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

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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_{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_{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_{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_{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_{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_{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_{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.

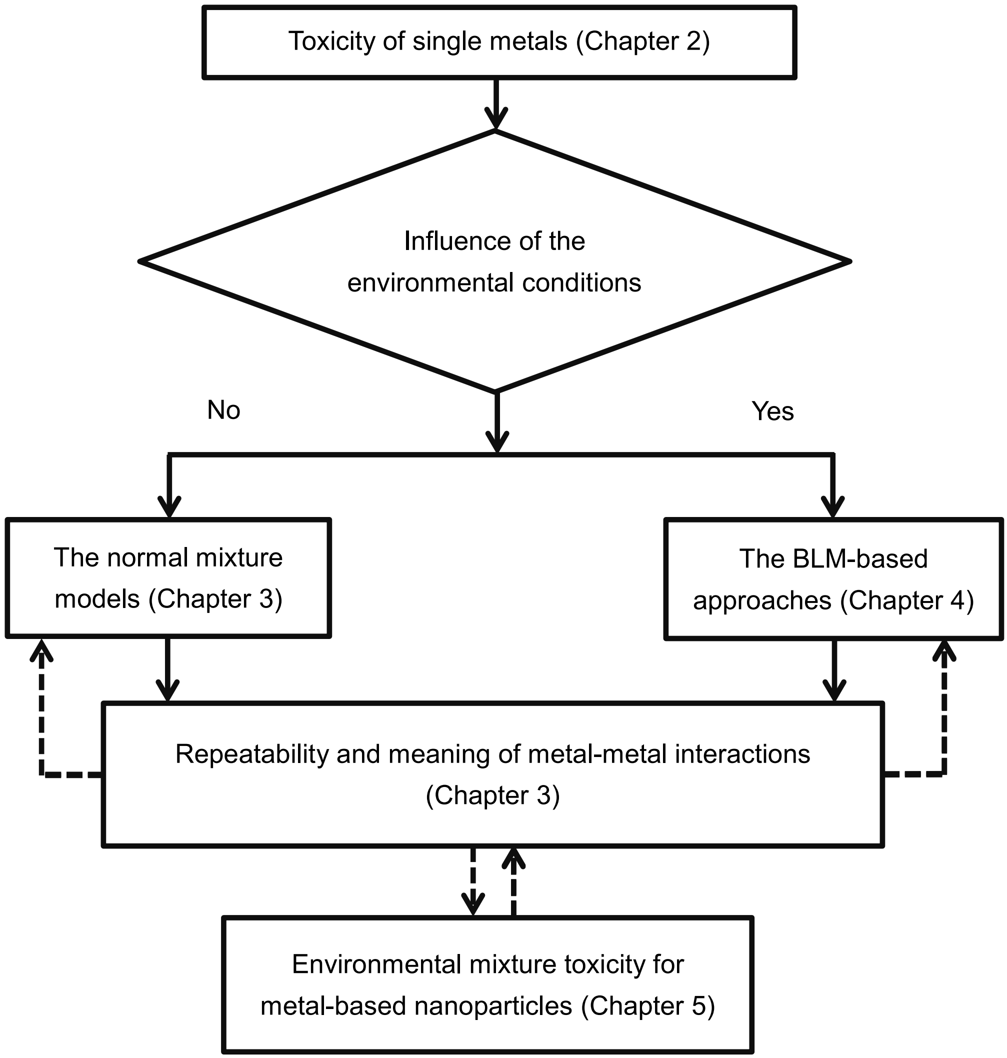

Figure 6.1 Scheme of approaches for assessing toxicity of metal-based mixtures applied in this PhD thesis.

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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