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.

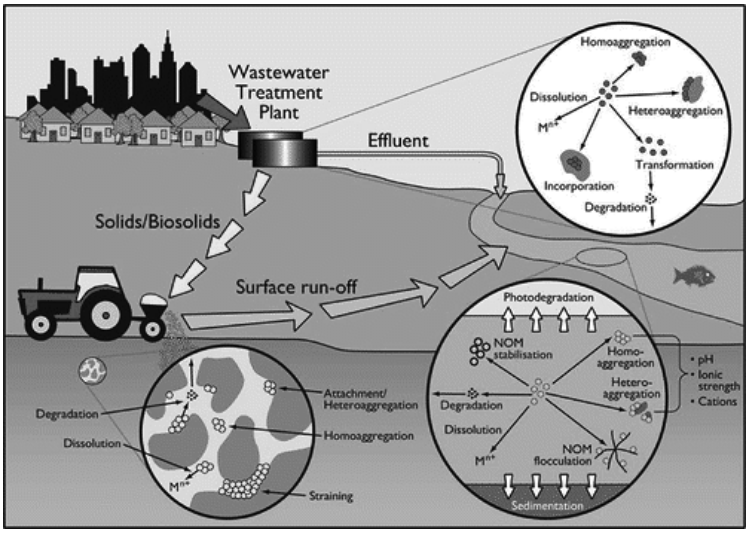

Figure 1.1 Pathways and transformations of nanomaterials in the environment. (Cited from Batley et al., 2012)

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:

$$\{M^{z+}\} + [L] \leftrightarrow [ML]\,; K_1 \times \{M^{z+}\} = \frac{[ML]}{[L]} \tag{1-1}$$

$$\{M^{z+}\} + \{-X_{cell}\} \leftrightarrow \{M-X_{cell}\}\,; \{M-X_{cell}\} = K_2 \times \{M^{z+}\} \times \{-X_{cell}\} \tag{1-2}$$

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

$$\{M-X_{cell}\} = K_3 \times \frac{\{-X_{cell}\} \times [ML]}{[L]} = K_1 \times K_3 \times \{-X_{cell}\} \times \{M^{z+}\} \tag{1-3}$$

where $\{M^{z+}\}$ 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; $\{-X_{cell}\}$ is the concentration of free surface sites on the cell membrane; $\{M\text{-}X_{cell}\}$ 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 $\{M\text{-}X_{cell}\}$ follow the change of $\{M^{z+}\}$ 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; β 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.

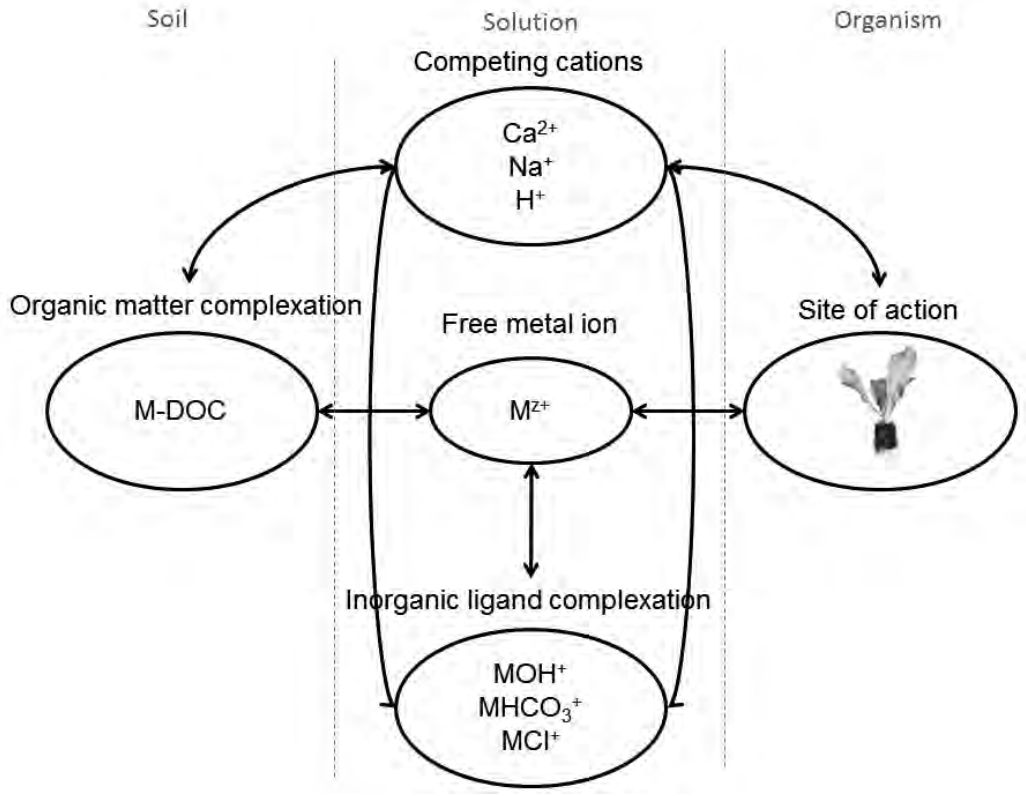

Figure 1.2 Diagram of the biotic ligand model framework in terrestrial ecosystems. (Adapted from Thakali et al., 2006)

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 (ψ_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)

$$EC_{x\text{mix}} = (\sum_{i=1}^{n} \frac{p_i}{EC_{xi}})^{-1} \quad (1\text{-}7)$$

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

The IA model can be defined as

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

where c_i is the concentration of the i_{th} component in the mixture; $E(c_{\text{mix}})$ 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_{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 (DA or CA)	Independent joint action (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)

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

where c_i is the concentration of the i_{th} component in the mixture; EC_{xi} is the standard effect concentration of the i_{th} component in the mixture, it can be the 50% effective concentration of the organisms (EC_{50}) or even the lowest observed effect concentration (LOEC). If the 50% toxic effects are observed based on 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 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.

$$MTI = 1 - \frac{\log M}{\log M_0} \quad (1\text{-}10)$$

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.

$$TEQ = \sum_{i=1}^{n} c_i \times TEF_i \quad (1\text{-}11)$$

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.

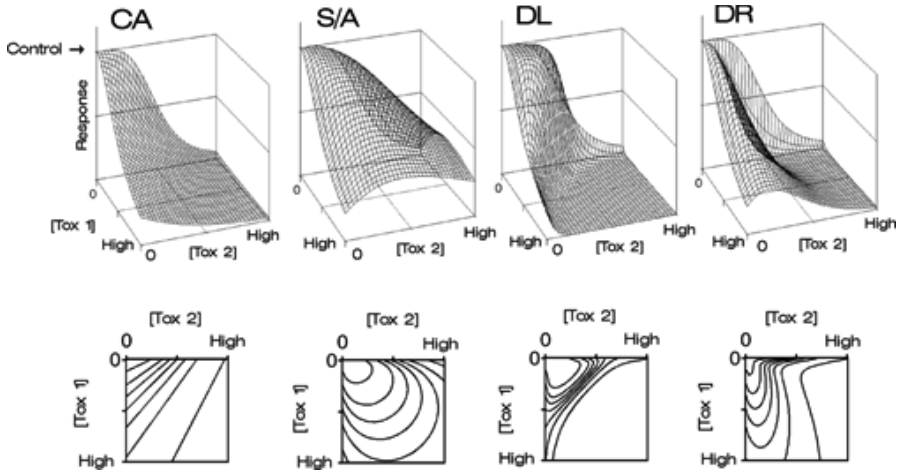

Figure 1.3 Three dimensional dose-response relationships (Top) of binary mixtures and isobologram (Bottom) illustrating interaction patterns from the additivity (CA): antagonistic deviation (S/A), dose level-dependent deviation (DL), and dose-ratio-dependent deviation (DR). (Cited from Jonker et al.,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

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

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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