(8) Will Cu NPs interact with ZnO NPs and influence the toxicity of each other to lettuce?

Prior to a synthesized discussion and a future outlook, answers to the research questions are given below.

## 6.1 Answers to research questions

*(1) How does water chemistry affect the toxicity of individual metals (Ni and Cd) to lettuce and how to quantify the influence of water chemistry?*

Based on the experimental results, it was shown that only $Mg^{2+}$ other than $H^+$, $K^+$, $Na^+$, and $Ca^{2+}$ was found to exert a significantly alleviative effect on the toxicity of Ni to lettuce, whereas no significant influence of these common cations was observed on the toxicity of Cd to root growth of lettuce. The effects of $Mg^{2+}$ on $Ni^{2+}$ toxicity to lettuce (*Lactuca sativa* L.) were quantified by calculating the affinity (the stability constants) of $Ni^{2+}$ for biotic ligands at the water-organism interface and the fraction of the total number of biotic ligands occupied by $Ni^{2+}$ according to the biotic ligand model (BLM) theory. (Chapter 2)

*(2) Can the toxicity-modifying factors of water chemistry be incorporated into toxicity models and will the prediction of acute toxicity of individual metals (Ni and Cd) to lettuce seedlings be improved because of incorporation of these factors in the*

*toxicity models?*

By incorporating the competition from $Mg^{2+}$ in a developed BLM, the prediction of Ni-toxicity was significantly improved from 50% to 80% of the explained variance in lettuce responses, as compared to the total metal model (TMM) and the free ion activity model (FIAM). Since the overall variations of $IC_{50}\{Cd^{2+}\}$ within the varied concentrations of $H^+$, $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$ in the solution were rather small, the TMM and the FIAM instead of BLM performed equally well in explaining the inhibitive effects of Cd on root elongation of lettuce. (Chapter 2)

*(3) What kind of statistically significant deviation patterns from additivity are induced in assessing the combined effects of metal mixtures (Cu-Cd, Ni-Cd and Cu-Ni) to lettuce?*

The statistically significant deviation patterns from additivity varied for specific binary mixtures of metals and for different base models applied. Using the MixTox model, statistically significant deviations were always found in predicting the toxicity of Cu-Cd, Ni-Cd and Cu-Ni mixtures to lettuce (*Lactuca sativa* L.) when the concentration addition (CA) model was used as the reference model. Deviations shifted from antagonism to synergism, the magnitude of which depended on the relative concentrations of the two metal components in the mixture and the dose levels across the whole tested ranges. However, no statistically significant deviations were found when the independent action (IA)-based models were applied to assess the overall toxicity of $Cu^{2+}$ and $Ni^{2+}$ to root growth of lettuce. Similarly, the BLM-based toxic unit (TU) method without considering ion-ion interactions was significantly superior to $f_{\mathrm{mix}}$ or TEF approaches in assessing the toxicity of Cu-Ni mixtures, which indicated no substantial deviations from additivity as well. (Chapter 3 and Chapter 4)

*(4) Can the statistically significant deviations from additivity be reproduced and how likely is it that metal ions ($Cd^{2+}$, $Ni^{2+}$ and $Cu^{2+}$) interact with each other?*

Dissimilar results or even contradictory deviation patterns were obtained when the datasets of Ni-Cd and Cu-Ni mixtures with lower concentrations of Ni and Cd were inserted into the MixTox model. The assessment of deviations strongly depended on the fitting of experimental data, the choice of mathematical models and the specific range of exposure concentrations. Thus, the toxic actions or interactions of

$Cd^{2+}$, $Ni^{2+}$ and $Cu^{2+}$ cannot be easily concluded based on these non-reproducible statistically significant deviations. Further measurements and modeling may assist in improving the mechanistic understanding of interactions between metals in a mixture especially at the internal process of organisms. (Chapter 3)

*(5) How to incorporate the impacts of environmental chemistry in assessing the toxicity of metal mixtures (Cu-Ni, Cu-Zn and Cu-Ag) to lettuce?*

According to the concepts of the BLM, the toxicity of metals to organisms is mainly determined by the fraction of the biotic ligands occupied by free metal ions. Thus, the affinities of $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Ag^{+}$ for biotic ligands at the water-organism interface were included in the toxicity assessment of Cu-Ni, Cu-Zn and Cu-Ag mixtures to lettuce (*Lactuca sativa* L.). This allowed not only to integrate the impacts of environmental chemistry (i.e. $Mg^{2+}$ and $H^{+}$) but also the interactions between $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Ag^{+}$ and roots of lettuce in toxicity modelling. By combining the BLM with the overall amounts of metal ions bound to the biotic ligands ($f_{\mathrm{mix}}$), competitions at the water-organism interface between each component in the binary mixtures for binding sites on the biotic ligands were also considered in estimating mixture toxicity. With the toxic equivalency factor (TEF) as a toxicity index, the different potencies of $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Ag^{+}$ relative to the most toxic metal (Cu) towards lettuce can be incorporated in modeling toxicity of metal mixtures as well. (Chapter 2 and Chapter 4)

*(6) Will the estimation of mixture toxicity be improved considering ion-ion interactions?*

Using the MixTox model, the predictive capabilities of extended mixture functions were compared with those of reference models (CA and IA). Extended mixture functions integrating ion-ion interactions were mostly better than the addition models for four of the five datasets. By the method of bootstrapping, the statistical significance of difference in predictive power was compared between different non-nested BLMs. The models considering ion-ion interactions were better than the BLM-based toxic unit (TU) approach and the strictly additive models for assessing the overall toxicity of Cu-Cd, Ni-Cd, Cu-Zn, Cu-Ag mixtures, apart from the combination of Cu-Ni. This may be caused by the different mechanisms of toxicity of diverse metal mixtures and suggests that joint toxicity of metal mixtures to terrestrial plants needs to be evaluated on a combination-specific basis. (Chapter 3 and Chapter 4)

*(7) Will the dissolved metal species and the particulate fractions of each type of metal-based NP act jointly according to the rules of additivity?*

Since most metal-based nanoparticles (NPs) are hydrophilic but slightly soluble, it was assumed that each type of metal-based NPs can be divided into two parts i.e. the soluble species and the undissolved particles, and both of them may play a role in inducing toxicity of Cu NPs or ZnO NPs to lettuce (*Lactuca sativa* L.). The dissolved concentrations of Cu NPs or ZnO NPs were expressed as the averaged values after 1 h and 24 h as the exposure media was refreshed every day. Antagonistic effects were indeed found between the dissolved Zn and the particulate Zn based on the toxicity data obtained for Zn-nanoZnO mixtures, which was not observed for Cu-nanoCu mixtures. This finding simultaneously explained the difference in predictive power (10%) when the IA model was used to predict the combined toxicity of Zn-nanoZnO ($R^2$=0.84) and Cu-nanoCu ($R^2$=0.94) mixtures respectively. (Chapter 5)

*(8) Will Cu NPs interact with ZnO NPs and influence the toxicity of each other to lettuce?*

The IA model explained 82% of the variance in the data of mixtures of Cu NPs and ZnO NPs to lettuce. To systematically detect how and where the discrepancy of modeling occurred, the experiments were designed with six nested combinations i.e. mixtures of Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO. The 50% effective concentrations of Cu NPs or ZnO NPs were found to be statistically significant increased by the raised amount of each other and by $Cu(NO_3)_2$ or $Zn(NO_3)_2$ in the solution. Besides the interactions between dissolved Cu and dissolved Zn (or $Cu^{2+}$ and $Zn^{2+}$), their particulate forms were also highly correlated with the overall toxicity of Cu NPs and ZnO NPs to lettuce. This indicated that the combined toxicity of Cu and Zn in nano-size was much more complex than the combined toxicity of their nitrate mixtures. Moreover, only the amount of dissolved Cu released from Cu NPs after 24 h was found to be consistently decreased by the added amount of $Zn(NO_3)_2$. These findings suggested that the small antagonistic effects between Cu NPs and ZnO NPs likely occurred at the organism level and therefore is responsible for the remaining variation (18%) in toxicity modeling. (Chapter 4 and Chapter 5)

## 6.2 Application of biotic ligand models in assessing toxicity of metals to terrestrial organisms

Understanding bioavailability and toxicity of metals in depth is necessary to derive environmental quality criteria and standards. Some researchers have proposed that the free metal ion activity, considering the influence of environmental factors on bioavailable fractions of metals, can establish a better link between effects and exposure of metals as compared to total metal or dissolved metal concentrations (Lexmond and Vorm, 1981). As an extension of free ion activity model (FIAM), the biotic ligand model (BLM), which integrates competitions from common cations in natural environment for binding to the biotic ligands (BL), has been suggested as a useful tool to address how metals interact with organisms in the aquatic environment. For instance, the US Environmental Protection Agency (EPA) has applied the aquatic biotic ligand model ((a)BLM) to outline Ambient Water Quality Criteria (AWQC) in surface water (EPA, 2007).

As compared with water systems, the exposure pathways of metals are much more complex in the soil phases for different terrestrial organisms (exposure via the pore water or the soil particles). Steenbergen et al. (2005) developed a terrestrial biotic ligand model ((t)BLM) to predict the toxicity of Cu to the earthworm *Aporrectodea caliginosa* and Lock et al. (2006) developed a (t)BLM to predict cobalt toxicity to the potworm *Enchytraeus albidus*. However, some scientists have shown that there is no single bioassay or organism that can be representative of all biota present in the ecosystem (Ince et al., 1999). Thus, food choice of higher plants may be a potential alternative in currently developed short-term toxicity testing methods to represent the bioavailability and toxicity of metals to soil biota. Thakali et al. (2006a, 2006b) developed (t)BLMs for assessing the ecotoxicity of Cu and Ni to higher plants, invertebrates, and microbes.

The development of (t)BLMs largely relies on the partitioning of metals between the soil and the solution phase, which is usually estimated by speciation models such as WHAM 6 and MINEQL+4.5. However, the accuracy of prediction for metal speciation in soil may be affected by the default assumptions of these models, e.g. by overestimating the binding capacity of humic substances with metals (Cloutier-Hurteau et al., 2007) and by ignoring precipitation removing metals from the solution in WHAM 6 (Thakali et al., 2006a). Higher plants are predominantly exposed to metals via the pore water (McLaughlin, 2000). To manipulate better the composition of the soil pore water and the metal concentrations to which organisms are exposed, hydroponic solutions were chosen as the exposure media in this study to overcome the above problems in the application of BLMs for terrestrial organisms. To avoid uncertainties in activity modeling, the free ionic form of Cu ($Cu^{2+}$) was directly measured by a Cu-ionic selective electrode (Cu-ISE) in this thesis (Chapter 3 and Chapter 4). In Chapter 2, it was proven that the total concentration of nickel cannot well account for its bioavailability and toxicity to lettuce and the site-specific competitions of other cations in solution helped to explain the variations in toxicity, which was consistent with the concept of BLM (Di Toro et al., 2001). The derived stability constants of metal ions for biotic ligand binding i.e. log $K_{MgBL}$ = 2.86, log $K_{NiBL}$ = 5.1, $f_{NiBL}$ = 0.57 may help scientists to estimate the intrinsic toxicity of individual metals and the sensitivity of terrestrial organisms to specific metals. However, similar results were not observed for cadmium, which suggested that the toxicity of metals to higher plants needs to be evaluated on a metal-specific basis.

Until now, most studies are focused on development and application of a (t)BLM for assessing metal toxicity in controlled water systems (Antunes and Kreager, 2009; Li et al., 2009; Lock et al., 2007), and validations in the field are further needed. Nevertheless, it is problematic to exactly determine the most influential soil characteristics affecting metal toxicity across different soils (Christiansen et al., 2015) based on the current level of knowledge and technology. This raises the difficulty of extrapolating the developed BLMs from solution to soil. As shown in Chapter 2, the values of $f_{NiBL}$ differed a lot in solution (0.57) and soil culture (0.05) even for the same plant species *Hordeum vulgare*. This was strongly correlated with the different toxicity-modifying factors (e.g. common cations) found in different conditions. Additionally, deviations of toxicity modeling can also be effects caused by other factors that are ignored in conventional BLMs, e.g. mixture, food quality or quantity, and life history of organisms (Verschoor, 2013). Integrating mixture factors can be not only helpful for further model validation but would also assist in obtaining accurate knowledge of underlying mechanism of metals. Thereupon, the BLM was extended with mixture effects for toxicity modeling in this thesis (Chapter 4). By combining BLMs with toxicity indices (i.e. TU, $f_{\mathrm{mix}}$ and TEQ), both the influence of other toxic metals in the surrounding environment and the different toxic potencies of each metal were included in toxicity assessment of metals. However, it remains to be determined whether to incorporate unfavorable conditions from the environment and the organism in risk assessment. In this thesis (Chapter 2 to 5), those variables were strictly controlled which allowed to focus the toxicity-modifying factors on mixture factors and water chemistry. To reduce the interference of nutritional deficiencies, the Steiner solution which has been proven to be sufficient for lettuce growth and rooting (Peijnenburg et al., 2000) was used as the culturing and testing media in the present study. To avoid individual differences, the 4 d seedlings were strictly chosen making sure that roots with a length greater than 3 cm were used for all experiments.

## 6.3 Interpretation of interactions in assessing toxicity of metal mixtures

Since metal mixtures are often found in the environment instead of single metals alone, the assessment of metal toxicity seems to be more relevant and accurate when mixture effects are considered. Metal speciation, competition and complexation, as well as interactions with organisms may help to construct a real scenario of bioavailability and toxicity for metal mixtures (Qiu, 2014). The mechanistic bioavailability models such as the BLM and the electrostatic toxicity model (ETM) may be expanded to increase the predictive power for the combined effects of metal mixtures. Until now, the concept of concentration addition (CA) is the mostly used method to extend the BLM for toxicity assessment of metal mixtures (Playle, 2004; Hatano and Shoji, 2008; Jho et al., 2011; Le, 2012). In this thesis (Chapter 4), the relative contributions of mixture components to the overall toxicity were expressed as three toxicity indexes i.e. toxic unit (TU), the overall amounts of metal ions bound to the biotic ligand ($f_{\mathrm{mix}}$), the toxic equivalency factor (TEF), and were added up to reflect inhibition of lettuce root elongation (*RRE*, %) by metal mixtures. The use of TU was based on the assumption that no competition occurs between toxic components in a mixture (Hewlett and Plackett, 1979) apart from the statistically significant impacts from the surrounding media e.g. the influence of $H^+$ and $Mg^{2+}$ on the toxicity of $Cu^{2+}$ (Le, 2012) and $Ni^{2+}$ (Chapter 2) respectively. Using the approach of $f_{mix}$, both competition between metal ions and competition with common cations in the surrounding media for the binding sites can be included in mixture modelling (Jho et al., 2011). On the basis of $f_{mix}$, the different toxic potencies of each metal relative to the most toxic one (Cu) can be considered by TEF (Van den Berg et al., 1998; Le, 2012). The best fitted models (i.e. the BLM-based $f_{mix}$ and TEF) explained 73% to 74% of the variance in inhibition effects of Cu-Zn and Cu-Ag mixtures on root elongation (see Table 6.1). As compared to the BLM-based methods, the ETMs showed a higher predictive power for Cu-Zn ($R^2$ = 0.92) and Cu-Ag ($R^2$ = 0.80) mixtures. This difference may be caused by the exclusion of physiological processes in simulating ion-ion interactions by BLMs e.g. the change of the electrostatic nature of the plant cell wall (Wang et al., 2010). At various levels, metals will interact with each other and with organisms, whereas only competitions for the binding sites at the water-organism interface are included in the BLMs and directly related to the combined toxicity of metal mixtures. However, without considering the toxic-kinetic mechanism of metal ions, the incorporated interactions (fitting factors) in the ETMs for assessing toxicity of metal mixtures largely depend on the mathematical fitting (Le, 2012) and therefore may not be applicable to complex mixtures. Based on the significance tests of bootstrapping, the BLM-based TU approach ($R^2$=0.86) provided the best prediction of the overall toxicity of Cu-Ni mixtures regardless of the potential competitions between $Cu^{2+}$ and $Ni^{2+}$. Similar to the BLM-based TU method, the FIAM also explained 85% of the variance in toxicity of Cu-Ni mixtures based on the concept of independent action (IA) and the assumption of no substantial interactions. It was thus concluded that the underlying mechanisms of mixture toxicity are different across diverse metal combinations, as indicated by the best fitting model.

The extended BLMs based on the concept of CA integrated the influence of environmental chemistry on the toxicity of each metal in a mixture, which allowed them to be applied for complex mixtures containing more than two metals. The binding constants derived from single exposure of metals were applicable to metal mixtures under the same experimental conditions, which reduced the amounts of measurements for all the combinations of metals. However, the inclusion of affinity of metal ions for the biotic ligands sometimes becomes a problem for specific metals. In this PhD thesis (Chapter 2), it was impossible to empirically fix the key parameters of the BLM (e.g. $f_{50}$ and $K_{\mathrm{CdBL}}$) for cadmium due to the lack of a statistically significant relationship between the $Cd^{2+}$ toxicity (median effective concentrations) and the concentrations of other common cations in the solution. Therefore, the conventional models (i.e. CA and IA) were extended for assessing the combined toxicity of metal mixtures with Cd (Chapter 3). As shown in the study of Jonker et al. (2005), the deviations from 'additivity' can be quantified by the additional parameters in the extended CA or IA model. Statistically significant antagonistic effects were commonly found for Cu-Cd and Ni-Cd mixtures by the MixTox model and their changes of magnitude were dependent on the relative concentration levels across the whole range and the concentration ratios of mixture components. However, similar deviation patterns were not observed when the mixture models were fitted to the toxicity data of mixtures with lower concentrations of $Ni^{2+}$ or $Cd^{2+}$. This implied that the statistically significant deviations may not necessarily be the biologically relevant interactions, which proved the arguments of Cedergreen et al. (2007) and EFSA (2011). Alternatively, the assessment of deviation patterns strongly depended on the different metal combinations, the diverse predictive methods applied and the mathematical fitting results. The MixTox model overcomes the shortcoming of BLM that the binding constants of each metal should be fixed separately beforehand, and refines the complex deviation patterns not limited to overall antagonism or synergism. However, the intricate calculation process relying on empirical isotherms and the lack of insight into the mechanisms of the interaction would hinder the wide-scale applicability of the MixTox model. This raises the question of how to balance the mathematical data-fittings with the explanations of possible mechanisms in which interactions of metals would occur in toxicity assessments of metal mixtures. Generally, our findings provided the comparison of existing models in assessing combined toxicity of different metal mixtures, pointed out the technical problems in interpreting statistically significant departures from classic 'additivity', and proposed a possible future of developing alternative models.

Table 6.1 Assessment of interactions in Cu-Ni, Cu-Zn, Cu-Ag mixtures by the biotic ligand model (BLM), the free ion activity model (FIAM), and the electrostatic toxicity model (ETM) on the basis of concentration addition (CA) and independent action (IA) concepts.

| Methods | Cu-Ni mixtures | Cu-Zn mixtures | Cu-Ag mixtures |
|---|---|---|---|
| Assumption of FIAM or ETM (CA) | DR or DL dependent interactions | Interactions | Interactions |
| Goodness of fitting (Chapter 3; Le, 2012) | $R^2 = 0.55$ | $R^2 = 0.92$ | $R^2 = 0.80$ |
| Assumption of FIAM or ETM (IA) | No substantial interactions | Interactions | No substantial interactions |
| Goodness of fitting (Chapter 3; Le, 2012) | $R^2 = 0.85$ | $R^2 = 0.92$ | $R^2 = 0.80$ |
| Assumption of BLM-based TU (CA) | No substantial interactions | No substantial interactions | No substantial interactions |
| Goodness of fitting (Chapter 4) | $R^2 = 0.86$ | $R^2 = 0.58$ | $R^2 = 0.69$ |
| Assumption of BLM-based $f_{\text{mix}}$ (CA) | Interactions | Interactions | Interactions |
| Goodness of fitting (Chapter 4) | $R^2 = 0.58$ | $R^2 = 0.73$ | $R^2 = 0.58$ |
| Assumption of BLM-based TEF (CA) | Toxic potency dependent interactions | Toxic potency dependent interactions | Toxic potency dependent interactions |
| Goodness of fitting (Chapter 4; Le, 2012) | $R^2 = 0.76$ | $R^2 = 0.65$ | $R^2 = 0.74$ |

TU: toxic unit index; $f_{\text{mix}}$: the overall amounts of metal ions bound to the biotic ligands; TEF: the toxic equivalency factor; DR: dose ratio; DL: dose level; $R^2$: the determination coefficient.

## 6.4 Extrapolation of mixture models to nano-toxicity

Due to the decreased size, some metal-based NPs are showing increased toxicity to organisms as comparted to their bulk forms, even for inert elements such as Ag, Au and Cu (Schrand et al., 2010), which has gained increasing attention from people. However, precise knowledge should be gained before establishing the standards to assess the hazards of metal-based NPs. Physical and chemical properties of metal-based NPs keep changing over time when particles are released into the environment. Inadequate information is currently available for metal-based NPs to quantify the processes of dissolution, agglomeration or aggregation (Tourinho et al., 2012). Thus, as an extension of this thesis (Chapter 5), we tried to increase the understanding of behavior and effects of metal-based NPs in liquids based on a newly designed toxicity testing method and the conventional mixture models applied in previous chapters.

Due to the high uncertainties in calculating $EC_{50}$s for engineered metal-based NPs, the most frequently used independent action model (IA) was applied for assessing toxicity of Cu NPs and ZnO NPs other than the concentration addition (CA) model. More than 80% of the variation in combined toxicity was explained by the IA model for nanoCu-nanoZnO mixtures. To identify where and how the variations left in toxicity modeling occurred, a comprehensive experiment was designed with six nested combinations i.e. Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO. Copper or zinc nitrates were mixed with ZnO NPs or Cu NPs to mimic changing concentrations of dissolved species of metal-based NPs. To date, the dissolution, agglomeration or aggregation of metal-based NPs are found to be dynamic processes which result in an intermediate state of bulk and molecular for metal-based NPs (Misra et al., 2012). It was thus assumed that each type of metal-based NP was a mixture containing a part of dissolved metal species and a part of undissolved particles in the present study. In exploring whether these two parts would impact the toxicity of each other, increasing concentrations of $Zn(NO_3)_2$ in the solution were found to strongly correlate with the $EC_{50}$ values of ZnO NPs, and vice versa. This finding emphasized the importance of particulate forms in inducing the toxicity of metal-based NPs to environmentally relevant organisms and suggested that searching a dominant metal species may not be appropriate to truly reflect the adverse effects of NPs. Similar effects were not observed for Cu NPs and $Cu(NO_3)_2$, which was consistent with the result that 94% of the variation in toxicity of Cu-nanoCu mixtures can be explained by the IA model. The increasing concentrations of dissolved or particulate Cu or Zn were also substantially associated with reduced toxicity of $Zn(NO_3)_2$ or $Cu(NO_3)_2$ to lettuce. The above results succeeded in explaining the difference in the 2D isobolic representations between nanoCu-nanoZnO mixtures and $Cu^{2+}$-$Zn^{2+}$ mixtures. Small antagonistic effects were found between Cu NPs and ZnO NPs by using linear relationships, whereas these mutual impacts between metal-based NPs were much complex than interactions occur among metal ions.

Based on the current knowledge, the concentrations of particulate forms can be roughly estimated by the total concentrations minus the dissolved concentrations. However, the toxic effects that resulted from the particulate forms alone cannot be easily separated from the total effects of Cu NPs or ZnO NPs following the rules of additivity because of the potential interactions between dissolved metal species and non-dissolved particles. The way of quantifying the biological responses caused by the non-dissolved particles of metal-based NPs seems to be beneficial to further application of mixture models in toxicity assessment of metal-based NPs. Ideally the toxicological studies will be more accurate if testing is performed at intermediate points in time instead of a standardized exposure time (Baas et al., 2010). However, it is difficult to get data over time and continue the experiments for 4 d since lettuce seedlings are very sensitive to the environment out of water and easy to be hurt while manually measuring length. This problem may be solved by an automatic image measuring instrument, while experimental costs would be greatly increased and the measurement error due to the curling roots is difficult to avoid. Although there is still much room for improvement, our research no doubt established a more realistic scenario which would enrich the rapid evolving field of nano-toxicology and helps scientists to develop approaches to predict the potential impacts of metal-based NPs on eco-systems.

## 6.5 Implications

Ecological risk assessments of chemicals are supposed to evaluate how likely it is that the environment may be impacted as a result of exposure to these environmental stressors. The information and tools developed from ecological risk assessments can be used to create criteria and management means by government agencies or industry for chemical stressors before application or release into the environment (Van Gestel, 2012). Generally, the derivation of limit values accounting for soil or water quality has strong links with the eco-toxicological data. However, the laboratory conditions have been well-standardized far from potentially exposed ecosystems in most studies concerning effect assessments of chemicals (Arvidsson et al., 2011). This reduces the interference from the complex nature of the environment in toxicological experiments and therefore adds a high uncertainty in actual consequences of chemical stressors in the environment (EC, 2013). In this thesis, relatively realistic scenarios were developed in effects assessments of metals and metal-based NPs by incorporating the factors of environmental chemistry, bioavailability and mixtures. A range of health issues such as the neuro-developmental disorders are suspected to be related to cumulative stress of heavy metals (Løkke et al., 2013). Thus, lettuce (*L. sativa* L.) as one of the main food items on the table was chosen to be a biomarker of early life exposure to metals and metal-based NPs in this study. Data of measurements on exposures to individual metals and mixtures of metals in Chapter 2, 3 and 4 enrich the database on adverse effects of multiple metals on edible plants. The critical values (e.g. $EC_{50}$ of metals) calculated at specific conditions can be used for setting environmental risk limits (e.g. negligible concentrations) for metals. The affinity of metals may help distinguish interactions occurring at the membrane surface or at the internal process. The bioavailability models developed in Chapter 2 and 4 help toxicologists to understand how and why metals interact and the approaches used in Chapter 3 assist in quantifying and characterizing the uncertainty in current methodologies for searching interactions between metals. Since laboratory work is not feasible to be carried out for all the possible combinations of metals, this thesis investigated five most likely combinations of metals in the terrestrial environment (Han et al., 2002), and developed a scheme as shown in Figure 6.1 to assess the combined effects of metals for specific combinations. First of all, the bioavailability and toxicity of each metal in a mixture should be investigated separately. If the variability in median effective concentrations of metals could be sufficiently described with no impact of common cations, then the normal mixture models (CA or IA) can be used to estimate the overall toxicity of metal mixtures to organisms and the extended mixture functions can be used to quantify the deviations of modelling from 'additivity'. If cations ($H^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Na^+$ etc.) are found to significantly alleviate the toxicity of single metals, it is better to incorporate the influence of environmental chemistry in modelling the joint toxicity of multiple metals in terms of competitive binding for the biotic ligand. In that case, models with a mechanistic basis are recommended for a relatively effective and accurate risk assessment of metal mixtures e.g. the extended BLM in diverse ways for describing deviations or interactions. Based on the current scientific knowledge, it is still difficult to directly determine the underlying mechanisms of interactions as an organism is a complex entity. This also hinders the way to distinguish deviations from interactions. The enhancement of statistically-based tools (Van Genderen et al., 2015) and the improvement of bioavailability models such as combining BLM and ETM may additionally explain how and where metal-metal interactions occur, and may advance the mixture modelling. Engineered metal-based nanoparticles are a new source of environmental contamination, while the information is scarce on their release, fate and toxicity, especially under their co-exposure. In Chapter 5, we first proposed that the well-known independent action (IA) model can be preliminarily used to assess the combined toxicity of mixtures with metal-based nanoparticles based on good fitting results ($R^2$=0.82-0.94). This indicates that our study provided a way to roughly calculate environmental quality standards (EQS) for metal-based NPs which is essential to protect and sustain the quality of surface water and soils. The variations left in toxicity modeling of Cu NPs and ZnO NPs (up to 18%) were exactly explained by a novel experimental setup with six nested combinations. This experimental design assisted in searching mutual impacts between different types of metal-based NPs and tracing down where these mutual impacts took place. Further measurements and modeling can be focused on verifying these statistically small antagonistic effects. If the underlying mechanism of metal-based NPs can be determined across different exposure conditions, the specific assessing framework can be generated for evaluating the potential impacts of metal-based NPs on eco-systems.

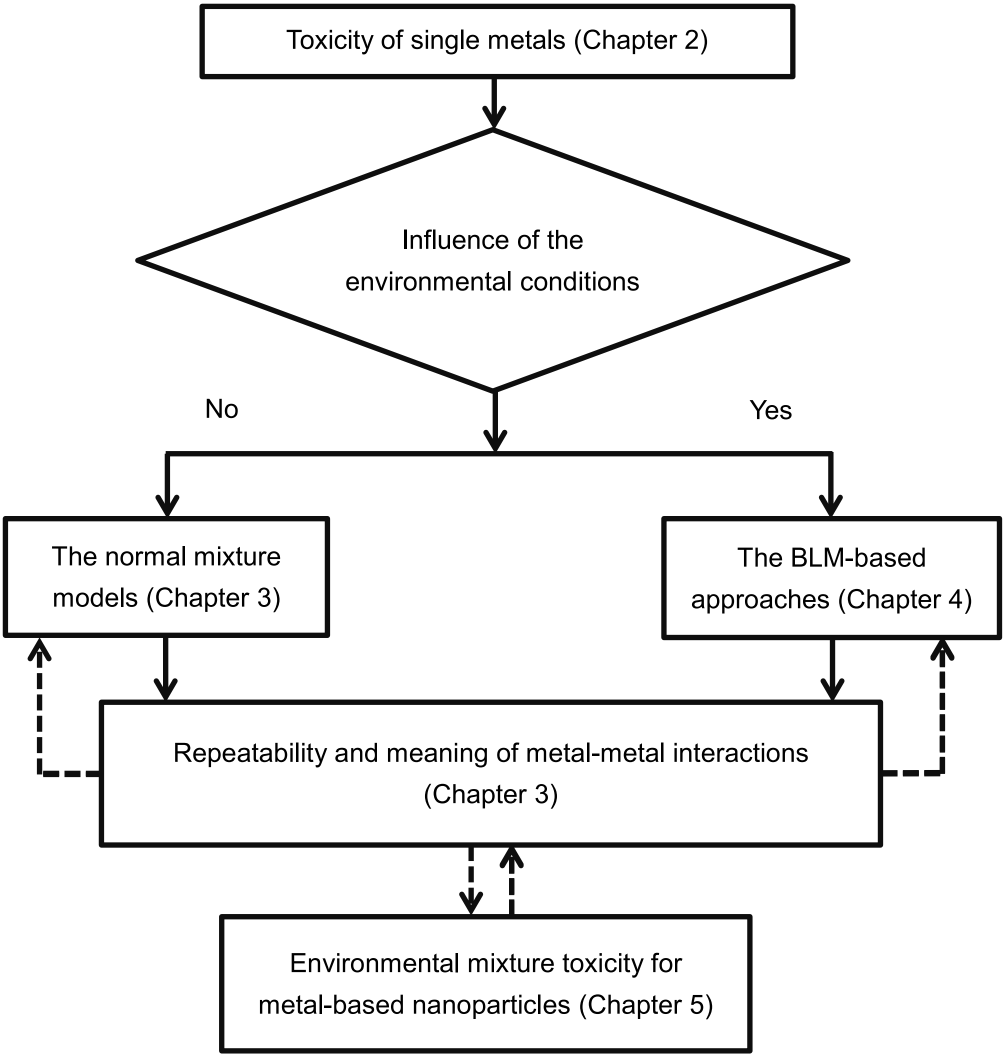

Figure 6.1 Scheme of approaches for assessing toxicity of metal-based mixtures applied in this PhD thesis.

## 6.6 Future outlook and recommendations

In this PhD thesis, two of the most important toxicity-modifying factors (i.e. environmental chemistry and mixture effects) were incorporated into the assessment of adverse effects of metals and metal-based NPs on terrestrial plants in different ways. To improve the risk assessment procedures for metals and metal-based NPs, the observed toxic effects and the mutual impacts found among metals or metal-based NPs were interpreted by means of considering several processes. It is recommended that a series of validation and extrapolation studies are performed in the future for further strengthening the models and conclusions developed in our research.

Compared to organic compounds with a known mode of action, the toxicity of metals and the underlying mechanisms are much more complex. This may be specific across different conditions. As a starting point for looking into the mixture toxicity, the bioavailable fractions of each metal in different surrounding environments were linked to toxicity by TMM, FIAM and BLM. In the natural environment, the water chemistry (common cations) is not the only potential stressor. Other factors e.g. temperature, oxygen, and light may also affect the functioning of organisms and then affect the adverse effects of metals. It may be favorable to work with these multiple stressors and integrate them in explaining toxicity of metals under natural conditions.

To deal with the impacts of mixtures on toxicity assessment of metals, metals and their mixtures were exposed in a simplified system—a hydroponic solution, to avoid the interactions in the soil compartment and to manipulate the exposure concentrations. Different metals may share the same uptake route and likely interact at the water-organism interface (Bongers, 2007). It was observed that $Mg^{2+}$ and $H^{+}$ did compete with respectively $Ni^{2+}$ and $Cu^{2+}$ for the binding sites on lettuce roots. Based on the concept of concentration addition, BLMs considering competition from common cations were extended to describe the combined toxicity of metal mixtures and several parameters (e.g. $TEQ_{50}$) were derived for mixtures of Cu-Ni, Cu-Zn and Cu-Ag. Toxicity of metal mixtures with Cd was assessed using the extended additivity models (CA or IA) with additional parameters. However, the biological meaning of such parameters was not completely clarified given the large variability of statistical significance and of bioavailability and sensitivity of metals to specific organisms. To improve mixture toxicity principles, it is necessary to intensively identify relationships between these parameters and the ‘intrinsic’ toxicity of metal mixtures. Although the bioavailability models developed in this study explained chemical-chemical interactions which may affect the combined toxicity of metal mixtures before entering organisms, the mechanisms of interaction of metals present in mixtures inside the organisms are poorly understood. Metal accumulation in organisms does not always correlate well with observed toxic effects (Lanno et al., 2004) as organisms have created many mechanisms to process metal stressors (Tangahu et al., 2011). It is therefore suggested to further investigate the observed mutual impacts between multiple metals by advanced monitoring tools such as patch clamp, proteomics and genomics.

The developed models for mixtures of Cu-Ni, Ni-Cd, Cu-Cd, Cu-Zn, Cu-Ag are recommended to be further validated in real soils and extrapolated for other higher plants. For a better extrapolation from water to soil, it is essential to increase the understanding of toxicokinetics and toxicodynamics of metals (Van Gestel, 2012). Toxicity of metals was already found to be time-dependent (Alda Álvarez et al., 2006; Baas et al., 2010). Evaluating mixture toxicity and interactions may also benefit from a better understanding of such dynamic processes, especially for metal-based NPs, the toxicity of which is known up to now as a consequence of aggregation, agglomeration and dissolution processes that vary over time. The combined effects of mixtures of metal-based NPs were found to be different from those of metal mixtures in the sense that mutual impacts as observed between metal-based NPs were much more complex than interactions among metal ions. Besides dissolved metal species, the fractions of undissolved particles also played an important role in inducing toxicity of metal-based NPs to higher plants. Although further studies are still needed for selecting a representative endpoint or biomarker, we made the first step to unravel the fate and toxicity of metal-based NPs and their complex mixtures for terrestrial plants. Death and growth are often regarded as multi-step processes. In parallel with growth, other physiological endpoints such as pigment content, primary chlorophyll and carotenoids which can be directly associated with the health of the plants, may be helpful to describe internal interactions over time and evaluate the joint toxicity of nanoparticles and their mixtures. Properly evaluating the effects of mixture interactions on modulating the combined toxicity can help authorities to determine how to incorporate the issue of mixtures into the risk assessment of exposures to metals and metal-based nanoparticles.

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# Summary

Metals are widely present in oceans and in the crust of the earth. Some of them provide organisms on this planet with the necessary nutrients to sustain proper functioning. However, excessive quantities of metals can be toxic. The physicochemical properties of water and soil in the natural environment, such as hardness, pH and dissolved organic carbon, may affect the bioaccumulation of metals and complicate their risk assessment in aquatic and terrestrial ecosystems. Integrating these factors in the development of mechanistic models allows the variability in predicting bioavailability and toxicity of metals to be reduced.

The study presented in Chapter 2 investigated the influence of $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Na^+$ and pH on the acute toxicity of Ni and Cd to butter-head lettuce seedlings (*Lactuca sativa* L.). It was shown that only $Mg^{2+}$ and not $H^+$, $K^+$, $Na^+$, and $Ca^{2+}$, exerts a significant alleviative effect on the toxicity of Ni to lettuce, whereas no significant influence of any of these common cations was observed on the effect of Cd on the root growth of lettuce. Based on the biotic ligand model (BLM), the competition of $Mg^{2+}$ with $Ni^{2+}$ for binding sites at the biotic ligand at the water-organism interface was incorporated in the prediction of Ni toxicity. This greatly improved the predictive power ($R^2$=0.80) in assessing the toxic effects of Ni at varying concentrations of $Mg^{2+}$ in the solution, compared to the total metal model (TMM) ($R^2$=0.49) and the free ion activity model (FIAM) ($R^2$=0.60). As regards Cd, since the overall variations of IC50$\{Cd^{2+}\}$ at the different concentrations of $H^+$, $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$ in the solution were rather small, the TMM performed just as well as the FIAM in explaining the inhibition of root elongation of lettuce by Cd. We therefore suggest that mechanistically underpinned models for assessing the toxicity of metals to higher plants should be generated on a metal-specific basis according to the toxicological data.

Another toxicity-modifying factor, namely mixture effects, also plays an important role in the assessment of adverse effects of metals on terrestrial plants. Humans and other organisms living in the natural environment are often exposed to a variety of substances. Toxicity of metal mixtures to organisms may deviate significantly from the added effects of individual metals because of interactions. Hence, researchers are constantly improving the accuracy of methods for toxicity assessments for multiple metals.

Chapter 3 reports on a study to investigate the joint toxicity of binary metal mixtures, i.e. Cu-Cd, Ni-Cd, and Cu-Ni, using concentration addition (CA) based models and independent action (IA) based models. Inhibition of the root elongation of lettuce by Cu-Cd, Ni-Cd and Cu-Ni mixtures was quantified by statistical software, i.e. the MixTox model. The toxicity of these binary metal mixtures was equally well predicted by the CA-based and IA-based models. Statistically significant deviations from additivity were often found in the toxicity modeling of these three metal mixtures, and the deviation patterns were variable for specific combinations and for different base models. To examine whether these statistically significant deviations were reproducible, other datasets derived from independent experiments using Ni-Cd and Cu-Ni mixtures were used as input in the same mixture models. However, the deviations patterns were found to be inconsistent or even contradictory across various ranges of metal concentrations and different base models. We therefore recommended that a statistically significant deviation from a standard model must be interpreted with caution, and does not necessarily reflect a biologically relevant interaction. Finding statistically significant deviations may be a starting point for further measurements and modeling to improve the understanding of non-additive interactions occurring inside organisms.

In Chapter 4, the biotic ligand model (BLM) was extended to predict the overall toxicity of Cu-Ni, Cu-Zn, and Cu-Ag mixtures to lettuce (*Lactuca sativa* L.) in three approaches based on the concept of additivity, namely the toxic unit approach (TU), the toxic equivalency factor approach (TEF) and the approach of determining the fraction of the total number of biotic ligand sites bound by metal ions in mixtures ($f_{\text{mix}}$). The impacts of environmental chemistry and ion-ion interactions can be incorporated in the assessment of both bioavailability and toxicity of metal mixtures by combining the BLM with toxicity indexes. Using the method of bootstrapping, the predictive capabilities of these non-nested BLM-based approaches for each combination were compared, and the best fitted model was found to be dependent on the specific composition of the mixtures. This finding may be attributable to diverse physiological properties of individual metals with regard to higher plants, and different underlying mechanisms of metal mixtures in lettuce.

Engineered metal-based nanoparticles (NPs) are a new source of environmental contamination, but the information concerning their release, fate and toxicity is limited. It is therefore difficult to assess the potential effects of metal-based NPs in the environment.

The study presented in Chapter 5 systematically evaluated the combined effects and mutual impacts of Cu NPs and ZnO NPs, using six nested combinations, namely mixtures of $Cu(NO_3)_2$-$Zn(NO_3)_2$, $Cu(NO_3)_2$-Cu NPs, $Zn(NO_3)_2$-ZnO NPs, $Cu(NO_3)_2$-ZnO NPs, $Zn(NO_3)_2$-Cu NPs, and Cu NPs-ZnO NPs. No substantial differences were found in the aggregation or agglomeration of Cu NPs or ZnO NPs and their mixtures with nitrates. More than 80% of the variability in the combined effects of mixtures of $Zn(NO_3)_2$-ZnO NPs and Cu NPs-ZnO NPs was explained by the independent action (IA) model. The variations left in toxicity modeling of Cu NPs and ZnO NPs can be explained by small antagonistic effects found among dissolved metal species as well as non-dissolved particulate fractions of NPs. These results demonstrated that mutual impacts of soluble metals and non-dissolved particles can affect the combined toxicity of Cu NPs and ZnO NPs, which cannot be easily explained by a simple combination of $Cu(NO_3)_2$ and $Zn(NO_3)_2$.

In conclusion, our study emphasizes the importance of two toxicity-modifying factors (the composition of the surrounding exposure media and mixture effects) in the assessment of toxic effects of metals and metal-based NPs on higher plants. Based on the affinity of metals for binding sites on the biotic ligand at the water-organism interface, the mechanistic models we developed provide better links with the toxicity of metal mixtures. We also recommend that finding a statistically significant deviation from additivity can be the starting point for further mechanistic research concerning toxicologically relevant interactions between substances, instead of the endpoint of research used so far. As an extension of the research discussed in the third chapter of this thesis, the commonly known model for the toxicity of mixtures was proven to be suitable for preliminarily assessing the effects of metal-based NPs on terrestrial organisms. The experimental design of nested combinations helps establish a more realistic exposure scenario for the environment and makes it possible to identify where and how chemical-chemical interactions occur with metal-based NPs. Consequently, our findings enrich the rapidly evolving field of toxicology regarding metals and metal-based NPs.

## Samenvatting

Overal in de oceanen en de aardkorst bevinden zich metalen, waarvan er sommige als voedingsstoffen dienen die de organismen op onze planeet in staat stellen hun normale functies te blijven vervullen. Hoge concentraties aan metalen kunnen echter ook toxisch zijn. De fysisch-chemische eigenschappen van water en bodem in het natuurlijke milieu, zoals hardheid, pH en opgelost organisch koolstof, kunnen van invloed zijn op de bioaccumulatie van metalen, en bemoeilijken daarmee de bepaling van de risico's van metalen in aquatische en terrestrische ecosystemen. Wanneer deze factoren worden meegenomen bij de ontwikkeling van mechanistische modellen, kan dit de variabiliteit bij het voorspellen van de biologische beschikbaarheid en de toxiciteit van metalen verminderen.

Het in Hoofdstuk 2 beschreven onderzoek had betrekking op de effecten van $Ca^{2+}$, $Mg^{2+}$, $K^{+}$, $Na^{+}$ en de pH op de acute toxiciteit van Ni en Cd voor zaailingen van sla (*Lactuca sativa* L.). Gevonden werd dat alleen $Mg^{2+}$, en niet $H^{+}$, $K^{+}$, $Na^{+}$ of $Ca^{2+}$, de toxiciteit van Ni voor sla vermindert; en geen van deze veel voorkomende kationen had een significante invloed op het effect van Cd op de wortelgroei van sla. Op basis van het biotische ligand model (BLM) werd de competitie tussen $MG^{2+}$ en $Ni^{2+}$ voor de bindingsplaatsen aan de biotische ligand op het grensvlak tussen water en organisme meegenomen in de voorspelling van de toxiciteit van Ni. Dit leidde tot een aanzienlijke verbetering van de voorspellende waarde ($R^2$=0,80) voor wat betreft het toxische effect van Ni in combinatie met verschillende concentraties $Mg^{2+}$ in de oplossing, in vergelijking met het totaal-metaal model (TMM) ($R^2$=0,49) en het vrije-ion activiteitsmodel (FIAM) ($R^2$=0,60). Voor wat betreft Cd, aangezien de totale variatie in IC50{$Cd^{2+}$} bij de toegepaste concentraties $H^{+}$, $K^{+}$, $Na^{+}$, $Ca^{2+}$ en $Mg^{2+}$ in de oplossing vrij klein was, deden het TMM het even goed als het FIAM als het erom ging de remming van de lengtegroei van de wortels van sla door Cd te verklaren. Aanbevolen wordt daarom om per metaal op basis van de toxicologische data mechanistische modellen te genereren voor het bepalen van de toxiciteit van metalen voor hogere planten.

Een andere eigenschap die van invloed is op de toxiciteit, namelijk mengings-effecten, speelt ook een belangrijke rol bij het bepalen van negatieve effecten van metalen op terrestrische planten. Mensen en andere organismen staan in hun natuurlijke omgeving bloot aan allerlei stoffen, en de toxiciteit van mengsels van metalen voor organismen kan als gevolg van interacties aanzienlijk afwijken van de som van de biologische effecten van elk van die metalen afzonderlijk. Daarom werken onderzoekers steeds aan de verbetering van de nauwkeurigheid van methoden voor het bepalen van de toxiciteit van mengsels van metalen.

Het in Hoofdstuk 3 beschreven onderzoek had betrekking op de gecombineerde toxiciteit van binaire metaalmengsels, te weten Cu-Cd, Ni-Cd en Cu-Ni, bestudeerd met op concentratie-additie (CA) gebaseerde modellen en op onafhankelijke werking (independent action, IA) gebaseerde modellen. De remming van de lengtegroei van de wortels van sla door mengsels van Cu-Cd, Ni-CD en Cu-Ni werd gekwantificeerd met behulp van statistische software, het MixTox model. De toxiciteit van deze binaire metaalmengsels bleek even goed te worden voorspeld door de CA-modellen als door de IA-modellen. Bij het modelleren van de toxiciteit van deze drie metaalmengsels werden vaak statistisch significante afwijkingen van additiviteit gevonden, en de patronen in deze afwijkingen verschilden voor de specifieke combinaties en verschillende soorten modellen. Om te onderzoeken of deze statistisch significante afwijkingen reproduceerbaar waren, werden andere datasets, verkregen uit onafhankelijke experimenten met Ni-Cd en Cu-Ni mengsels, als invoer gebruikt in dezelfde modellen voor mengsels. De patronen in de afwijkingen bleken echter inconsistent of spraken elkaar zelfs tegen bij verschillende metaalconcentraties en de verschillende soorten modellen. Aanbevolen wordt daarom om voorzichtig te zijn bij de interpretatie van statistisch significante afwijkingen van een standaardmodel, aangezien deze niet per se wijzen op een biologisch relevante interactie. Het zoeken naar statistisch significante afwijkingen kan een uitgangspunt vormen voor verdere metingen en modellen ter bevordering van het inzicht in niet-additieve interacties die optreden binnen organismen.

Het in Hoofdstuk 4 besproken onderzoek omvatte de uitbreiding van het biotische ligand model (BLM) met het voorspellen van de totale toxiciteit voor sla van Cu-Ni, Cu-ZN en Cu-Ag mengsels. Hiertoe werden drie benaderingen toegepast, gebaseerd op het concept van additiviteit, namelijk de toxische eenheid (toxic unit, TU) benadering, de toxische equivalentiefactor (TEF) benadering en de benadering waarbij wordt gekeken naar de fractie van het totale aantal biotische ligandplaatsen dat bezet is door metaalionen uit mengsels ($f_{\text{mix}}$). De invloeden van het chemische milieu en de ion-ion interacties kunnen worden meegenomen in de bepaling van zowel de biologische beschikbaarheid als de toxiciteit van metaalmengsels, door het BLM te combineren met de toxiciteits-index. Met behulp van bootstrapping werd de voorspellende waarde van deze niet-geneste op BLM gebaseerde benaderingen vergeleken voor elke combinatie, waarna het model met de beste fit afhankelijk bleek van de specifieke samenstelling van het mengsel. Dit kan wellicht worden toegeschreven aan de uiteenlopende fysiologische eigenschappen van de verschillende metalen in hogere planten en verschillende onderliggende mechanismen van metaalmengsels in sla.

Kunstmatige metallische nanodeeltjes vormen een nieuwe bron van milieuverontreiniging, waarvoor nog weinig informatie beschikbaar is aangaande hun lozing, verdere gedrag en toxiciteit. Dit maakt het moeilijk om de potentiële effecten van metallische nanodeeltjes in het milieu te bepalen.

In het in Hoofdstuk 5 beschreven onderzoek werden de gecombineerde effecten en de wederzijdse beïnvloeding van Cu-nanodeeltjes en ZnO-nanodeeltjes systematisch bestudeerd met behulp van zes geneste combinaties, namelijk mengsels van $Cu(NO_3)_2$ met $Zn(NO_3)_2$, $Cu(NO_3)_2$ met Cu-nanodeeltjes, $Zn(NO_3)_2$ met ZnO-nanodeeltjes, $Cu(NO_3)_2$ met ZnO-nanodeeltjes, $Zn(NO_3)_2$ met Cu-nanodeeltjes en Cu-nanodeeltjes met ZnO-nanodeeltjes. Er werden geen significante verschillen gevonden in de mate van aggregatie or agglomeratie van de Cu- of ZnO-nanodeeltjes en de mengsels daarvan met nitraten. Meer dan 80% van de variabiliteit van de gecombineerde effecten van mengsels van $Zn(NO_3)_2$ met ZnO nanodeeltjes en Cu- en ZnO-nanodeeltjes kon worden verklaard met het IA-model. De resterende variaties in de toxiciteitsmodellen van Cu-nanodeeltjes en ZnO-nanodeeltjes kunnen worden verklaard door kleine antagonistische effecten die werden gevonden bij opgeloste metalen en niet-opgeloste deeltjesfracties van nanodeeltjes. Deze resultaten laten zien dat de wederzijdse beïnvloeding tussen opgeloste metalen en niet-opgeloste deeltjes van invloed kan zijn op de gecombineerde toxiciteit van Cu- en ZnO-nanodeeltjes, hetgeen niet gemakkelijk kan worden verklaard uit een simpele combinatie van $Cu(NO_3)_2$ en $Zn(NO_3)_2$.

Geconcludeerd kan worden dat dit onderzoek het belang heeft onderstreept van twee factoren die van invloed zijn op de toxiciteit van metalen en metallische nanodeeltjes op hogere planten; te weten de samenstelling van het omringende milieu waarin de blootstelling plaatsvindt en de effecten van mengsels. Op basis van de affiniteit van metalen voor de bindingsplaatsen op de biotische ligand kunnen de ontwikkelde mechanistische modellen zorgen voor betere links met de toxiciteit van metaalmengsels. Aanbevolen wordt om wanneer statistisch significante afwijkingen van additiviteit worden gevonden, dit als uitgangspunt voor verder mechanistisch onderzoek naar toxicologisch relevante interacties tussen stoffen te nemen. Als vervolg op het in de derde hoofdstuk van dit proefschrift beschreven onderzoek, is aangetoond dat het algemene model voor mengseltoxiciteit van stoffen geschikt is om een eerste schatting te geven van de effecten van metallische nanodeeltjes op terrestrische organismen. De op geneste combinaties gebaseerde onderzoeksopzet helpt bij het opstellen van een meer realistisch scenario voor blootstelling in het milieu en maakt het ook mogelijk te bepalen waar en hoe onderlinge chemische interacties optreden bij metallische nanodeeltjes. Onze bevindingen betekenen daarmee een verrijking van het zich snel ontwikkelende onderzoeksterrein van de toxicologie van metalen en metallische nanodeeltjes.

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## Curriculum Vitae

Yang Liu was born in Kunming, Yunnan province, China on 5th September 1987. In 1999, the 12-year-old girl studied a junior basic education in No. 3 middle school of Kunming and she studied an advanced basic education in No. 8 middle school of Kunming from September 2002. Afterwards, Yang graduated with high marks and went to College of Water Resources & Civil Engineering, China Agricultural University (CAU, Beijing) in 2005 to study Agricultural Structure Environment & Energy Engineering. Four years later, she obtained a Bachelor Degree of Engineering with an outstanding academic performance and was thus recommended by the college as a postgraduate candidate exempt from admission exams to study Agricultural Biological Environment & Energy Engineering at CAU in 2009. Yang spent two years to finish her Master's project entitled 'Evaluation of methods and indexes for heat preservation of walls in solar greenhouses'. In addition to research and study, she also actively participated in various activities and has been awarded as the merit student several times during her stay at CAU. From 2011 to 2015, she was recommended by CAU and awarded a government scholarship by China Scholarship Council (CSC) to follow the Ph.D. program which was performed at the department of Conservation Biology (CB), Institute of Environmental Sciences (CML), Leiden University, the Netherlands. After obtaining her Ph.D., Yang will continue her scientific research career.

# Publication List

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