Modelling bioaccumulation and toxicity of metal mixtures

T.T. Yen Le

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T.T. Yen Le
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Promotoren:
Prof. dr. ir. A.J. Hendriks
Prof. dr. ir. W.J.G.M. Peijnenburg (UL)

Copromotoren:
Dr. R.S.E.W. Leuven
Dr. M.G. Vijver (UL)

Manuscriptcommissie:
Prof. dr. M.A.J. Huijbregts
Prof. dr. J.G.M. Roelofs
Dr. C.A.M. van Gestel (VU Amsterdam)

Paranimfen:
Codrut Constanin Paun
Aniek Thissen

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by

T.T. Yen Le
Born on December 10, 1985
in Hung Yen (Vietnam)

Supervisors:
Prof. dr. ir. A.J. Hendriks
Prof. dr. ir. W.J.G.M. Peijnenburg (LU)

Co-supervisors:
Dr. R.S.E.W. Leuven
Dr. M.G. Vijver (LU)

Doctoral Thesis Committee:
Prof. dr. M.A.J. Huijbregts
Prof. dr. J.G.M. Roelofs
Dr. C.A.M. van Gestel (VU Amsterdam)

Paranymphs:
Codrut Constanin Paun
Aniek Thissen

Contents

Chapter 1. General introduction 7

1.1. Introduction 8

1.2. Principles of metal exchange 9

1.3. Metal toxicity 13

1.4. Affinity for biological ligands and metal toxicity 16

1.5. Objectives of the thesis 25

1.6. Outline of the thesis 26

References 29

Chapter 2. Modeling metal bioaccumulation in the invasive mussels *Dreissena polymorpha* and *Dreissena rostriformis bugensis* in the rivers Rhine and Meuse 47

2.1. Introduction 48

2.2. Methods 49

2.3. Results 52

2.4. Discussion 54

References 58

Supplementary information 61

Chapter 3. Predicting effects of cations on copper toxicity to lettuce (*Lactuca sativa*) by the Biotic Ligand Model 71

3.1. Introduction 72

3.2. Methods 73

3.3. Results 75

3.4. Discussion 77

References 79

Supplementary information 82

Chapter 4. Interactions in Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures affect toxicity to lettuce *Lactuca sativa* 85

4.1. Introduction 86

4.2. Methods 87

4.3. Results 91

4.4. Discussion 95

References 99

Supplementary information 104

Chapter 5. Modeling toxicity of binary metal mixtures (Cu^{2+}–Ag^{+}, Cu^{2+}–Zn^{2+}) to lettuce, *Lactuca sativa*, with the Biotic Ligand Model 107

5.1. Introduction 108

5.2. Methods 109
5.3. Results 112
5.4. Discussion 116
References 118
Supplementary information 120
Chapter 6. Integrating ion-ion interactions in modelling rhizotoxicity of metal mixtures to lettuce *Lactuca sativa* based on the electrostatic approach 121
6.1. Introduction 122
6.2. Methods 123
6.3. Results 128
6.4. Discussion 135
References 138
Supplementary information 141
Chapter 7. Synthesis 149
7.1. Introduction 150
7.2. Affinity of metals for biological ligands in modelling toxicity of single metals 152
7.3. Contribution of the integration of affinity of metals for biological ligands to modelling toxicity of metal mixtures 155
7.4. Comparison of the Biotic Ligand Model and the Electrostatic Toxicity Model in modelling metal toxicity 157
7.5. Conclusions 158
7.6. Recommendations 160
References 164
Summary 167
Samenvatting 171
Acknowledgements 175
Curriculum vitae 177
List of publications 179

Chapter 1
General introduction

1

1.1. Introduction

Environmental pollution with metals is a serious problem in many areas in the world. Metals can be accumulated at high concentrations in organisms, resulting in effects on organisms and on humans directly and indirectly. Therefore, assessment of metal bioaccumulation and toxicity is of high concern.

This PhD thesis aimed to model metal bioaccumulation and toxicity by different methods (Fig. 1.1). Metal uptake, subcellular distribution, and toxicity depend on behaviour and characteristics of each individual metal (*Section 1.2* of this thesis). Because of this high specificity, mechanistic models simulating metal bioaccumulation, bioavailability, and toxicity are scare and poorly validated. Interactions between metal ions and biological surfaces (*Section 1.3.1* of this thesis) and between different metal ions (*Section 1.3.2* of this thesis) complicate metal uptake, subcellular distribution, and toxicity further. In this thesis, affinity of metals for biological ligands has been used as a unifying factor in generalisation of metal bioaccumulation, bioavailability, and toxicity, allowing integration of metal ion-organism interactions in predictions (*Section 1.4.1* of this thesis). Moreover, the incorporation of metal-specific affinity for biological ligands into modelling potentially facilitates delineating ion-ion interactions and taking into account the interactive effects in predicting bioaccumulation and toxicity of metal mixtures (*Section 1.4.1* of this thesis). In this PhD thesis, the affinity of metals for biological ligands was integrated in three different modelling approaches: the mechanistic bioaccumulation modelling (*Section 1.4.2* of this thesis), the Biotic Ligand Modelling (*Section 1.4.3* of this thesis), and the electrostatic toxicity modelling (*Section 1.4.4* of this thesis). By applying these approaches, this thesis aimed to model bioaccumulation and toxicity of metal mixtures, taking into account ion-ion and ion-organism interactions (*Sections 1.5 and 1.6* of this thesis).

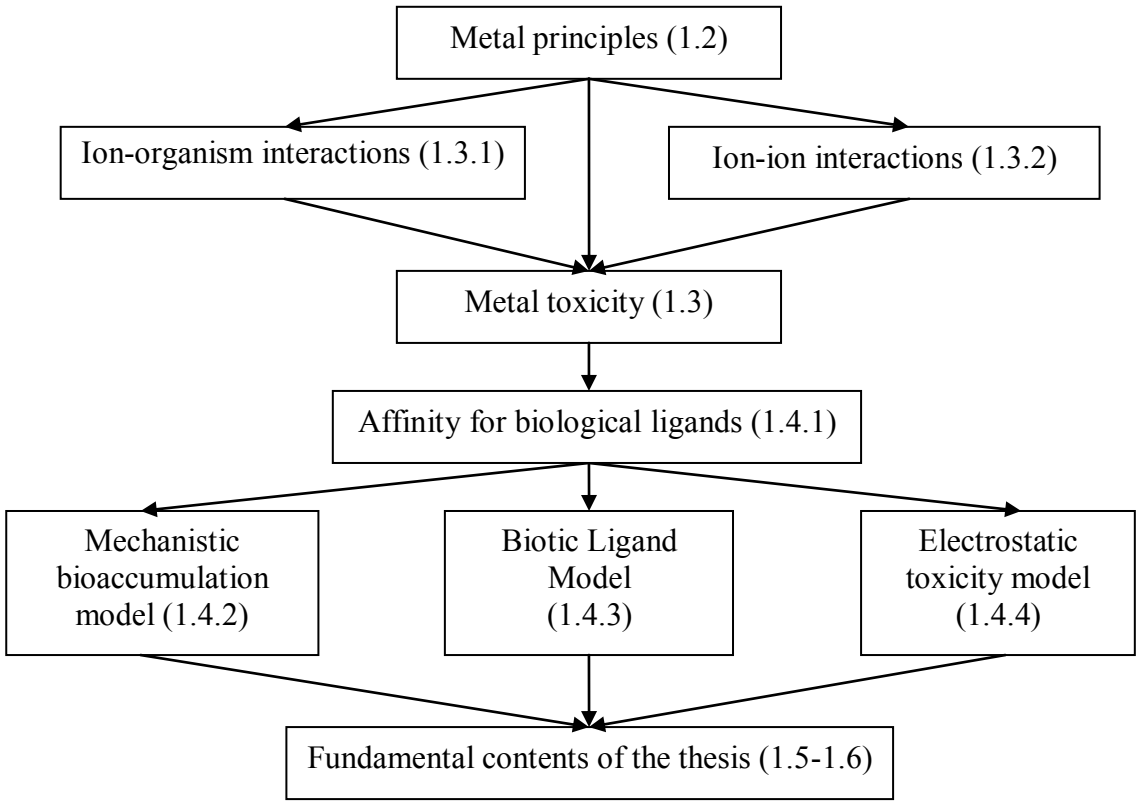

Figure 1.1. The flow chart describes the issues presented in the *Introduction* Chapter of this PhD thesis

1.2. Principles of metal exchange

1.2.1. General principles

Metals have a number of unique properties that are largely different from characteristics of organic substances (Fairbrother et al., 2007). For example, metal bioavailability and toxicity are influenced by the speciation in the environment, which is in turn heavily affected by the environmental chemistry. Metals are naturally occurring in the environment with varying background concentrations in different geographic regions. Additionally, some metals are nutritionally essential for the growth of organisms. Therefore, organisms have developed various regulatory mechanisms for uptake, metabolism, distribution, storage, and elimination of metals in response to metal exposure in the one hand and to maintain metal homeostasis on the other hand. Furthermore, metals occur in the environment in mixtures. On the one hand, these principles influence the toxicokinetics and toxicodynamics of metals and contribute to complications in metal behaviour (i.e., transport, trafficking, subcellular distribution, and binding to potentially sensitive sites). On the other hand, they complicate the use and interpretation of bioaccumulation data as well as the toxicity of bioaccumulated metals (Fairbrother et al., 2007). Therefore, these metal-specific properties should be addressed and taken into account in assessment of metal bioaccumulation and toxicity. Ignorance of these characteristics may lead to inaccurate estimations of metal bioaccumulation, bioavailability, and toxicity.

1.2.2. Bioaccumulation

Metal bioaccumulation is of high concern because of its relations to direct effects on organisms accumulating the metal and indirect effects on their predators through trophic transfer (Fairbrother et al., 2007). The bioaccumulation of metals is complicated as it results from a variety of uptake and depuration mechanisms (OECD, 2001; see below). Metal accumulation is related to various mechanisms, e.g., natural background uptake, homeostasis and internal detoxification, storage, and elimination (McGeer et al., 2002, 2003). Metal accumulation at background concentrations is a natural phenomenon and may be non-toxic. Detoxification and elimination of accumulated metals represent the ability of organisms to acclimate to metal exposure (McGeer et al., 2003). Physiological processes that influence metal bioaccumulation are presented in *Sections 1.2.3–1.2.5*.

The tendency of substances to build up in organisms is often expressed by the bioaccumulation factor (BAF) and the bioconcentration factor (BCF), representing the ratio of the residue in the organism versus the concentration in water at equilibrium, including and excluding uptake from food, respectively (Mackay, 1982; Bysshe, 1982; Barron et al., 1990; Newman, 1995). For metals, BCF and BAF depend on exposure conditions. Specifically, these accumulation factors vary widely and are inversely related to the external water concentration (Chapman et al., 1996; McGeer et al., 2002, 2003). The wide variability in BCF and BAF for metals and the inverse correlation with environmental concentrations are attributed to highly complex and specific mechanisms of metal uptake and accumulation, e.g., the saturable uptake and the ability of organisms to store and regulate accumulated metals within certain ranges (McGeer et al., 2003; Borgmann et al., 2004; Chapman et al., 1996). Therefore, BCF and BAF are not intrinsic properties of metals.

These characteristics of BCF and BAF for metals invalidate the use of single and generic values of BCF and BAF in assessing hazard potentials of metals (Chapman et al., 1996; McGeer et al., 2003; Fairbrother et al., 2007; US EPA, 2006). Firstly, BCF and BAF for metals do not meet the most important criteria for the applicability of accumulation factors in hazard identification, i.e., the independence on exposure conditions, and are therefore not

relevant indicators of potential hazards of metals (OECD, 2001). Secondly, the potential for extrapolation of single values of BCF and BAF for metals across different conditions is limited (Fairbrother et al., 2007). There are substantial uncertainties in metal assessment based on these data resulting from the differences in metal bioavailability among various conditions and from complex metal toxicokinetics. Another reason that accounts for the inappropriateness of the application of single and generic values of BCF and BAF in lab-field extrapolation is the potential importance of ingestion in total metal uptake (Davies and Dobbs, 1984). Thirdly, BCF and BAF are not necessarily reflective of ambient contamination or indicative of metal bioaccumulation level or potential toxicity. Particularly, these values usually do not reflect the steady state and are therefore not truly accurate indicators of bioaccumulation levels (Chapman et al., 1996). Moreover, the accumulation from natural background and of essential elements is not distinguished from the toxicologically potential accumulation in these indicators. Regulations based on these generic accumulation values may lead to misleading results about metal toxicity (Chapman et al., 1996; McGeer et al., 2002, 2003). For example, decreasing BCF and BAF values at elevated exposure levels reveal a reduction in impacts, contrasting to toxicological data. Due to these limitations of the use of single and generic values of BCF and BAF, these accumulation factors should be expressed in relation to media chemistry and the exposure concentration for particular species (Allen and Hansen, 1996; Fairbrother et al., 2007).

1.2.3. Uptake and elimination

A number of specific transport mechanisms are involved in metal uptake. Metal uptake can occur via passive diffusion, facilitated transport, active transport, or endocytosis (Simkiss and Taylor, 1995). Only lipid soluble metal species, such as neutral, inorganically complexed metals and alkyl-metal compounds, can be taken up by organisms via passive diffusion through the membrane. Most ions are highly hydrophilic and mainly taken up by organisms via membrane transport proteins, i.e., channels, carriers, and pumps. As a result of these specific transport mechanisms, the bioaccumulation of metals may involve saturable uptake kinetics. In a number of cases, it was reported that uptake via saturable kinetics-exhibiting mechanisms is substantially more common and toxicologically relevant than passive diffusion (Newman, 1995; McKim, 1994; Simkiss and Taylor, 1989; McDonald and Wood, 1993; Kiss and Osipenko, 1994; Wood, 2001). Similar to uptake processes, elimination is controlled by physiological processes (McGeer et al., 2002, 2003). Cellular pathways for the elimination of metals involve passive and active processes by the similar mechanisms as for metal uptake (Roesijadi and Robinson, 1994; Rainbow, 2007; Sokolova and Lannig, 2008). However, the relative importance of these mechanisms in metal elimination may be different from that in the metal uptake (Sokolova and Lannig, 2008). Although a thorough understanding of mechanisms of metal elimination is lacking, different affinities of metals for biological materials may account for the variations in their elimination (Veltman et al., 2008).

In plants, metal uptake may result from ion-exchange and other surface interactions with metal ions through binding at the polymers (Flemming, 1995; Kapoor and Viraraghavan, 1997; Tsezos et al., 1997; Diels et al., 1995; Tsezos and Remoundaki, 1997; Kratochvil and Volesky, 1998). Particularly, the cell wall consists of biopolymers, e.g., proteins, polysaccharides, and nucleic acids (Beveridge and Murray, 1976, 1980; Flemming, 1995; Kapoor and Viraraghavan, 1997; Tsezos et al., 1997; Tsezos, 1983). As functional groups, e.g., carboxyl, sulphate, and amino, are included in the chemical structure of these biopolymers, negative charges exist on the plant cell wall, leading to electronic interactions at the membrane surface (Kratochvil and Volesky, 1998; Tobin et al., 1984). Generally, the adsorption of metal ions by the cell can occur via a number of processes: complexation, chelation, coordination, ion exchange, precipitation, and reduction (Tsezos et al., 2006).

Among them, the binding of metal ions to the biopolymers may occur via two major mechanisms: ion-exchange and complex formation. In the first mechanism, the sorption of metal ions occurs as a result of the replacement of protons, alkali, alkaline earth, or other cations by the toxic metal ions (Schneider et al., 2001; Tsezos et al., 2006). In the second mechanism, complexes are formed between organic molecules and metal ions (Tsezos et al., 2006). Although ion-exchange is extensively reported as the main pathway, the relative importance of these mechanisms is metal-specific. For instance, ion-exchange at the cell wall is the major pathway for the accumulation of Cd and Cu by hairy roots while its role in Ni uptake is uncertain (Nedelkoska and Doran, 2000).

Metals can be accumulated in or eliminated from organisms via both the dissolved and particulate phases (Wang and Fisher, 1999; Xu and Wang, 2002). It is important to take into account and distinguish between these pathways in estimating metal bioaccumulation (Wang and Fisher, 1999). Firstly, separation of these two exposure routes provides a better understanding of factors controlling metal bioavailability and more reliable indicators for setting water and sediment quality criteria. Secondly, a distinction between the two pathways provides insights into the metal trophic transfer and biogeochemical cycling. Additionally, the exposure type determines internal distribution and eventually toxicity. Contribution of the dietary source to the total metal uptake depends on the bioaccumulation in the prey, the type of prey organism, and the ingestion rate of the predator. For example, diatoms are digested by bivalves at higher assimilation efficiency than sediments. In addition, the difference in the assimilation efficiency for these two food items highly varies among predating bivalve species (Wang and Kong, 2003). It is evident that dietary exposure can substantially contribute to the total metal uptake (Griscom et al., 2002; Rainbow and Wang, 2001; Wang et al., 1996; Chong and Wang, 2001; Ke and Wang, 2001; Wang and Ke, 2002; Xu and Wang, 2002). For trace elements in anionic forms, e.g., As and Se, dietary exposure dominates the total uptake while for metals that are mainly taken up via transport proteins, their accumulation predominantly results from the dissolved phase (Wang and Fisher, 1999). Different processes affecting metal bioavailability and toxicity are disentangled in *Sections 1.2.4 and 1.2.5*.

1.2.4. Regulation

Organisms have developed physiological and/or anatomical mechanisms to adapt to metal exposure (Fairbrother et al., 2007). They are able to actively regulate metal bioaccumulation and maintain homeostasis over a range of exposure via exclusion or increased elimination (Hamilton and Mehrle, 1986; Chapman et al., 1996; Wood, 2001). Additionally, organisms have ability to regulate internal metal concentrations through sequestration, detoxification, and storage (George et al., 1980; Mason and Nott, 1981; Rainbow et al., 1980; Simkiss, 1981; White and Rainbow, 1982; Rainbow, 1988; Viarengo, 1989; Depledge and Rainbow, 1990; Mason and Jenkins, 1995; McDonald and Wood, 1993). The metals accumulated can be stored in detoxified forms, such as in inorganic granules or in complexes with metallothionein-like proteins, and may therefore become nonbioavailable for organisms and their predators (Mason and Jenkins, 1995; Noel-Lambot et al., 1980; Roesijadi, 1980; Langston and Zhou, 1986; Hylland et al., 1994; Nott and Nicolaidou, 1990, 1993, 1994). Because of the natural occurrence of metals in the environment, these physiological processes have evolved over time, allowing organisms to adapt to excess metals and to accumulate essential metals at required levels.

Similar to aquatic organisms, plants have developed multiple mechanisms to increase their tolerance to metal exposure. Main detoxification mechanisms in plants include the distribution of metals to apoplast tissues like trichone and cell walls, chelation of the metals by different ligands, and the sequestration of the metal-ligand complex into the vacuole (Memon and

Schroder, 2009; Yang et al., 2005). In plants, cell walls are of importance in detoxifying some metals, e.g., Ni, Zn, Cd, and Mn (Kramer et al., 2000; Li et al., 2006; Memon et al., 2001; Memon and Yatazawa, 1984). As a result of this allocation, metal storage in metabolically active compartments, e.g., cytosol, mitochondria, and chloroplast, is limited. Metals may form complexes with extracellular organic acids released from roots or intracellular low-molecular weight proteins, e.g., metallothioneins (MTs) and phytochelatins (PCs) (Yang et al., 2005; Memon and Schroder, 2009; Clemens et al., 1999; Cobbett, 2000; Cobbett and Goldsbrough, 2002; Sarry et al., 2006; Zenk, 1996; Robinson et al., 1993, 1997; Rauser, 1999). In addition to detoxification, PCs and MTs are involved in the homeostasis, regulating metal availability in plant cells (Thomine et al., 2000). Besides MTs and PCs, heat shock proteins are produced in plants under stress of metal contamination (Wollgiehn and Neumann, 1995; Neumann et al., 1994; Memon et al., 2001). They were suggested to have protective effects on the membrane by facilitating the formation of complexes between metals and proteins. Yet, the importance of these heat shock proteins is not fully understood. Compartmentalisation of intracellular metals into the vacuole acts as another sequestration mechanism in plants (Tong et al., 2004; Kramer et al., 2000). The vacuole is considered the main storage site for metals in plant cells and the ultimate storage site for metal ions entering the cytosol (Salt et al., 1995; Memon et al., 2001). Metal complexation may occur in the vacuole with the involvement of metal-binding molecules, e.g., anthocyanins and organic acids (Kramer et al., 2000; Pilon-Smits and Pilon, 2002).

1.2.5. Subcellular distribution

Subcellular partitioning of metals is of importance in assessing adverse effects as it provides a mechanistic understanding of metal toxicity and tolerance (Wallace et al., 2003; Wang and Rainbow, 2006). Metals may form complexes with proteins or other carrier molecules for distribution to particular organs or for sequestration or excretion (Fairbrother et al., 2007). Metal distribution in organisms is dependent on the primary uptake route (Selck and Forbes, 2004; Fisher et al., 1996; Long and Wang, 2005; George et al., 1986; Wang et al., 1996; Wang and Fisher, 1998; Reinfelder and Fisher, 1994). This specificity of metals in internal distribution complicates the issue further. The accumulation of metals within a certain tissue may partly result from the presence of specific binding sites and may be influenced by detoxification mechanisms within that tissue. Cadmium, for example, may preferentially bind to the sites that are associated with calcium concretions in gills of freshwater mussels (Pynnonen et al., 1987). Consequently, cadmium concentrations are lowest in the shell and muscle and highest in the digestive glands, gills, and kidney (Adams et al., 1981; Hemelraad et al., 1986; Herwig et al., 1989).

Similarly, cellular and subcellular distribution of metals should be considered in assessing metal bioavailability and toxicity to plants. The allocation pattern of metals into different plant organs is metal- and species- specific and provides a better understanding of environmental consequences and reflects the sensitivity of organisms (Castro et al., 2009). For example, metal distribution into the belowground or aboveground tissues is related to the availability of metals in the environment and the tolerance of organisms (Doyle and Otte, 1997; Weis and Weis, 2004; Weis et al., 2002; Windham et al., 2003; Ramos et al., 2002; Ouariti et al., 1997). The importance of metal subcellular partitioning is additionally demonstrated by statistically significant relationships found between the distribution pattern and the response (Li et al., 2011). In general, in hyperaccumulating plants, metals are mainly found in cell walls and vacuoles (Wierzbicka, 1998; Hu et al., 2009; Frey et al., 2000; Kupper et al., 2001; Robinson et al., 2003). For example, in salt marsh plants, cell walls and membranes are the main storage sites for metals, limiting the accumulation in the cytoplasm (Ramos et al., 2002; Zornoza et al., 2002; Castro et al., 2009). By contrast, in sensitive

species, a large proportion of the metals accumulated is distributed in the organelle-containing fractions (Wu et al., 2005). The subcellular partitioning of metals is, in turn, determined by detoxification and sequestration mechanisms mentioned above or acts as a detoxification mechanism. For instance, as a result of sequestration by organisms, a high fraction of metals accumulated is allocated in the vacuoles in leaves, cell walls, or epidermal layers (Vogeli-Lange and Wagner, 1990; Chardonnens et al., 1998, 1999; Frey et al., 2000; Tian et al., 2009, 2010).

1.3. Metal toxicity

1.3.1. Ion-organism interactions

Bioavailability and toxicity of metals are strongly affected by the chemical speciation in the environment (Fairbrother et al., 2007; Pagenkopf et al., 1974; Sunda and Guillard, 1976; Pagenkopf, 1986; Allen et al., 1980; Luoma, 1983). Particularly, environmental conditions as well as biological or chemical processes influence the forms of metals (i.e., species, compound, matrix, and particle size), which subsequently determine metal bioaccessibility, bioavailability, fate, and effects (Fairbrother et al., 2007). The importance of the geochemical speciation of metals has been recognised and taken into account in metal assessment by linking metal bioavailability to the free metal ion (Parker et al., 1995; Wilkinson and Buffle, 2004; Parker and Pedler, 1997). Based on this relationship, the free ion activity model (FIAM) was developed with the assumption that free ions are the main reactive species of metals, determining metal bioavailability and toxicity (Morel, 1983; Pagenkopf, 1983; Sunda and Huntsman, 1983). Additionally, a number of chemical speciation models, e.g., Windermere Humic Aqueous Model (WHAM), have been developed to integrate effects of the environmental chemistry in assessment of metal bioavailability (Tipping, 1998).

However, toxicokinetics and toxicodynamics of metals are highly dynamic and complicated, depending on the metals, speciation of the metals, exposure routes and conditions, and regulation and storage of the metals by organisms (Fairbrother et al., 2007). The organ or tissue affected may be different from the organ or tissue in which metals are accumulated. The target organ or tissue is species-specific and dependent on the kinetics of metals, i.e., the differences in absorption, distribution, and excretion. Moreover, trafficking processes of metals such as binding to ligands and competition for receptor sites have effects on both the uptake level and the internal fate of accumulated metals (McGeer et al., 2003). These processes influence metal availability at different levels, i.e., in the aquatic medium, at the biological membrane in the vascular or intercellular transfer, and the intracellular matrix (Chapman, 1996; Newman and Jagoe, 1994; Langston and Bryan, 1984; Pagenkopf, 1983; Hamilton and Mehrle, 1986; Hering and Morel, 1990; Bergman and Dorward-King, 1997; Campbell, 1995; Mason and Jenkins, 1995; Stumm and Morgan, 1994; Chapman et al., 1996; Playle, 1998). Binding of metals to sites on the membrane determines the pertinent mass balance in the environment on the one hand and metal internal speciation, and subsequent metal bioavailability, on the other hand (Parker and Pedler, 1997). Furthermore, after metals are taken up into the cell, the binding of the metals to efflux transporters as well as the complexation of the metals with intracellular and extracellular ligands induced by the organism in response to metal bioaccumulation influence the reactivity of the metals accumulated (Worms et al., 2006). A number of families of proteins have been found to be involved in these transport processes of metals in both plants and aquatic organisms. In general, they function as cation efflux transporters, carrying cation efflux out of the cytoplasmic compartment, across the plasma membrane to the cell exterior, or across endomembranes into the intracellular compartments, e.g., the vacuole (Maser et al., 2001). For

example, the heavy metal (CPx-type) ATPases, e.g., the H^+-ATPases, the Na^+/K^+-ATPases, and the Ca^{2+}-ATPases, involve in the transport of cations across the cellular membrane in both plants and fish (Yang et al., 2005; Hall and Williams, 2003; Bury et al., 1999a; Havelaar et al., 1998; Axelsen and Palmgren, 2001). Therefore, metal ions, e.g., Cu^{2+} and Ag^+, will inhibit the uptake of Na^+ or Ca^{2+} for these specific binding sites at the gills, for instance, leading to toxic effects (Morgan et al., 1997; Bury et al., 1999b; Wood et al., 1996). Consequently, these proteins are considered the toxic sites of the biotic ligands (Niyogi and Wood, 2003). In general, based on the physiological mechanism of toxicity, metals can be classified into three categories (Niyogi and Wood, 2003). Monovalent metals, e.g., Cu^+ and Ag^+, disrupt the uptake of Na^+ and Cl^-. Divalent metals, e.g., Cd^{2+} and Zn^{2+}, disrupt Ca^{2+} uptake. And, metals like aluminum and nickel increase the diffusion distance, thus compromising the ability of the gill to take up O_2 and excrete CO_2 (Playle et al., 1989; Pane et al., 2003).

Because of the multitude of processes affecting metal uptake and internal metal distribution, the relationship between the chemical speciation of metals in the environment and their bioavailability is complicated and not well developed (van Leeuwen, 1999; Pinheiro and van Leeuwen, 2001). In addition to the geochemical speciation of metals in the environment, reactions of metals with different binding sites at the biological surface and activities of organisms control metal bioavailability. This accounts for a number of exceptions from the FIAM that have been summarised (Parker et al., 1995; Parker and Pedler, 1997; Kalis et al., 2006). A biological response only occurs as a result of interactions of metals with the cell membrane surface (Brown and Markich, 2000). Toxicity of metals is dependent on their affinity for biological ligands (Seregin and Kozhevnikova, 2006). The role of metal-organism interactions in determining metal bioavailability has been increasingly noticed (Worms et al., 2006; Ahlf et al., 2009). The organisms should be included in the speciation system in order to obtain a better understanding of factors controlling metal bioavailability (Kalis et al., 2006). Specifically, interactions at the biological ligands, such as sites of action of toxicity and other binding sites, need to be integrated together with chemical speciation in assessment of metal bioavailability (Allen and Hansen, 1996; Di Toro et al., 2001). In other words, formation of both the metal-ligand complex in solution and the metal-cell surface complex should be simultaneously taken into account in the assessment (Parker and Pedler, 1997). From a chemistry perspective, a binding site at the biological surface is considered a biotic ligand, which competes with abiotic ligands, e.g., organic molecules, in the water phase for metal ions, determining metal bioavailability. Reactions in the immediate proximity of the biological surface are a key determinant of metal bioavailability (Worms et al., 2006).

Some new methods that take into account interactions at the biological surface have been developed from the FIAM in assessments of metal bioavailability. The fish gill surface interaction model is one example of extension of the FIAM (Playle, 1998). This model is based on the integration of conditional metal-gill surface binding constants into a geochemical speciation model. In the model, effects of competition and complexation on metal binding to freshwater fish gills are incorporated, providing mechanistic-based estimations of metal bioavailability. However, the model has been developed to investigate toxicity of only a limited number of metals at the known gills of rainbow trout or fathead minnows. Then, Brown and Markich (2000) combined the concepts of the FIAM and the biological receptor theory to obtain more reliable estimations of metal bioavailability. In the approach, the interactions of chemical species at biological receptor sites are more precisely quantified. The extended FIAM shows better potential for assessment of metal-organism interactions. More recently, the Biotic Ligand Model (BLM) has been developed on the basis of the fish gill surface interaction model to increase the potential for application to a wide range of species, metals, and exposure chemistry conditions. Both the fish gill surface interaction model and

the BLM are based on the assumption of competition among ions for binding sites as the mechanism of interaction, but a more general concept of binding sites, i.e., biotic ligands, was introduced into the BLM (Di Toro et al., 2001). Additionally, ion-organism interactions at the membrane surface have been integrated in metal assessment in another approach that is based on the role of the electrical potential at the plasma membrane (PM) surface in the ion transport (Kinraide et al., 1998). Particularly, the surface potential influences the metal activity at the membrane surface as well as the transport of the ions across the membrane (Kinraide, 2006; Wang et al., 2011). According to the electrostatic approach, ameliorative effects of competing ions on metal bioavailability are expressed by their influence on the depolarisation of the PM surface (Kinraide, 2006).

1.3.2. Ion-ion interactions

In the environment, toxic metals are present in mixtures with major cations and other toxic metal ions. Significant progress has been made in investigating effects of major cations on toxicity of detrimental ions. For example, according to the concept of the BLM, major cations, e.g., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, might compete with toxic ions, e.g., Cu^{2+} and Zn^{2+}, for binding sites at the biotic ligands (Di Toro et al., 2001). These interactions result in a decrease in the accumulation of toxicants at the biotic ligands, and subsequently reduce metal toxicity. Based on the electrostatic theory, additions of the major cations reduce the negativity of the electrical potential at the membrane surface. The change in the potential subsequently leads to variations in the activity of toxic ions at the PM surface as well as the transport of the ions via the membrane, therefore affecting toxicity (Kinraide et al., 1998; Kinraide, 1998). However, limited developments have been obtained in incorporating ion-ion interactions in predicting bioaccumulation and toxicity of mixtures of toxic metals.

The low progress in estimating bioaccumulation and toxicity of metal mixtures is ascribed to the complex interactions between different metals. In addition to interactions of ions with the cellular or organismal system, toxicity of metal mixtures is influenced by interactions between different ions in the mixtures (Dardenne et al., 2008). Interactions between different metals in their mixtures contribute to adverse effects following exposure to metal mixtures even when these substances are present at concentrations below the environmental quality guideline levels of individual components (Cooper et al., 2009). The interactions occur at different levels, i.e., in the environment, at the root surface, and within the plant (Kabata-Pendias and Pendias, 1984; Phalsson, 1989). At the environmental-chemical level, in combination with physicochemical conditions of the environment, interactions outside organisms determine the environmental availability of metals. Subsequently, at the toxicokinetic phase, interactions between different substances influence uptake of single substances by organisms. At the toxicodynamic phase, interactions occur at target sites in organisms, affecting joint toxicity. Metal binding and uptake are highly influenced by competition between different ions (Kalis et al., 2006). Major cations, e.g., H^+, Na^+, Ca^{2+}, and Mg^{2+}, may compete with toxic metal ions, e.g., Cu^{2+}, for binding sites at the organism-water interface, such as fish gills (Pagenkopf, 1983; Zitko and Carson, 1976; Playle et al., 1992, 1993; Campbell and Stokes, 1985; Santore et al., 2001). Furthermore, once accumulated in organisms, metals may compete for binding sites on specific enzymes or receptors during the absorption, excretion, or sequestration, or at the target site (Fairbrother et al., 2007). For example, Cu(I) and Ag(I) were found to compete for the transporter Ctr1 (Lee et al., 2002; Boyle et al., 2011). Consequently, the chemical and toxicological reactions between different metals following exposure to their mixtures have effects on their reactivity, bioavailability, and toxicity. These interactions together with interactions of ions with the cellular systems, e.g., uptake, transport, and receptor binding, may result in different patterns of joint toxicity, e.g., additive, synergistic, or antagonistic. Toxicity of metal mixtures may vary widely and

biological actions of metal mixtures may deviate significantly from the actions of single metals (Norwood et al., 2003; Otitoloju, 2002; Manzo et al., 2010). Bioaccumulation of metal mixtures is a function of the specific components and their proportions in mixtures (Shuhaimi-Othman and Pascoe, 2007). These observations emphasise the importance of integrating interactions between different ions in metal assessment. The exclusion of the interactions in interpreting the relation between metal speciation in the environment and metal uptake may lead to incorrect assessment of bioavailability. The incorporation of the joint biological action of mixtures provides more reliable estimations of toxicity (Otitoloju, 2002).

While interactions between different metals in the environment are well assessed by chemical speciation models, e.g., WHAM, toxicological interactions, i.e., at the biological ligands at the toxicokinetic and toxicodynamic phases, are usually excluded or not properly addressed in current models for assessment of mixture toxicity. For example, the concepts of concentration addition and response addition (or response multiplication) are based on the assumption that the presence of one metal does not affect the biological action of another (Bliss, 1939; Hewlett, 1979). Consequently, deviations from the ideal behaviour of substance mixtures (i.e., additivity), e.g., more than and less than additive effects, cannot be quantified by these models or incorporated in estimating toxicity of the mixtures.

1.4. Affinity for biological ligands and metal toxicity

1.4.1. Affinity for biological ligands

Metal uptake, bioaccumulation, and bioavailability are highly influenced by ligand binding and competitive interactions at the receptor site as described in the previous section and reported by Alsop et al. (1999) and Hollis et al. (2000). Toxicity of metals is dependent on their affinity for biological ligands (Seregin and Kozhevnikova, 2006). Binding of metal ions to the cell wall varies as a function of the affinity of the ions for ligands, e.g., polygalacturonic acid (Rudakova et al., 1988; Merce et al., 2001). Therefore, the use of the affinity of metals for the biological ligands may create significant progress in modelling bioaccumulation, bioavailability, and toxicity of metal mixtures. On the one hand, the inclusion of the interactions between ions and biological materials as expressed by the metal-specific affinity for the biological ligands provides more reliable estimations of metal bioaccumulation and bioavailability. On the other hand, the integration of the metal-specific affinity in predicting metal bioaccumulation and bioavailability allows taking into account interactions between different metals in estimating toxicity of metal mixtures. Moreover, this modelling approach is based on an intrinsic property of metals, i.e., independent of environmental conditions, thus facilitating extrapolation to different conditions.

In this PhD thesis, the use of the affinity of metals for biological ligands in metal assessment was studied in three different approaches: the mechanistic bioaccumulation model, the Biotic Ligand Model (BLM), and the electrostatic toxicity model (ETM) (Fig. 1.2). Besides the potential for integrating interactions between metals and organisms and between different metals, these approaches show other significant advantages in estimating metal bioaccumulation and toxicity. Mechanistic bioaccumulation models overcome limitations of the use of single and generic values of accumulation factors BCF and BAF by delineating physiological processes in simulating metal uptake and elimination. By including the interactions at sites of toxic action, the BLM, at least in principle, provides a mechanistic understanding of the relation between metal bioaccumulation and toxicity. The ETM provides insights into other mechanisms of interactions besides the competitive binding as included in the BLM. A detailed description of these approaches is presented in *Sections 1.4.2–1.4.4.*

1.4.2. Mechanistic bioaccumulation models

Kinetic-based models have been recommended as an alternative to the use of single and generic values of BCF and BAF in assessment of metal bioaccumulation (Luoma and Rainbow, 2005; Wang and Zauke, 2004; Kahle and Zauke, 2003; Chang and Reinfelder, 2000; Reinfelder et al., 1998). These models provide reliable estimations of metal accumulation in organisms with the integration of different exposure routes (e.g., water vs. diet) and the dynamic nature of bioaccumulation processes (Fairbrother et al., 2007; Ahlf et al., 2009). Another advantage of the models is that the distinction of metal bioaccumulation from different pathways, which determines internal distribution and subsequent toxicity, is addressed in the models. The models were found to accurately predict metal bioaccumulation from different uptake routes for a number of metals and organisms (Luoma and Rainbow, 2005; Luoma et al., 1992; Thomann et al., 1995; Wang et al., 1996, 1997, 1998; Wang and Fisher, 1998; Hendriks and Heikens, 2001; Veltman et al., 2007a,b).

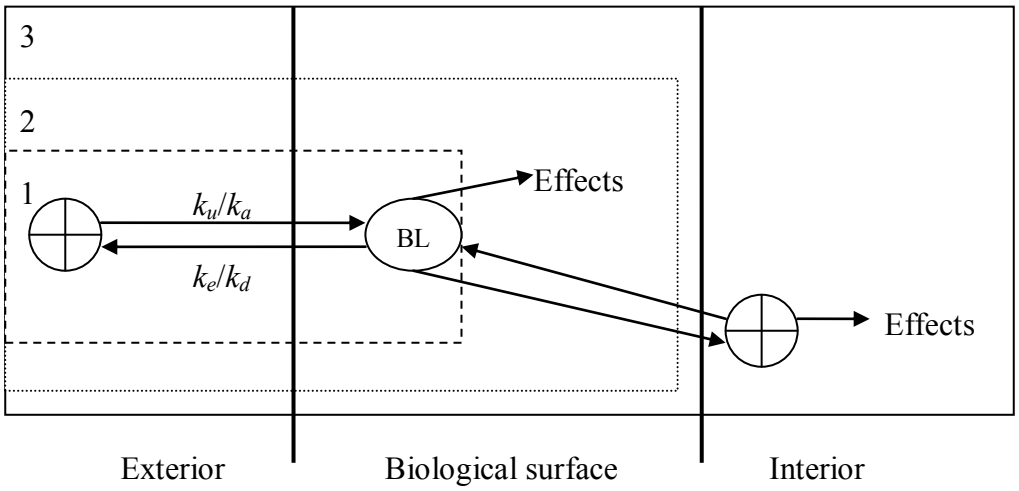

Figure 1.2. The diagram describes the principles of: (1) the mechanistic bioaccumulation model (denoted by the dashed rectangle), (2) the Biotic Ligand Model (BLM) (denoted by the dotted rectangle), and (3) the electrostatic toxicity model (denoted by the solid rectangle). All these models are based on the binding of metals (denoted by the circles with a plus sign) to the biological ligands (BL). The biological ligands are transport proteins in the bioaccumulation model, biotic ligands at the water-organism interface in the BLM, and the binding sites at the membrane surface in the electrostatic toxicity model. In the mechanistic bioaccumulation model, the internal concentration of metals in organisms is a function of kinetics of uptake (k_u) and elimination (k_e) via different exposure pathways. According the BLM concepts, metal accumulation at the biotic ligands results from the association (k_a) and dissociation (k_d) processes. In the BLM, one more step is modelled, i.e., the bioaccumulation of metals at the biotic ligands is related to toxic effects. In the electrostatic toxicity model, both the bioaccumulation of metals at the biological surface and the transport of metals through the membrane might be predicted and incorporated in modelling toxic effects. As such, the interactions between metal ions and biological ligands are integrated in modelling toxicity of metal mixtures.

The kinetic-based bioaccumulation model is based on a biodynamic delineation of metal accumulation processes that provides a detailed understanding of the extent of and contribution to the variability in the amount of different metals accumulated in various species

and environmental conditions (Luoma and Rainbow, 2005). This biokinetic model is developed on the basis of the concept of biodynamics originated by Riggs (1963) that accumulation of chemical substances is the result of a balance of different fluxes: uptake from food, uptake from the dissolved phase, and losses (Luoma and Rainbow, 2005). Although the model is based on the assumption of steady-state conditions, it can be used to assess accumulation of substances in temporally varying conditions (Wang and Fisher, 1999). Another assumption of the model is that the rate of these fluxes can be determined by realistic, controlled experiments at various exposure concentrations and in different conditions (Luoma and Rainbow, 2005). Therefore, site-specific exposure concentrations and conditions as well as empirical metal-specific physiological parameters can be integrated into the model in order to estimate metal concentrations in organisms (Luoma and Rainbow, 2005; Wang and Fisher, 1999). As such, different bioaccumulation processes are distinguished, quantitatively modelled, and balanced to give an estimation of the site-specific bioaccumulation level (Luoma and Rainbow, 2005). Metal uptake from both the dissolved phase and the dietary source is taken into account and distinguished in the model. These sources can be further divided. Uptake from the dissolved phase, for example, can be from pore or overlying water. Food can include detritus, phytoplankton, inorganic particles, and sediments. Uptake from each pathway is quantified based on an assumed proportional relationship between the uptake and the metal concentration in that exposure phase (Wang and Fisher, 1999). Based on the concept of Riggs (1963), Thomann (1981) developed a model estimating accumulation of substances in aquatic food chains. In the model, the influx rate from the dissolved phase was determined by multiplying the substance concentration in water by the absorption rate constant (Factor 1 in Equation 1.1). The absorption rate constant is a function of the filtration rate of organisms and the absorption efficiency of the dissolved substance by the organisms. The influx rate from food was quantified as a product of the assimilation efficiency and the amount of substances ingested, which is a function of the concentration of the substance in the food and the ingestion rate (Factor 2 in Equation 1.1). The elimination rate of metals via both pathways and the growth dilution were also incorporated (Factor 3 in Equation 1.1). Metal uptake over time is presented by the first-order physiological process (Eqn. 1.1) (Wang and Fisher, 1997, 1999; Thomann, 1981; Landrum et al., 1992; Luoma and Fisher, 1997):

$$\frac{dC}{dt} = (k_u \times C_w) + (AE \times IR \times C_f) - (k_e + g) \times C \qquad (1.1)$$

where C (μg/g) is the metal concentration in the organism; k_u (L/g/d) is the uptake rate constant from the dissolved phase (or the absorption constant); C_w (μg/L) is the metal concentration in water; AE (mg/g/d) is the metal assimilation efficiency from ingested particles; IR (g/g/d) is the ingestion rate of the organism; C_f (μg/mg) is the metal concentration in the ingested food; k_e (1/d) is the efflux rate constant; and g (1/d) is the growth rate constant.

However, the application of the kinetic models in extrapolating to different environmental conditions has been suggested to be limited. Particularly, several parameters describing physiological processes, e.g., the metal assimilation efficiency from ingested food, the metal absorption rate from water, the metal elimination rates, and the growth rate, which are highly influenced by environmental conditions, need to be empirically measured (Wang and Fisher, 1999). Moreover, the measurements need to be carried out in environmentally realistic conditions for each combination of a particular metal and one species. This limitation in extrapolation potential can be avoided by integrating diverse phenomena into unifying concepts. The biokinetic models based on a unifying concept are considered “mechanistic” from the perspective that uptake and elimination kinetics are derived from chemical-specific properties and species-specific physiological characteristics (Hendriks and Heikens, 2001;

Veltman et al., 2008). For organic substances, fugacity is widely considered such a unifying concept as it provides insight into the extent of variability in bioaccumulation among organisms, chemicals, and environmental conditions as well as factors contributing to this variability (Mackay, 2004; Luoma and Rainbow, 2005). Consequently, the approach allows extrapolating to a number of chemicals, organisms, and environmental conditions, without the necessity for case-specific calibration. Accordingly, the octanol-water partition coefficient K_{ow} that reflects the partitioning of lipophilic organic compounds into the fatty tissue is widely used together with the lipid content as inputs to first-order mechanistic bioaccumulation models (Gobas, 1993; Hendriks, 1995; Hendriks et al., 1998, 2001). Particularly, absorption and elimination rate constants are estimated based on these chemical- and species-specific properties.

The development and validation of a mechanistic bioaccumulation model for metals based on metal-specific properties lag far behind the assessment for organic chemicals. This low progress is mainly caused by the complex and specific behaviour of metals in the environment and in organisms as mentioned in previous sections. Bioaccumulation of metals is influenced by metal species, environmental chemistry, and highly-specific physiological uptake mechanisms (Allen et al., 1980; Rainbow, 1991). For example, the metal assimilation efficiency varies widely, depending on food quantity and quality and chemical composition (Wang et al., 1995; Decho and Luoma, 1994; Wang and Fisher, 1996a,b; Wang and Kong, 2003). The uptake rate constant is dependent on environmental chemistry, e.g., the concentration of dissolved organic carbon (DOC) and salinity, and species-specific features, e.g., the filtration rate (Wright, 1995; Wang et al., 1996; Wang and Fisher, 1999). Efflux rate constants are influenced by the exposure route (Wang and Fisher, 1998). Because of this high specificity, no generic value was found to be able to describe physiological parameters for each species-metal combination (Wang and Fisher, 1999). Metal uptake and elimination rate constants need to be quantitatively linked to metal-specific properties and species-specific physiological characteristics in order to facilitate extrapolation to a wide range of metals and species (Veltman et al., 2008). The influence of this metal specificity, environmental conditions, the exposure route, and species-specific characteristics on metal bioaccumulation cannot be explained by any simple generalisation (Luoma and Rainbow, 2005).

Findings during the last two decades indicate potential for developing mechanistic models delineating metal bioaccumulation. In particular, metal absorption and elimination rates can be explained in relation to the filtration rate and the species weight, respectively (Baines et al., 2006; Hendriks and Heikens, 2001). Furthermore, as presented in previous sections metal uptake involves different membrane transport proteins. The affinity of proteins is metal-specific, i.e., depending on metal charge and atomic radius, and preferences for coordination and ligands (Bell et al., 2002; Handy and Eddy, 2004; Veltman et al., 2008). The uptake rate constant from the dissolved phase may therefore be a function of metal properties that show affinity for proteins (Veltman et al., 2008). This theory was supported by a correlation found between the absorption efficiency and the binding to membrane transport proteins (Bryan, 1984). Although a quantitative explanation for this relationship is currently unavailable, efforts have been put into integrating metal affinity for biological ligands in bioaccumulation and toxicity models, e.g., quantitative structure-activity relationships (Veltman et al., 2008; Jones and Vaughn, 1978; Newman and McCloskey, 1996; McCloskey et al., 1996; Tatara et al., 1997; Walker et al., 2003). Significant relationships were found between the covalent index and metal toxicity (Newman and McCloskey, 1996; McCloskey et al., 1996; Tatara et al., 1997). Recently, the metal absorption rate constant was reported to be a function of the metal-specific covalent index and the species-specific filtration rate (Veltman et al., 2008).

These results imply potential for developing a mechanistic model to estimate bioaccumulation for a number of metals and species based on these metal- and species-specific characteristics.

Although the kinetic-based bioaccumulation models may provide significant progress in predicting metal bioaccumulation, some disadvantages have been recognised. Firstly, the models do account for neither the influence of metal speciation on bioaccumulation nor the relationship between metal bioaccumulation and toxic effects (Paquin et al., 2002). Secondly, tissue compartmentalisation, which is of importance in metal bioavailability and toxicity, is excluded in the models (Fairbrother et al., 2007). In addition, detoxification, which influences the tolerance of organisms to metals, is ignored in the models. In other words, different fractions of metals accumulated, i.e., the detoxified metal fraction and the metabolically reactive fraction, are not distinguished by this approach.

1.4.3. Biotic Ligand Models

The Biotic Ligand Model (BLM) has been developed as a mechanistic-based approach for estimating metal bioavailability, taking into account interactions at the water-organism interface (Di Toro et al., 2001; Santore et al., 2001; US EPA, 2000). The conceptual framework for the BLM was developed from the gill surface interaction model and the FIAM model (Pagenkopf et al., 1974; Pagenkopf, 1983; Playle et al., 1992, 1993; Janes and Playle, 1995; Hollis et al., 1996, 1997; Playle, 1998; Richards and Playle, 1998; Wood et al., 1999; Morel, 1983; Morel and Hering, 1993; Campbell, 1995). According to the BLM concept, together with chemical speciation in the environment, interactions of toxic metals with competing cations at target sites of toxicity influence metal toxicity (Pagenkopf, 1983; Meyer, 1999). Therefore, both aquatic geochemistry and toxicology principles are integrated in defining the fraction of metals that produces adverse effects (Playle, 1998; McGeer et al., 2000; Di Toro et al., 2001). Toxic effects occur as a result of the binding of free metal ions to physiologically active binding sites at the site of action, which is described by the formation of metal-biotic ligand complexes according to a normal mass reaction equation (Eqn. 1.2):

$$[\mathrm{MBL}] = K_{\mathrm{MBL}} \times [\mathrm{M}^{n+}] \times [\mathrm{BL}] \tag{1.2}$$

where [MBL] (mol/L) is the concentration of the cation-biotic ligand complex; K_{MBL} (L/mol) is the stability constant of the cation-biotic ligand binding; $[\mathrm{M}^{n+}]$ (mol/L) is the concentration of the free metal ion in the solution; and [BL] (mol/L) is the concentration of unbound biotic ligands (Di Toro et al., 2001).

On the one hand, the biotic ligand competes with aqueous ligands, especially dissolved organic matter, for metal ions. On the other hand, the toxic metal ions compete with other cations in the solution, e.g., H^+, for binding to the biotic ligand. The extent of toxic effects is determined by the concentration of the metal-biotic ligand complex. Toxic effects occur when this concentration exceeds a critical level. As such, both chemical speciation and cationic competition for binding sites are integrated in estimating metal toxicity (Di Toro et al., 2001; Santore et al., 2001; Deleebeeck et al., 2007). The inclusion of these interactions distinguishes the BLM and the FIAM, which are both based on the assumption that free ions are the main reactive species of metals. For fish, the biotic ligand was suggested to be the Na^+ or Ca^{2+}–channel proteins on the surface membrane of the gill as metal binding at the sites disrupts ionoregulatory processes, e.g., transport of sodium across the gill, and eventually results in adverse effects (McDonald et al., 1989). For a number of other species, methods allowing direct measurements of the density of the target sites of toxicity are not available. Therefore, the site of action of toxicity is generalised to the biotic ligand in order to facilitate the applicability to a variety of species (Di Toro et al., 2001). In other words, it is assumed that the toxicology principles for fish are applicable to any other species for which the target site

of toxicity is in direct contact with the external aqueous environment. The BLM parameters include the stability constants of binding of cations to the biotic ligand and the total density of binding sites. In fish, these parameters are available for several metals, including copper, cadmium, and silver, from direct empirical measurements while for other species they are determined by fitting the model to the experimental toxicity data (Playle et al., 1993; Janes and Playle, 1995; Di Toro et al., 2001). The most important criterion for the validity of the BLM is the constancy of the critical concentration over different environmental conditions (Di Toro et al., 2001). With the advantages mentioned above, the BLM is increasingly applied in setting water quality criteria and objectives (Fairbrother et al., 2007). The BLM has been successfully applied to a number of aquatic organisms, e.g., fish, algae, and water flea (De Schamphelaere and Janssen, 2002, 2004; De Schamphelaere et al., 2002, 2003, 2004; Di Toro et al., 2001; Heijerick et al., 2000, 2002; Santore et al., 2001; McGeer et al., 2000).

A number of difficulties are encountered in developing and applying the BLM to plants as this approach was originally developed for aquatic organisms (Antunes et al., 2006). These challenges are met in the determination of concentrations of free ions in soil solutions and of ligands, incorporation of nonequilibrium dissociation into the equilibrium BLM, and metal estimation and speciation in plant roots. These difficulties are attributed to complications of chemical speciation as well as metal uptake by organisms in the terrestrial ecosystem (Steenbergen et al., 2005). Firstly, the uptake routes in terrestrial organisms are more complicated than those in aquatic biota. Uptake from the pore water and from soil particles can significantly contribute to metal accumulation in terrestrial organisms. Secondly, it is difficult to control the composition of the water phase as well as metal concentrations in the pore water because of the dynamic equilibrium. Any changes in the soil properties, such as additions of metal salts, will interrupt the equilibrium. Despite these difficulties, recent findings show that the application of the BLM is theoretically and empirically feasible to terrestrial organisms. In particular, the assumption that the free metal ion is the main reactive species is applicable to terrestrial ecosystems as it is empirically evident that responses of plants and other soil organisms to metal exposure can be explained by variations in the concentration of this metal species in the water phase of soil (Spark, 1995). Moreover, toxicity mechanisms of aquatic and terrestrial organisms are assumed to be similar (Peijnenburg et al., 2007; Steenbergen et al., 2005). Influence of the environmental chemistry on metal bioavailability and toxicity is dependent on the properties of metals and competing components in the water phase as well as root characteristics (Wu and Hendershot, 2009). The BLM is therefore suggested to be applicable to plants in which metal binding sites to roots of the apoplasm are considered biotic ligands (Steenbergen et al., 2005; Voigt et al., 2006; Antunes et al., 2006). The potential applicability of the BLM to terrestrial organisms was proven by recent studies on plants. For example, Thakali et al. (2006) found the median effective concentration predicted by the BLM within a factor of ± 2 of the measurements, demonstrating that this approach is a promising method in assessing metal toxicity to plants.

With the principles mentioned above the BLM may result in significant progress in metal assessment. Firstly, interactions between ions with other abiotic ligands and biotic ligands are included in the predictions of metal bioavailability by this approach. The interactions between ions with these ligands influence metal bioavailability and toxicity. Specifically, the complexation of metals with abiotic ligands in the solution reduces the amount of metals in available species, i.e., environmental availability of metals. In addition, competition of toxic metals with other cations for binding sites at the biotic ligand leads to decreases in the accumulation of metal ions at these sites, i.e., potentially toxic bioavailability of metals. By including the interactions between metal ions with abiotic and biotic ligands, the BLM therefore provides more reliable estimations of metal bioavailability and toxicity. Secondly,

the potentially toxic accumulation can be, in principle, distinguished from the total body burden and the environmental availability (Fairbrother et al., 2007). Thirdly, as environmental characteristics, e.g., pH and concentrations of DOC, K^+, Na^+, Ca^{2+}, and Mg^{2+}, are included in estimations of metal bioavailability and toxicity, the BLM allows extrapolation to different exposure conditions (Fairbrother et al., 2007). Furthermore, the BLM may offer mechanistic explanations about metal binding and more detailed analysis and interpretation of toxicity of metal mixtures (Playle, 2004). This approach possesses potential for addressing interactions among metals and predicting toxicity of metal mixtures because of its assumption about the competitive binding at the biotic ligands (Norwood et al., 2003; Chen et al., 2010). According to Norwood et al. (2003), interactions between metals can be predicted if their stability constants are known. If two metals compete for binding to the same site of toxic action, the total amount of metals bound to the site would be a key factor, determining mixture toxicity. Alternatively, if metals in mixtures bind to different target sites of toxicity, estimations of bioavailability of individual metals might be a reliable predictor of mixture toxicity through the response addition model. This potential was confirmed by higher predictive power of the BLM compared to the FIAM and the total metal concentration model in estimating toxicity of metal mixtures (Hatano and Shoji, 2008). Furthermore, the higher capacity of the BLM is ascribed to the inclusion of competition between different ions for binding to transport sites at the biotic ligands. However, the applicability of the BLM to toxicity of metal mixtures needs to be further investigated as the validity of applying equilibrium constants for metals determined from single exposure to the metals following exposure to their mixtures is not verified yet (Chen et al., 2010).

A number of disadvantages of the BLM resulting from its assumptions have been recognised. According to the BLM concept, metal internalisation is a rate-limiting process because of the assumed equilibrium between metals in the bulk solution and metals bound to the biotic ligands (Antunes and Hale, 2006). In other words, transport of metal ions from the bulk solution to the biotic ligands is hypothesised to be faster than the actual uptake rate by organisms. This assumption is still unidentified, especially as metals can be resupplied by soil in response to decreasing concentrations of metals in the species of free ions surrounding the biotic ligand or in the bulk medium which are caused by rapid internalisation. In these cases, labile complexes may become a source of metals that eliminates diffusion restrictions and depletion in the bulk medium and consequently increases the total metal uptake. This possibility is supported by substantial contribution of metal complexes, e.g., metal-dissolved organic carbon, to the total uptake observed in both aquatic organisms and plants (Martin and Goldblatt, 2007; Ferreira et al., 2008; Pinheiro and van Leeuwen, 2001; Campbell et al., 2002; Pinheiro et al., 2004; McLaughlin et al., 1998; Antunes and Hale, 2006; Degryse et al., 2006). The exclusion of this source of metal uptake in the BLM led to inaccurate estimates of metal toxicity, e.g., large deviations between measurements and predictions of copper toxicity to *Daphnia magna* at low pH of 5.5 and high levels of Fe and Al (De Schamphelaere and Janssen, 2004). In addition, neither active regulations (controlling metal uptake) nor subcellular regulations (e.g., detoxification, homeostasis, and sequestration) were included in the model. Other limitations of the BLM are related to the assumption about the competitive binding at the biotic ligand. It is difficult to verify the assumption that toxicity occurs only when cations bind to the hypothetical ligand and alleviative effects, by H^+ and Ca^{2+} for example, only happen when these cations and toxic ions bind to the same biotic ligand (Kinraide, 2006). According to the BLM principles, interactions among different metals occur only via competitive inhibition of binding of metals to the biotic ligands, ignoring other possibilities, such as non-competitive, anti-competitive, and mixed forms of inhibition (Laidler and Bunting, 1973). Relatively constant or increased accumulation of several metals with additions of others as reported by Norwood et al. (2007) cannot be interpreted in terms of

competition in the BLM. Among the different types of joint toxicity, i.e., additive, synergistic, or antagonistic, complete additivity and complete antagonism might be explained by the two- or single-binding-site BLM, respectively, but not synergistic interactions. Another disadvantage of the BLM is induced by the assumption about the independence of binding sites to each other while metals can be absorbed by organisms via various transport systems depending on physiochemical conditions. These assumptions potentially account for a number of observed deviations from the BLM predictions (Hassler et al., 2004; Campbell, 1995; Campbell et al., 2002; Wilkinson and Buffle, 2004; Slaveykova and Wilkinson, 2002).

1.4.4. Electrostatic toxicity models

The electrostatic approach is increasingly considered an alternative in estimating metal bioavailability and toxicity to plants. This method originates from the observation that there are considerable differences between ion concentrations at the root plasma membrane (PM) surface and concentrations in the external medium because of a negative charge at the PM surface (Wagatsuma and Akiba, 1989; Kinraide, 1998). The surface potential is affected by the ionic composition of the bulk-phase medium and plays an important role in the transport of ions through the membrane (Kinraide, 2001). Firstly, the potential has influence on the activity of ions at the PM surface via electrostatic attraction or repulsion. Secondly, the potential impacts the difference in the electrical potential across the membrane, which is a driving force for the transport of ions through the membrane. Effects of the electrical potential at the PM surface on cation uptake as well as on physiological effects have been reported in a number of studies (Gimmler et al., 1991a,b; Gimmler et al., 2001; Kinraide, 2001; Zhang et al., 2001; Nagata and Melchers, 1978; Wagatsuma and Akiba, 1989; Suhayda et al., 1990; Kinraide, 1994, 1998, 1999; Krab et al., 2000; Ahn et al., 2001). Recent developments in the measurement of the plant PM potential and the surface charge density facilitate the application of the surface potential in estimating metal toxicity (Nagata and Melchers, 1978; Moller et al., 1984; Gibrat et al., 1985; Abe and Takeda, 1988; Oka et al., 1988; Obi et al., 1989a,b). Together with measurements of ion-PM binding affinities, these approaches allow the computation of the PM surface potential by the Gouy-Chapman-Stern model (Kinraide, 1998). In the model, the PM potential ψ_0 is determined based on the assumption of the existence of negatively charged and neutral sites at the membrane, which can bind to metal ion M^{n+} (Kinraide et al., 1998). The principal effect of the surface potential is controlling ion activities at the surface (Nobel, 1991). This effect can be computed by the Nernst Equation:

$$\{M^{n+}\}_0 = \{M^{n+}\}_b \times \exp\left(\frac{n \times F \times \psi_0}{R \times T}\right) \quad (1.3)$$

where $\{M^{n+}\}_0$ and $\{M^{n+}\}_b$ (mol/L) are the activities of free ion M^{n+} at the PM surface and in the bulk phase medium, respectively; n (dimensionless) is the charge on the ion; F (J/mV) is the Faraday constant (F = 96.485); R (J/mol/K) is the universal gas constant (R = 8.314); and T (K) is the temperature (Nobel, 1991).

Based on the above principles, the electrostatic approach shows both advantages and disadvantages in evaluating metal uptake and toxicity. By integrating electrical properties of the cell membrane, the electrostatic approach may lead to significant progress in assessment of metal toxicity to plants. Particularly, this method allows investigating plant-ion interactions and incorporating impacts of these interactions in predicting metal bioavailability, thus providing more reliable estimates of metal toxicity (Wang et al., 2010). Moreover, the interactions between different ions can be addressed by the electrostatic approach through determining changes in the surface potential with varying ionic composition of the solution. The varying electrical potential at the PM surface subsequently influences the partition of ions

between the PM surface and the bulk phase medium. For example, decreases in the negativity of the surface potential resulting from additions of cations reduce the activity of cations while increasing the activity of anions at the PM surface. This principle has been used to study effects of major cations, e.g., H^+, Na^+, Ca^{2+}, and Mg^{2+}, on toxicity of toxicants, e.g., Cu^{2+} and Zn^{2+} (Kinraide, 2006). As the addition of H^+, Na^+, Ca^{2+}, or Mg^{2+} leads to a reduction in the negativity of the surface potential and subsequent decreases in the activity of the toxic cations at the PM surface, these major cations potentially have alleviative effects on metal uptake and toxicity (Kinraide, 2006). Opposite effects were reported on the uptake and toxicity of anions, e.g., SeO_4^{2-} (Kinraide, 2003). Therefore, the electrostatic approach might provide additional explanations for the impact of ion-ion interactions on metal toxicity besides the competitive binding usually assumed in toxicological studies in general and in the BLM in particular. According to the electrostatic approach, three different mechanisms were found to account for ameliorative effects of Ca^{2+} on metal toxicity **(**Kinraide, 1998).

1) Mechanism 1: The reduction in metal toxicity occurs as a result of the electrostatic displacement of toxic cations at the PM surface by Ca^{2+} induced by the decreasing surface potential. Moreover, the intensity of effects caused by different cations on metal toxicity by this mechanism can be compared (Kinraide et al., 2004).
2) Mechanism 2: The alleviation results from the restoration of Ca^{2+} at the cell surface in response to low levels of surface Ca^{2+} since the low contents of Ca^{2+} at the cell surface inhibit the growth of plants.
3) Mechanism 3: This mechanism involves interactions between Ca^{2+} and the toxicant at the cell surface, but not the displacement interactions like mechanisms 1 and 2. For instance, effects of Na^+ are caused by the blockade of the ion channel (Tyerman, 1997).

Although the relative importance of these mechanisms is toxicant- and cation- specific, generally mechanism 1 occurs in all cases and the contribution of mechanism 2 to the alleviation is minor **(**Kinraide, 1998). In addition, mechanism 3 is toxicant specific, e.g., H^+ has moderate alleviative effects while Al^{3+} does not affect toxicity of toxicants **(**Kinraide, 1998). Relative influence of Ca^{2+} and Mg^{2+} on toxicity of toxic metal ions is ion- and species-specific **(**Kinraide, 1998). For example, in soybean, Ca^{2+} has higher alleviative effects on Al^{3+} toxicity (Silva et al., 2001a,b). In wheat, Mg^{2+} is more effective than Ca^{2+} in inhibiting Zn^{2+} toxicity while Mg^{2+} does not influence Al^{3+} toxicity (Kinraide, 1998; Pedler et al., 2004). These findings demonstrate that the electrostatic approach is a promising method of quantitatively evaluating interactions between toxicants and ameliorative ions. Furthermore, it is implied that although competition may act as a mechanism of interactions between different ions, the surface potential should be included in assessment of metal toxicity. Another advantage of the electrostatic approach over the FIAM and the BLM is the potential for investigating effects of anions on metal toxicity (Kinraide, 2006). The activity at the PM surface is a more reliable predictor of the uptake and toxicity of selenate (SeO_4^{2-}) than the activity in the bulk phase medium (Kinraide, 2003). The increase in the toxicity of SeO_4^{2-} caused by Ca^{2+} cannot be interpreted in terms of competition for binding sites while this observation can be explained in relation to changes in the surface potential (Kinraide, 2006). Particularly, the presence of Ca^{2+} or Mg^{2+} reduces the negativity of the surface potential, therefore increasing the uptake and toxicity of SeO_4^{2-}. With these advantages, the electrostatic approach has been increasingly used to assess effects of common cations (e.g., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}) on metal toxicity through variations in the surface potential (Wang et al., 2008, 2011; Kopittke et al., 2011). Yet, the approach has not been applied to predict interactions in and toxicity of metal mixtures. Moreover, the electrostatic approach has some limitations. For instance, regulations, which have profound influence on uptake kinetics as well as subcellular allocation of metals, are not addressed in the electrostatic approach.

Additionally, the amount of accumulated metals that produces toxic effects is not determined by this approach.

1.4.5. Comparison of the different approaches

Although interactions between metal species and biological materials are included in the mechanistic bioaccumulation model, the BLM, and the ETM as described in *Sections 1.4.2–1.4.4*, there are fundamental differences in these models.

1) Endpoints: The final endpoint of the bioaccumulation model is the internal concentration of metals. In the BLM, the endpoint is toxic effects, which are linked to the accumulation of metals at the biotic ligands at the water-organism interface. In the electrostatic approach, toxicity is linked not only to the accumulation of free metal ions at the PM surface, but also to the transport of the ions via the membrane (Fig. 1.2).
2) Metal forms: In the BLM and the electrostatic approach, free ions are considered the main reactive species of metals, determining the extent of toxic effects whereas in the kinetic-based bioaccumulation model, the total accumulation of metals in different species is estimated, excluding metal speciation in predicting metal bioavailability.
3) Expression of the affinity of metals for biological ligands: In the bioaccumulation models, the interactions between metals and biological materials are included in the determination of the rate constants of the physiological processes. In the BLM and the electrostatic approach, the affinity of metals for the biological ligands is included in the stability constant of the binding of metal ions to the biological ligands.
4) Mechanisms of interactions: In the BLM, effects of one metal ion on another are related to the competition for binding sites. In the electrostatic approach, these effects are assumed to be associated with electrical reactions that are induced by changes in the surface potential.

1.5. Objectives of the thesis

Understanding of metal bioaccumulation and toxicity is lagging behind the progress for organic chemicals. The limited development in metal assessment is ascribed to the complex physiological processes, which determine metal bioaccumulation, bioavailability, and toxicity. These processes lead to high specificity in metal bioaccumulation and toxicity among different metals, across different exposure conditions, and across different organisms. The metal-specific selectivity of biota results in difficulties in estimating metal bioaccumulation, bioavailability, and toxicity. In addition to the chemical speciation in the environment, interactions between metal ions and organisms at the biological surface and between different ions at different phases control metal bioavailability, and subsequently influence metal toxicity. While effects of environmental chemistry and the ion-ion interactions in the environment have been well assessed in speciation models, interactions between ions and biological ligands as well as interactions between different ions at toxicokinetic and toxicodynamic phases are usually poorly addressed. The general objective of this PhD thesis is to integrate the interactions between metals and biological materials in modelling bioaccumulation and toxicity of metal mixtures. The integration is carried out by modelling approaches that are based on the affinity of metals for the biological materials (ligands). This integration is expected to provide more accurate estimations of metal bioaccumulation and bioavailability, to allow extrapolation across different exposure conditions and to different metals, and to create significant progress in assessment of metal bioavailability and toxicity following exposure to metal mixtures.

The thesis aims to answer the central question: Can the variability in bioaccumulation and toxicity of metal mixtures be explained by the metal-specific affinity for biological ligands?

To answer the central question, the following subquestions need to be addressed:

1) What are the relationships between kinetics of metal uptake and the covalent index and to what extent can the variability in metal bioaccumulation be explained by these relationships? (*Chapter 2*)
2) How do common cations, i.e., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, interact with Cu^{2+} and subsequently affect Cu^{2+} toxicity and how could these effects be modelled based on the stability constant of the binding of these ions to biotic ligands? (*Chapter 3*)
3) How could the ion-ion interactions be integrated in estimating toxicity of metal mixtures by an assessment based on the free ion activity of metals in the solution (interactions between metal ions and the biological ligands are not addressed)? (*Chapter 4*)
4) How could the interactions between ions and biotic ligands at the water-organism interface be incorporated in predicting ion-ion interactions and toxicity of metal mixtures (*Chapter 5*)?
5) How could the interactions between ions and the membrane surface be integrated in estimating ion-ion interactions and toxicity of metal mixtures? (*Chapter 6*)
6) Does the integration of interactions between ions and biological ligands in modelling ion-ion interactions and toxicity of metal mixtures improve the estimation accuracy in metal assessment? (*Chapter 7*)
7) What are the relationships between ion-ion interactions and metal toxicity? (*Chapter 7*)

1.6. Outline of the thesis

In this chapter, the problem statement and goals of the PhD thesis have been outlined. A detailed description of different modelling approaches to obtain the objectives mentioned in *Section 1.5* is given in next chapters (*Chapters 2–6*) (Fig. 1.3). In addition, a chapter synthesising results obtained by applying these methods is included (Fig. 1.3).

In *Chapter 2*, the development and validation of a semi-mechanistic model delineating metal bioaccumulation based on a metal-specific property and a species-specific characteristic are presented. With the involvement of transporters and other metal-binding proteins, a metal-specific property that reflects the affinity for these biological ligands may be able to explain the variability in metal bioaccumulation. Previous studies indicate significant relationships between metal bioaccumulation and toxicity with the covalent index, a metal-specific property (Newman and McCloskey, 1996; McCloskey et al., 1996; Tatara et al., 1997; Veltman et al., 2008). In this PhD thesis, the metal-specific covalent index and the species-specific size-based filtration rate are integrated in developing a semi-mechanistic model simulating metal bioaccumulation. In other words, the covalent index is considered a unifying factor in modelling metal bioaccumulation. Different physiological processes, i.e., uptake, elimination, and growth, are included in estimating the internal concentrations of metals. Uptake and elimination of metals from both the dissolved phase and the dietary source are integrated in the accumulation model. The potential of the developed model for predicting metal bioaccumulation is then assessed by comparing estimations with field measurements.

In *Chapter 3*, the development of a BLM for predicting Cu^{2+} toxicity to lettuce *Lactuca sativa*, taking into account effects of H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, is given. The BLM was originally developed and has been widely applied to aquatic organisms, considering protein

channels on the gills as the sites of action of toxicity. Recent findings indicate that the BLM is principally and empirically applicable to terrestrial organisms like plants. In *Chapter 3*, the BLM principles are used to assess Cu^{2+} toxicity to lettuce. According to the BLM concept, the accumulation of Cu^{2+} at the biotic ligands is a key indicator, determining Cu^{2+} toxicity. Moreover, H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} may compete with toxic metal ions for binding to transport sites at the biotic ligands. This competitive binding is expected to reduce the accumulation of Cu^{2+} at the biotic ligands, thus inhibiting Cu^{2+} toxicity.

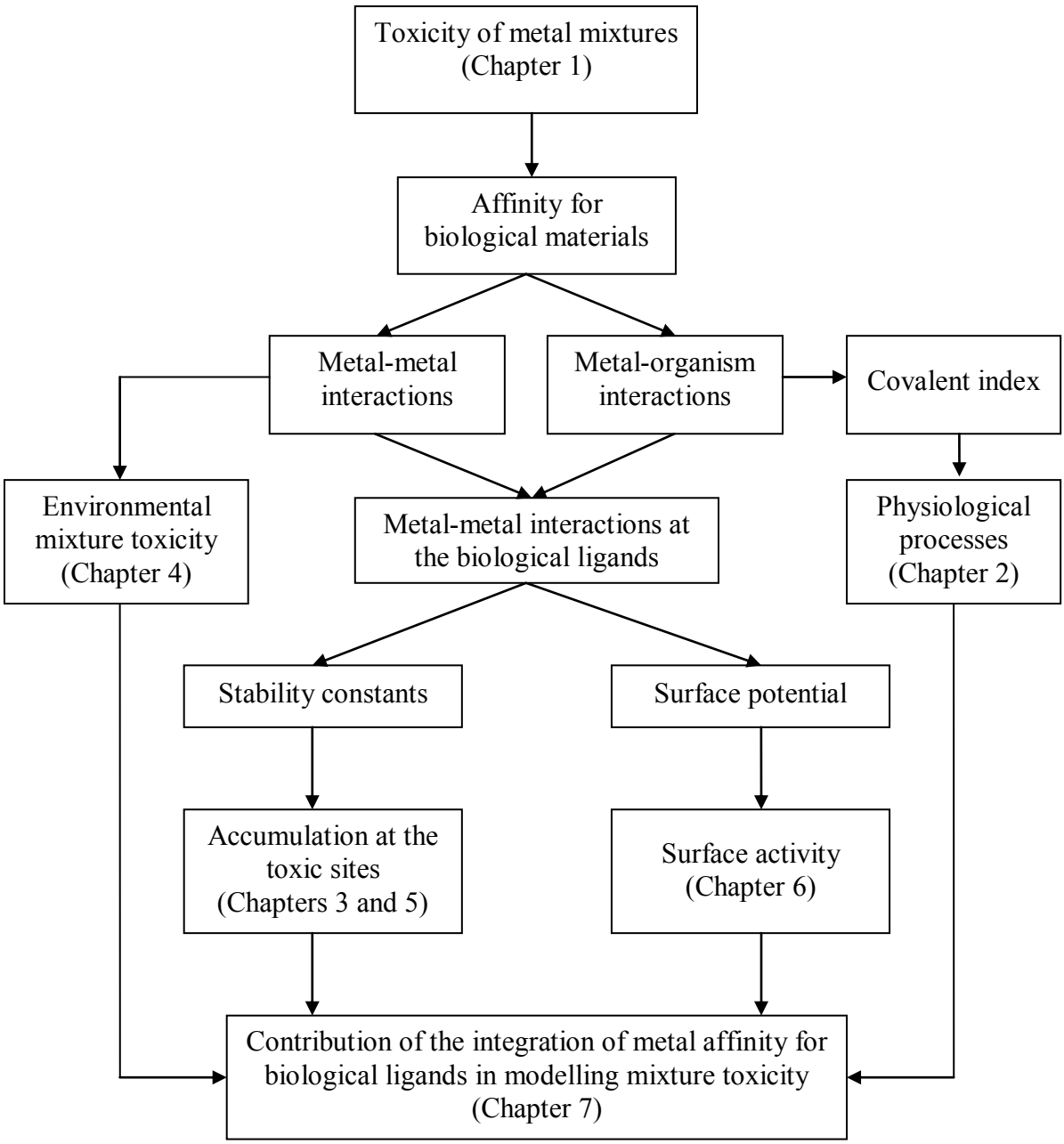

Figure 1.3. The main approaches for incorporating the affinity of metals for biological ligands in estimating toxicity of metal mixtures applied in this PhD thesis

In *Chapter 4*, an approach for modelling ion-ion interactions and integrating these interactions in estimating toxicity of Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+} mixtures is demonstrated.

1

Interactions between different metal ions are usually excluded in current models for assessment of metal mixtures, e.g., concentration addition and response multiplication (or response addition). The concept of these models is based on the assumption that substances in mixtures do not interact with each other, i.e., the presence of one metal does not affect the toxicity of another. In *Chapter 4*, these models are extended to incorporate ion-ion interactions in predicting toxicity of the mixtures.

In *Chapter 5*, a method of applying the BLM concept to mixture toxicity is shown. The interactions between different metal ions and between ions and the biotic ligands at the water-organism interface are incorporated into the toxic equivalency factor (TEF) approach for modelling toxicity of binary metal mixtures (Cu^{2+}–Ag^{+} and Cu^{2+}–Zn^{2+}). According to the BLM principle, the accumulation of metal ions at the biotic ligands is the key indicator of toxicity of single metals. Recent results indicate that this principle might be applicable to toxicity of metal mixtures. In *Chapter 5*, the fraction of the biotic ligands occupied by metal ions is used as the basic unit for the TEF approach.

In *Chapter 6*, an application of the electrostatic approach (i.e., the electrostatic toxicity model) is presented in assessing interactions between major cations, e.g., Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, and Cu^{2+} and interactions in mixtures of Cu^{2+}–Ag^{+} and Cu^{2+}–Zn^{2+}. According to the electrostatic theory, a negative potential exists at the PM surface. This surface potential has dual effects on metal toxicity by influencing the activity of ions at the PM surface and the transport of ions via the membrane. The interactions between ions and plants are assessed at different levels. Firstly, interactions occur at the proximate outside of the membrane surface (surface interactions). Additions of one metal reduce the negativity of the surface potential, thus leading to decreasing activities of others at the PM surface. Secondly, interactions occur between ions adsorbed, affecting metal toxicity (internal interactions). These surface and internal interactions might be related to the two functions of the electrical potential at the PM surface.

In *Chapter 7*, the main results of *Chapters 2–6* are linked to each other and synthesised in order to give an overview about the contribution of the integration of the affinity of metals for biological ligands in modelling bioaccumulation and toxicity of metal mixtures. Furthermore, the predictive power of different approaches studied in this thesis is compared. Based on this synthesis, *Chapter 7* accordingly provides recommendations for potential applications of the modelling approaches investigated in this thesis and for further research.

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Chapter 2

Modeling metal bioaccumulation in the invasive mussels *Dreissena polymorpha* and *Dreissena rostriformis bugensis* in the rivers Rhine and Meuse

T.T. Yen Le, Rob S.E.W. Leuven, A. Jan Hendriks

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Abstract—The metal-specific covalent index and the species-specific size-based filtration rate were integrated into a biokinetic model estimating metal bioaccumulation in mussels from the dissolved phase and phytoplankton. The model was validated for zebra (*Dreissena polymorpha*) and quagga (*Dreissena rostriformis bugensis*) mussels in the rivers Rhine and Meuse, the Netherlands. The model performed well in predicting tissue concentrations in different-sized zebra mussels from various sampling sites for ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{82}Se, ^{111}Cd, ^{118}Sn, and ^{208}Pb (r^2 = 0.71–0.99). Performance for ^{52}Cr, ^{63}Cu, ^{66}Zn, ^{68}Zn, and ^{112}Cd was moderate (r^2 < 0.20). In quagga mussels, approximately 73 to 94% of the variability in concentrations of ^{82}Se, ^{111}Cd, ^{112}Cd, and ^{208}Pb was explained by the model (r^2 = 0.73–0.94), followed by ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{60}Ni, and ^{63}Cu (r^2 = 0.48–0.61). Additionally, in both zebra and quagga mussels, average modelled concentrations were within approximately one order of magnitude of the measured values. In particular, in zebra mussels, estimations of ^{60}Ni and ^{82}Se concentrations were equal to 51 and 76% of the measurements, respectively. Higher deviations were observed for ^{52}Cr, ^{59}Co, ^{55}Mn, ^{56}Fe, ^{111}Cd, ^{63}Cu, and ^{112}Cd (underestimation), and ^{66}Zn, ^{68}Zn, ^{208}Pb, and ^{118}Sn (overestimation). For quagga mussels, modelled concentrations of ^{66}Zn and ^{68}Zn differed approximately 14% from the measured levels. Differences between predictions and measurements were higher for other metals. Environ. Toxicol. Chem. 2011;30:2825–2830.

Keywords — Bivalve Modeling Bioconcentration Factor River Rhine River Meuse

2.1. Introduction

Because of its widespread distribution and high efficiency in filtering particulate matter, the zebra mussel (*Dreissena polymorpha*) has been used in numerous monitoring programs (Kraak et al., 1991; Cope et al., 1999). Biomonitoring studies showed high metal concentrations in mussels from the rivers Rhine and Meuse (Kraak et al., 1991). Recently, attention has been drawn to the displacement of the zebra mussel by the quagga mussel (*Dreissena rostriformis bugensis*) (Zhulidov et al., 2010). Quagga mussels are more tolerant to unfavorable conditions, such as bad water quality or low availability of food, and metal pollution (Stoeckmann, 2003). Understanding metal bioaccumulation in these two species may provide insight into differences in tolerance to metal exposure. This may be one of the driving forces for the ongoing changes in the population structure of dreissenid mussels. This information is also useful in assessing potential effects on their predators, such as benthivorous fish and diving ducks.

With recognized difficulties in assessing metal bioaccumulation and toxicity, kinetic-based models have been recommended as a potential method for predicting metal bioaccumulation (Fairbrother et al., 2007). The models also enable distinguishing accumulation from dissolved and dietary sources. This distinction is important because exposure type determines internal distribution and eventually toxicity (Borgmann and Norwood, 1997). In mechanistic bioaccumulation models for organic chemicals, absorption, assimilation, and elimination rate constants are usually related to substance-specific properties, such as the octanol–water partition coefficient, and physiological features such as size (Hendriks et al., 2001). The advantage of these models lies in the potential for extrapolation to a wide range of pollutants, species, and conditions without case-specific calibration (Veltman et al., 2008). Yet, similar models for metals are rare because of the complex chemistry of metals in the environment (Veltman et al., 2008; Rainbow, 2002; Luoma and Rainbow, 2005). So far, parameters in metal models, that is, the physiological rate constants, should be experimentally measured on a case-by-case basis. Relating these constants to metal-specific properties increases

extrapolation potential for a number of metals. Recent studies indicate possibilities of integrating the metal-specific covalent index into modeling metal bioaccumulation. In particular, significant relationships were found between the covalent index and metal bioaccumulation in mollusks (van Kolck et al., 2008). In addition, variability in metal absorption rate constants between mussels and other species was shown to be primarily a function of the filtration rate (Veltman et al., 2008). The integration of the species-specific filtration rate as well as other physiological processes may provide a better understanding of the differences in the sensitivities of the zebra and quagga mussels to metal exposure.

In the present study, we aimed to integrate the covalent index and the size-based filtration rate into a kinetic model simulating metal bioaccumulation in zebra and quagga mussels in the rivers Rhine and Meuse. The developed model was then validated by using data from field measurements in these rivers.

2.2. Methods

2.2.1. Specification and parameterization of the model

Trace metals can be accumulated in mussels from the dissolved phase and particulate matters or phytoplankton (Wang et al., 1996; Klerks and Fraleigh, 1997; Roditi et al., 2000). In the present study, phytoplankton was considered the main source of food for mussels. Metal concentrations in mussels C_m (mg/g dry wt) were regarded to be determined by uptake via water (the first factor) and food (the second factor) and by losses through elimination and growth dilution (the last factor) (Eqn. 2.1). These factors are specified later.

$$\frac{dC_m}{dt} = (p \times FR \times C_w) + (IR \times AE \times C_f) - (k_{ew} + k_{ef} + g) \times C_m \quad (2.1)$$

where p is the absorption efficiency; FR is the filtration rate; C_w is the dissolved metal concentration; IR is the ingestion rate; AE is the assimilation efficiency; C_f is the metal concentration in food; k_{ew} is the elimination rate via water; k_{ef} is the elimination rate via food; and g is the growth rate. A full list of all parameters included in the model is provided in Table S2.1, Supplementary information.

Uptake of metals from the dissolved phase

Metal uptake from water is a function of the dissolved metal concentration C_w (mg/L) and the absorption rate k_u (L/g dry wt/d). The absorption rate was considered metal- and species-specific (Borgmann and Norwood, 1997). It depends on biological factors, such as the filtration rate FR (L/g dry wt/d), and the metal-specific absorption efficiency p (%) (Roditi and Fisher, 1999; Thomann, 1981).

At low food levels, the filtration rate is generally independent of food concentration (Fanslow et al., 1995). After reaching certain levels, the rate will decrease as a function of food availability (Fanslow et al., 1995; Sprung and Rose, 1988). Therefore, the filtration rate was considered to be a function of food concentration F (in phytoplankton biomass, g/L), the maximum filtration rate FR_{max} (L/g dry wt/d), and the saturation constant K_m (g/L) (Eqn. 2.2). The saturation constant 0.04 g/L was derived from the value of 20 mg C/L determined by Descy et al. (2003) by using the conversion factor of 2 between organic carbon content and phytoplankton biomass suggested by Roditi et al. (1996). The filtration rate is mainly measured per individual (e.g., L/mussel/h) and depends on the mussel size, or dry weight, according to the power function $a.W^b$ with various values of a and b reported (Kryger and Riisgard, 1988; Lei et al., 1996). For zebra mussels, the maximum individual-based filtration

rate FR_m (L/mussel/h) and dry weight W (g) were taken from the widely applied relationships found by Kryger and Riisgard (1988) (Eqns. 2.3 and 2.4). The filtration rate found by Kryger and Riisgard (1988) was high because of the optimal experimental conditions (MacIsaac et al., 1992) and therefore considered maximum in the present study. For quagga mussels, the allometric equations from the study by Baldwin et al. (2002) were used to determine the FR_m (L/mussel/h) and dry weight W (g) (Eqns. 2.5 and 2.6). These individual-based filtration rates were transferred into the mass-specific form FR_{max} (L/g dry wt/d) applied in the model. The pseudo feces production, which may act as a mechanism to clear excess particles or to reject some particle types, was excluded in the current model (Sprung and Rose, 1988) (see *Discussion* section).

$$\mathrm{FR} = \frac{\mathrm{FR}_{\max} \times K_{\mathrm{m}}}{K_{\mathrm{m}} + F} \tag{2.2}$$

$$\mathrm{FR}_{\mathrm{m}} = 6.82 \times W^{0.88} \tag{2.3}$$

$$W = 1.54 \times 10^{-5} \times \mathrm{SL}^{2.42} \tag{2.4}$$

$$\mathrm{FR}_{\mathrm{m}} = 0.7866 \times W^{0.6266} \tag{2.5}$$

$$W = 0.0209 \times \mathrm{SL}^{2.53} \tag{2.6}$$

According to Wang and Fisher (1997), the metal absorption efficiency is independent of the filtration rate among mussels in different size classes. Available data on this efficiency for mussels, especially the quagga mussel, are limited. We therefore used the relationship between the metal absorption efficiency p and the covalent index ($X_m^2 r$) developed by Veltman et al. (2008) (Eqn. 2.7).

$$\log\left[\frac{p}{1-p}\right] = 0.57 \times \left[X_{\mathrm{m}}^2 r\right] - 4.37 \tag{2.7}$$

Uptake of metals from food

Metal uptake from food by mussels is a function of the ingestion rate IR (g/g dry wt/d), the metal assimilation efficiency AE (%), and the metal concentration in ingested food C_f (mg/g) (Thomann, 1981). All particles filtered by mussels were assumed to be ingested with greater than 90% retention efficiencies for different-typed particles (Roditi et al., 1996; Lei et al., 1996; Jorgensen et al., 1984) The ingestion rate (g/g dry wt/d) therefore equaled the amount of food (g/L) contained in the filtered water (L/g dry wt/d) (Eqn. 2.8).

$$\mathrm{IR} = \mathrm{FR} \times F \tag{2.8}$$

The assimilation efficiency is the percentage of ingested metals crossing gut lining. No statistically significant relationship was found between this parameter and the covalent index based on data from the study by Roditi et al. (2000). Therefore, available data collected from previous studies were applied directly to the model for Cd, Cr, Se, Co, Zn, and Pb (Table S2.2, Supplementary information).

Metal concentrations in phytoplankton C_f (mg/g) were calculated from bioconcentration factors (BCF) of phytoplankton BCF_p (L/kg) and dissolved metal concentrations C_w (mg/L) (Eqn. 2.9). The BCF_p is metal-specific and dependent on exposure concentrations (Hendriks and Heikens, 2001). However, the dependence on exposure concentrations was not included in modeling BCF_p to simplify the extrapolation for particular environmental conditions. To reduce uncertainties from this simplification, BCF_p data were included only if the dissolved

metal concentrations did not differ considerably from measurements in the rivers Rhine and Meuse (Table S2.3, Supplementary information). Collected BCF_p values were found not to be significantly correlated to the covalent index and, consequently, directly applied to the model.

$$C_f = BCF_p \times C_w \tag{2.9}$$

Elimination

Metals may be lost via water and food with elimination rates k_{ew} and k_{ef} (1/d), respectively (Roditi et al., 2000; Roditi and Fisher, 1999). Elimination rates are inversely proportional to species weight by a factor of (–0.25) (Hendriks and Heikens, 2001). Weight-corrected elimination rates are metal-specific, but studies relating these rates to the mussel size are limited (Veltman et al., 2008). Therefore, in the present study, elimination rates reported by Roditi et al. (2000) were considered weight-corrected for mussels with the standardized dry weight. This standardized dry weight was assumed to correspond to 20-mm shell length. The weight-corrected elimination rates were related to the covalent index (Table S2.4 and Fig. S2.1, Supplementary information). The elimination rates were therefore expressed as follows:

$$k_{ew} = 10^{-1.93} \times \left[X_m^2 r\right]^{0.91} \times \left(\frac{W}{W_s}\right)^{-0.25} \tag{2.10}$$

$$k_{ef} = 10^{-2.24} \times \left[X_m^2 r\right]^{1.44} \times \left(\frac{W}{W_s}\right)^{-0.25} \tag{2.11}$$

where W_s denotes the standardized dry weight.

Tissue concentrations also may decrease by growth dilution, which is proportional to the growth rate g (1/d). The growth rate depends on, for example, initial size, temperature, and food availability (Juhel et al., 2006; Jantz and Neumann, 1998). At the relatively constant environmental conditions, it was assumed that the initial mussel size is the decisive factor, determining the growth rate. For zebra mussels, the relationship between the shell length added per day (SLA) and the initial shell length (SL; mm) developed by Stoeckmann and Garton (1997) was used because of the wide range of size classes studied (Eqn. 2.12). The mass-based growth rate applied in the model was derived from this increase in shell length by using Equation 2.4 relating dry weight to shell length. According to Baldwin et al. (2002), the growth rate of quagga mussels is 4 to 19 times higher than that of zebra mussels. The difference between their growth rates increases with a decrease in food availability. The chlorophyll a concentrations measured in the rivers Rhine and Meuse were similar to the highest levels studied by Baldwin et al. (2002). As a result, a fourfold higher growth rate compared with that of the zebra mussel was assumed for the quagga mussel.

$$SLA = 0.0795 - 0.00347 \times SL \tag{2.12}$$

2.2.2. Sampling and chemical analysis

Mussel and water samples

Zebra and quagga mussels were collected from groyne stones at Lexkesveer in the River Rhine and at Middelaar in the River Meuse in April 2010. These sites were selected based on available evidence on the co-existence of the two species. At each site, river water samples were taken and filtered using a Whatman GFC Glass Microfiber Filter of 47 mm (cat. 1822-047). Filters were then dried for 24 h at 60 ^{o}C to determine the dry weight of suspended solids.

Sample preparation and analysis

In total, 424 and 688 individual mussels collected from the rivers Rhine and Meuse, respectively, were grouped into three size classes: small (< 15 mm), medium (15–22 mm), and large (≥ 22 mm). The fresh parts were separated and dried at 60 °C for 48 h. The dried fresh fraction was then digested with a mixture of HNO_3 65% and H_2O_2 in the Milestone Ethos-D microwave. For each sample, 0.2 g of the dried fresh weight was digested by 4 mL of HNO_3 and 0.5 mL of H_2O_2. Small- and medium-sized mussels showed higher abundance than larger ones. Therefore, for small- and medium-sized classes, two or three samples were measured to increase the representativeness of the examined samples. The cooled digests were made up to exactly 100 mL with high-quality deionized water. A similar procedure was performed for blank samples for corrections to determine metal concentrations in experimental samples. Diluted digests and filtered water samples were analyzed for ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{68}Zn, ^{82}Se, ^{111}Cd, ^{112}Cd, ^{118}Sn, and ^{208}Pb by inductively coupled plasma-mass spectroscopy.

2.2.3. Model validation

The model was validated by combining the measurement data in the present study and monitoring data from the Netherlands Monitoring Waterstaatkundige Toestand des Lands. Monitoring data measured at upstream monitoring stations Lobith and Belfeld in 2008 and 2009 were used for the sampling sites at Lexkesveer and Middelaar, respectively. Particularly, the measurements of suspended solids together with monitoring data about percentage of organic carbon in suspended matters (Table S2.5, Supplementary information) were used to calculate concentrations of organic carbon. Phytoplankton biomass was then derived from the organic carbon content using the conversion factor of 2 as described previously. Together with measurements of mussel shell length (Table S2.6, Supplementary information) and dissolved metal concentrations (Table S2.7, Supplementary information), the data on the phytoplankton biomass was used to determine modeled metal concentrations in mussel tissues. These results were compared with measured values for corresponding size classes (Table S2.8, Supplementary information). The explanatory power of the model was assessed by the coefficient of determination (r^2) between modeled and measured tissue concentrations for each metal. Data on BCF_p and assimilation efficiency were available for ^{52}Cr, ^{59}Co, ^{66}Zn, ^{68}Zn, ^{82}Se, ^{111}Cd, ^{112}Cd, and ^{208}Pb, so both the dissolved phase and food were taken into validation for these metals. For ^{55}Mn, ^{56}Fe, ^{60}Ni, ^{63}Cu, and ^{118}Sn, only water was included in model validation, because no available or derived data of these parameters were found. The relative contribution from food U_f (%) was calculated as the percentage of the uptake from food in total uptake (Eqn. 2.13).

$$U_f = \frac{IR \times AE \times C_f}{p \times FR \times C_w + IR \times AE \times C_f} \times 100\% \quad (2.13)$$

2.3. Results

2.3.1. Validation results

Approximately 71 to 99% of the variability in tissue concentrations of ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{82}Se, ^{111}Cd, ^{118}Sn, and ^{208}Pb in zebra mussels at different sampling sites and in various size classes was explained by the model (r^2 = 0.71–0.99; Fig. S2.2 and Table S2.9, Supplementary information). The explained variance in tissue concentrations was lower for ^{52}Cr, ^{63}Cu, ^{66}Zn, ^{68}Zn, and ^{112}Cd (r^2 < 0.20). For different-sized quagga mussels taken from

various sites, the best performance of the model was noted for ^{82}Se, ^{111}Cd, ^{112}Cd, and ^{208}Pb (r^2 = 0.73–0.94), followed by ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{60}Ni, and ^{63}Cu (r^2 = 0.48–0.61; Fig. S2.3 and Table S2.9, Supplementary information). In contrast, only 4 to 25% of the variability in tissue concentrations of ^{59}Co, ^{66}Zn, ^{68}Zn, and ^{118}Sn was explained by the model.

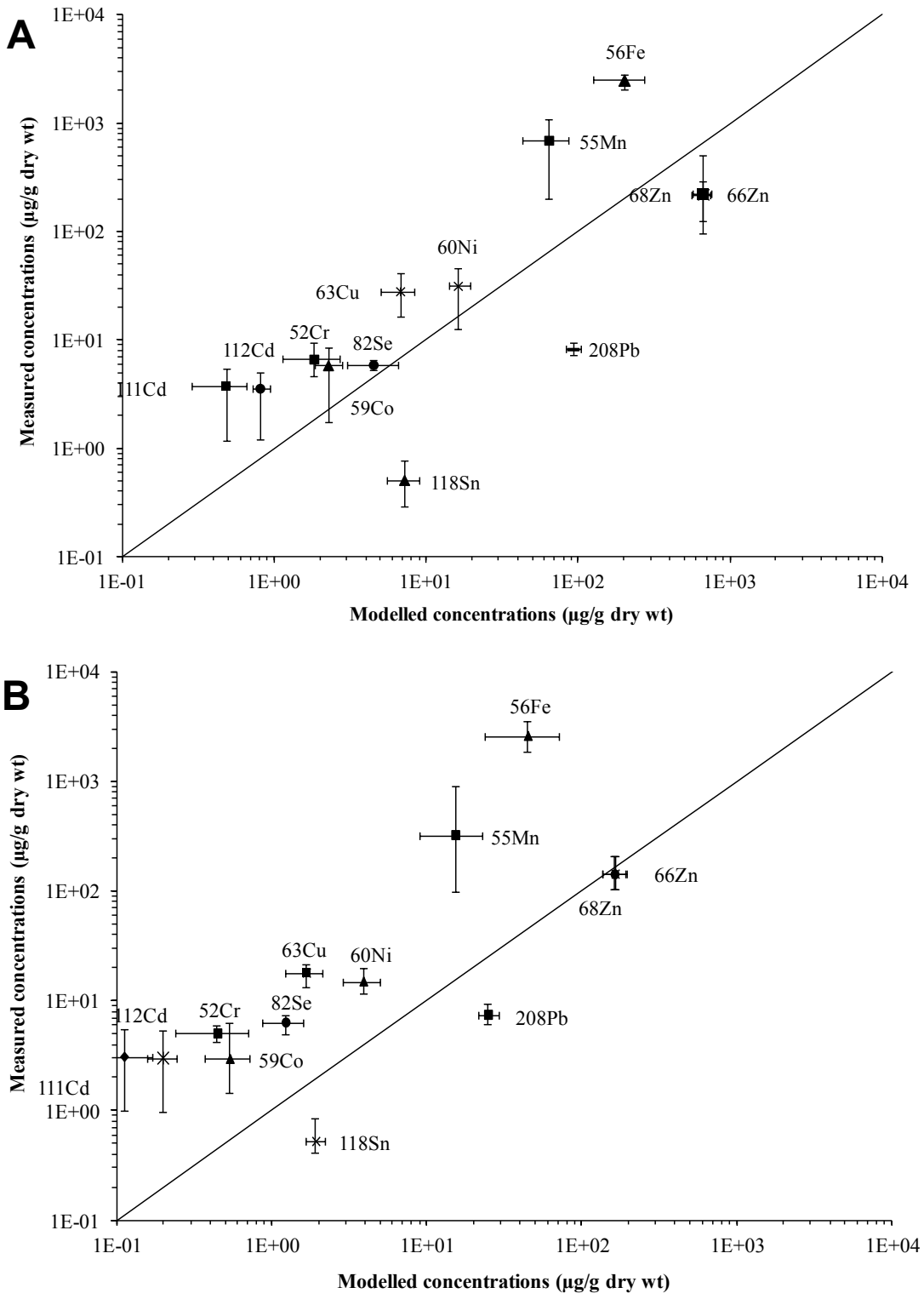

Figure 2.1. Comparison of modeled and measured metal concentrations in zebra mussels (A) and in quagga mussels (B) from the rivers Rhine and Meuse, the Netherlands

In general, average modeled concentrations for both the zebra and quagga mussels were below measured levels, except for ^{66}Zn, ^{68}Zn, ^{208}Pb, and ^{118}Sn, by approximately one order of magnitude (Fig. 2.1). In zebra mussels, estimations for ^{60}Ni and ^{82}Se concentrations agreed most, equaling 51 and 76% of the field measurements, respectively (Fig. 2.1A). Deviations between predictions and measurements were higher for other metals. Concentrations of ^{52}Cr, ^{59}Co, ^{55}Mn, ^{56}Fe, ^{111}Cd, ^{63}Cu, and ^{112}Cd were underestimated, whereas those of ^{66}Zn, ^{68}Zn, ^{208}Pb, and ^{118}Sn were overestimated. For quagga mussels, modeled concentrations of ^{66}Zn and ^{68}Zn differed from the measured levels by approximately 14% (Fig. 2.1B). Higher differences were found between predictions and measurements for other metals.

2.3.2. Metal bioaccumulation in mussels

In both rivers Rhine and Meuse, highly significant relationships were found between metal concentrations in zebra mussels and in quagga mussels ($p < 0.0001$; Table 2.1). Metal concentrations in the mussels in the river Rhine were significantly lower than those in the river Meuse ($p < 0.0001$). In addition, the relative contribution from the two uptake pathways was metal-specific, with a dominant fraction from food for ^{66}Zn and ^{68}Zn and from water for ^{52}Cr, ^{82}Se, ^{111}Cd, ^{112}Cd, and ^{208}Pb (Table S2.10, Supplementary information).

Bioconcentration factors and bioaccumulation factors were calculated as the ratio between metal concentrations in organisms, without and with uptake from food, respectively, versus metal concentrations in water. In both species, significant relationships were found between the BCF and bioaccumulation factors values ($p < 0.0001$; Table S2.10, Supplementary information). Bioconcentration levels were highest for ^{82}Se and ^{208}Pb and lowest for ^{95}Cr in both species. The BCF and bioaccumulation factors values of zebra mussels were significantly higher than those of quagga mussels ($p < 0.0001$).

2.4. Discussion

2.4.1. Metal bioaccumulation in zebra and quagga mussels

Metal concentrations in mussels from the river Rhine were generally lower than those in the river Meuse. Similar results were found in a previous study by Hendriks et al. (1998) for Cd and Zn, but not for the other metals (Table 2.1). This change may be attributable to a more significant improvement in water quality in the river Rhine. Metal concentrations in the zebra mussels taken from the rivers Rhine and Meuse were in the range reported for the Lawrence River (Kwan et al., 2003), except for ^{55}Mn and ^{208}Pb, with higher levels found in the present study (Table 2.1). The level of bioaccumulation from food modeled in the present study was lower than that derived by Roditi et al. (2000). This may be attributed to differences in food items included. These authors modeled bioaccumulation from total particulate metal concentrations (labile and refractory fraction) while we integrated metal uptake from phytoplankton.

The relative importance of uptake from the dissolved phase and from food, as reported in the literature, is inconclusive. DeForest et al. (2007) found inverse relations between the BCF and bioaccumulation factors and water concentrations, indicating a complex relationship between absorption and ingestion with metal levels in water and food. The metal-specific relative contribution of the two sources to tissue accumulation found in this study is consistent with results from some other studies (Wang et al., 1996; Klerks and Fraleigh, 1997; Roditi et al., 2000). The present study confirmed the findings by Mersch et al. (1993) that cadmium concentrations in zebra mussels were mainly determined by exposure to the aqueous phase.

Table 2.1. Average metal concentrations in zebra (*Dreissena polymorpha*) and quagga (*Dreissena rostriformis bugensis*) mussels in the rivers Rhine and Meuse (average ± standard deviation; mg/g dry wt) ([a]Secondary data from Hendriks et al. (1998))

Metals	Zebra mussels					Quagga mussels	
	Rhine	Meuse	Rhine[a]	Meuse[a]	Lawrence	Rhine	Meuse
^{52}Cr	4.60	7.68 ± 2.60	5.00	2.73	0.46-9.45	4.98 ± 0.84	5.10 ± 0.55
^{59}Co	1.76	7.88 ± 0.77				1.68 ± 0.21	4.23 ± 1.81
^{66}Zn	124.41	269.52 ± 30.99	241.67	418.18	129-340	114.78 ± 10.22	170.82 ± 32.70
^{68}Zn	123.76	269.32 ± 20.48				114.30 ± 10.29	170.06 ± 32.98
^{82}Se	5.26	6.23 ± 0.46			4.05-7.4	5.69 ± 0.69	6.99 ± 0.34
^{111}Cd	1.18	5.06 ± 0.45				1.12 ± 0.12	5.04 ± 0.43
^{112}Cd	1.22	4.76 ± 0.42	1.33	3.86	1.8-7.43	1.11 ± 0.13	4.90 ± 0.41
^{208}Pb	7.23	8.72 ± 1.15	3.92	2.91	0.31-1.78	6.75 ± 0.59	8.16 ± 1.18
^{55}Mn	200.89	947.33 ± 214.21	158.33	81.82	35-96	165.87 ± 68.64	482.40 ± 379.90
^{56}Fe	2,077.69	2,721.77 ± 88.95				2,107.57 ± 241.09	3,091.31 ± 384.24
^{60}Ni	12.68	41.01 ± 7.17	20.83	10.00	8.84-55.2	11.95 ± 0.19	18.08 ± 2.32
^{63}Cu	16.59	33.93 ± 10.94	22.50	17.27	14.2-35.9	17.91 ± 4.15	17.92 ± 2.78
^{118}Sn	0.76	0.38 ± 0.14				0.62 ± 0.20	0.42 ± 0.01
Study	Present study	Present study	Hendriks et al. (1998)	Hendriks et al. (1998)	Kwan et al. (2003)	Present study	Present study

2.4.2. Uncertainties

Some assumptions included in our model resulted in uncertainties. Important sources of uncertainties are related to food items, filtration rate, the BCF of phytoplankton, and kinetic rate constants. These factors are discussed in the following sections.

Food items

Mussels can consume a variety of food items, ranging from phytoplankton to bacteria, detritus, and small zooplankton. According to Bruner et al. (1994), uptake via algae is more important at the same exposure concentrations of algae and suspended particles. However, the contribution of algae and suspended sediment to metal bioaccumulation is determined by both the metal concentrations in these food items as well as their availability. Zebra mussels are able to filter particles as small as 0.7 mm, and the maximum retention efficiency is obtained at sizes larger than 5 mm (Sprung and Rose, 1988). In the Lower Rhine, small centric diatoms with sizes that are effectively cleared by mussels were the major component of the algal community (LWA, 1989–1993). Moreover, shell growth rates were strongly related to the chlorophyll a concentration, indicating phytoplankton as an important food source (Jantz and Neumann, 1998). In contrast, the mussel growth was found not to be correlated to the dissolved organic matter, total organic content, or biomass of seston of the river Rhine water (Admiraal et al., 1990). Similarly, no correlations were found between biomass of bacterial populations and chlorophyll a concentrations (LWA, 1988). These results may indicate that the fraction of bacteria in foods for the zebra mussel is insignificant (Jantz and Neumann, 1998).

Filtration rate

Filtration rate is an important physiological parameter, determining uptake from both water and food. Filtration is related to food selectivity and pseudofeces production, which were excluded in the present model. Similar filtration rates were found for different types of food as well as for various phytoplankton taxa in different sizes, suggesting that the influence of particle selection is negligible (Sprung and Rose, 1988; Roditi et al., 1996; Horgan and Mills, 1997). The exclusion of food selectivity in terms of size and types is therefore expected not to cause large uncertainties. Another factor influencing the filtration rate is pseudofeces production suggested to clear excess particles or to reject some particle types (Sprung and Rose, 1988). Clearing excess particles only occurs when food concentrations exceed a certain level, the so-called incipient limiting concentration. Different values of this concentration have been reported, but all were substantially higher than food levels measured in the rivers Rhine and Meuse (Sprung and Rose, 1988; Lei et al., 1996). Although rejection of particles by pseudofeces production is known to occur even at low food concentrations (Sprung and Rose, 1988), almost complete retention efficiency was reported for algae, the main food source in the present study (Lei et al., 1996; Jorgensen et al., 1984). As a result, uncertainties from the exclusion of the pseudofeces production in our model were assumed to be insignificant.

BCF of phytoplankton

The bioconcentration factor of phytoplankton, BCF_p, determines metal concentrations in food. But its value may decrease with the exposure concentrations as reported by Hendriks and Heikens (2001). This was not integrated in our approach to keep the model simple. This simplification was justified by choosing BCF_p values obtained at similar levels of metal exposure as in our sampling sites.

Kinetic rate constants

In the present model, metal absorption and assimilation efficiencies as well as elimination and growth rates were considered independent of environmental conditions, particularly metal and food exposure concentrations. This assumption may not be completely justified in all cases. In addition, the physiological processes may be influenced by metal-specific biological regulations by mussels. For example, essential metals, such as Cu and Zn, can be taken up at high amounts, and their tissue concentrations can be regulated biologically (Secor et al., 1993). However, measured concentrations of these essential metals did not deviate more from modeled levels than noted for other metals (Fig. 2.1). In addition, in the present study, the covalent index was used to model metal absorption efficiency and elimination rates. Yet, metal bioaccumulation is also related to other chemical properties, allowing further improvement of the estimations. For example, atomic weight was shown to considerably contribute to variations in metal tissue concentrations (Hendriks et al., 1998).

2.4.3. Recommendations

Validation in the present study showed the good potential of the model in estimating metal concentrations in zebra and quagga mussels. As noted by Veltman et al. (2008), integration of the covalent index thus may significantly improve modeling of metal bioaccumulation. By integrating this metal-specific property and the size-based filtration rate, metal bioaccumulation can be predicted for a number of metals without calibration for specific cases. Moreover, the difficulties and limitations in the application of bioaccumulation in metal risk assessment can be overcome because physiological processes influencing metal uptake kinetics can be included.

However, caution should be taken in applying the model, because assumptions that apply to the rivers Rhine and Meuse may not hold in other water systems. The estimation potential of the model can be improved by considering some additional factors, specifically, dependence of physiological rate constants on exposure concentrations, other chemical properties, such as molecular weight, and metal-specific behavioral characteristics of mussels, for example, biological internal regulations and sequestration.

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SUPPLEMENTARY INFORMATION

Table S2.1. Parameters used in the model

Parameters	Label	Unit	Values/equations
Metal concentration in mussels	C_m	µg/g dry wt	
Saturation constant	K_m	g/L	0.04
Shell length	SL	mm	Experimentally measured
Dry weight of the zebra mussel	W	g	$W = 1.54 \times 10^{-5} \times \mathrm{SL}^{2.42}$
Dry weight of the quagga mussel	W	g	$W = 0.0209 \times \mathrm{SL}^{2.53}$
Maximum FR for zebra mussels (individual)	$\mathrm{FR_m}$	L/mussel/h	$\mathrm{FR_m} = 6.82 \times W^{0.88}$
Maximum FR for quagga mussels (individual)	$\mathrm{FR_m}$	L/mussel/h	$\mathrm{FR_m} = 0.7866 \times W^{0.6266}$
Mass-specific maximum FR	$\mathrm{FR_{max}}$	L/g dry wt/d	$\mathrm{FR_{max}} = \frac{\mathrm{FR_m} \times 24}{W}$
Food concentration	F	g/L	F = organic carbon × 2 Organic carbon concentration was determined by the monitoring programme
Filtration rate	FR	L/g dry wt/d	$\mathrm{FR} = \frac{\mathrm{FR_{max}} \times K_m}{K_m + F}$
Absorption efficiency	p	%	$\log\left[\frac{p}{1-p}\right] = 0.57 \times \left[\mathrm{X_m^2} r\right] - 4.37$
Absorption rate	k_u	L/g dry wt/d	$k_u = p \times \mathrm{FR}$
Dissolved metal concentration	C_w	µg/L	Experimentally determined
Ingestion rate	IR	g/g dry wt/d	$\mathrm{IR} = \mathrm{FR} \times F$
Assimilation efficiency	AE	%	Data collected
Bioconcentration factor of phytoplankton	$\mathrm{BCF_p}$	L/kg	Data collected
Metal concentration in food	C_f	mg/L	$C_f = \mathrm{BCF}_p \times C_w$
Standardized dry weight	W_s	g	Corresponding to SL = 20 mm
Elimination rate via water	k_{ew}	1/d	$k_{ew} = 10^{-1.93} \times \left[\mathrm{X_m^2} r\right]^{0.91} \times \left(\frac{W}{W_s}\right)^{-0.25}$

Elimination rate via food	k_{ef}	1/d	$k_{ef} = 10^{-2.24} \times \left[X_m^2 r\right]^{1.44} \times \left(\frac{W}{W_s}\right)^{-0.25}$
Shell length added per day for zebra mussels	SLA	mm	$SLA = 0.0795 - 0.00347 \times SL$
Shell length after one day	SL_t	mm	$SL_t = SL + SLA$
The mass-based growth rate for zebra mussels	g	1/d	$g = \frac{W_t}{W}$
The growth rate for quagga mussels	g	1/d	4 times higher than the growth rate of zebra mussels

Table S2.2. Assimilation efficiency AE (%) of zebra mussels

Metal	**AE**	**References**
Cd	0.227	Roditi et al. (2000)
Cr(III)	0.015	Roditi et al. (2000)
Se(IV)	0.23	Roditi et al. (2000)
Co**	0.23	Reinfelder and Fisher (1994) Reinfelder et al. (1997) Wang and Fisher (1997) Gagnon and Fisher (1997) Wang and Fisher (1996) Griscom et al. (2000) Fisher et al. (1996)
Pb**	0.48	Thomann et al. (1995) Fisher et al. (1996)
Zn**	0.37	Reinfelder and Fisher (1994) Reinfelder et al. (1997) Wang and Fisher (1997) Wang and Fisher (1996) Griscom et al. (2000) Lee and Luoma (1998) Blackmore and Wang (2002) Fisher et al. (1996)

**Average values

Bioconcentration factor of phytoplankton (BCF_p) used to develop the model

The dependence of BCF_p on exposure concentrations was excluded in the model. To reduce uncertainties from this simplification, only findings from studies without significant differences in metal concentrations in the investigated conditions from conditions in the rivers Rhine and Meuse were considered valid. These data were used in the current study. In particular, results from the study by Lewis et al. (2004) were considered invalid since no information about metal concentrations in water was presented. Neither BCF_p values from the studies of Rick and Durselen (1995) nor of Goupta et al. (2001) were integrated because of considerably lower or higher metal exposure concentrations compared to measurements in the rivers Rhine and Meuse. Some BCF_p values, which were obtained in the studies by Wang and Dei (1998) and Kawai et al. (1984) with experimental conditions relatively similar to the Rhine and Meuse in terms of dissolved metal concentrations, were considered valid (See table S2.3). All results by Deniseger et al. (1986) and Muller et al. (1993) were used as the studied metal concentrations were in the range measured in the present study.

Table S2.3. Bioconcentration Factor of phytoplankton BCF_p (L/kg) used to develop the model

Study	Ni	Pb	Zn	Cu	Cd	Mn	Cr	Se	Co
Muller et al. (1993)	7900	8300	9200	13100	10800				
	6800	3600	12800	9700	6800				
	5800	7100	7600	21300	2200				
	6900	1900	3900	16200	5500				
	1400	1700	2800	1300	3000				
	1600	2100	2900	1000	5500				
	2100	5500	4000	900	1500				
	3400		3100	1300					
Deniseger et al. (1986)			52000	32000					
			56000	25000					
			21000	34000					
			20000	24000					
Wang and Dei (1998)			10000						
Kawai et al. (1984)					10900	4200	2500	150	
							3100		
Garnham et al. (1992)									60
Average	4487.5	4314.286	15792.31	14983.33	5775	4200	2800	150	60

Table S2.4. Standardised elimination rates via water and food (1/d) (from study by Roditi et al. (2000))

Element	Ag	Cd	Cr(III)	Cr(VI)	Hg	Se(IV)
Covalent index	4.284	2.713	1.708	0.716	4.080	3.251
Elimination rate via water	0.088	0.011		0.011		0.035
Elimination rate via food	0.07	0.012	0.019		0.05	0.026

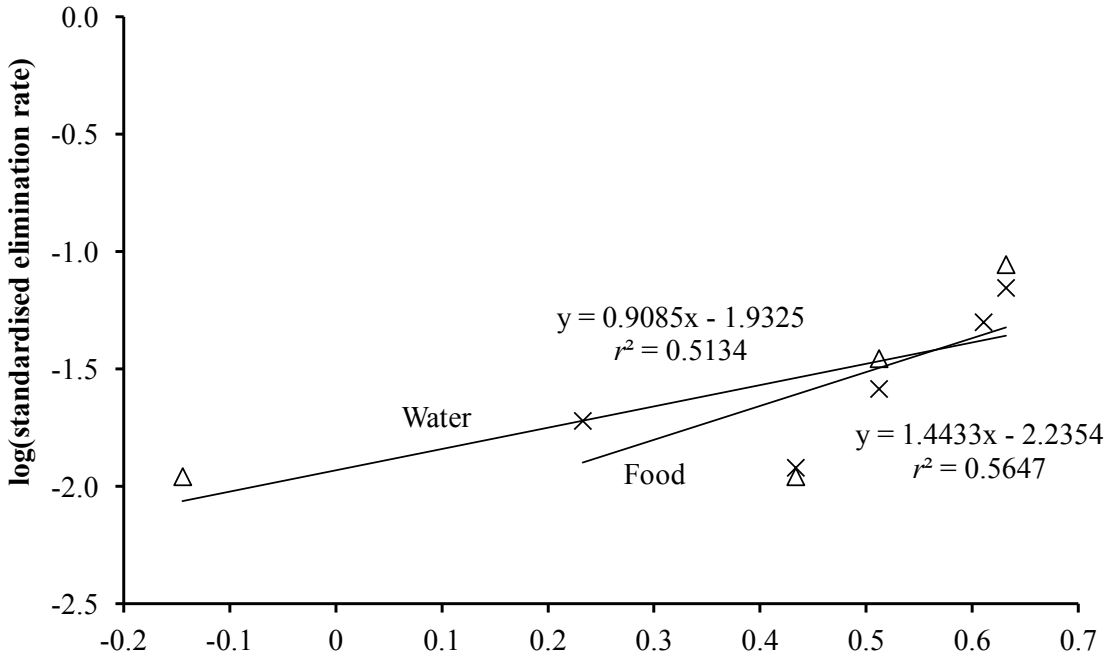

Figure S2.1. Relationships between elimination rates via water and via food versus the covalent index

Table S2.5. Percentage of organic carbon (%) in suspended solids at two monitoring sites Lobith and Belfeld (from water base)

Lobith (Rhine)		Belfeld (Meuse)	
Sampling date	**Percentage of organic carbon (%)**	**Sampling date**	**Percentage of organic carbon (%)**
15-Jan-09	5	14-Jan-08	7.2
29-Jan-09	4.3	10-Mar-08	6.3
12-Feb-09	6.4	6-May-08	9.9
24-Feb-09	4.8	30-Jun-08	7
11-Mar-09	4.6	25-Aug-08	6.4
26-Mar-09	5.3	20-Oct-08	7.3

09-Apr-09	4.7	15-Dec-08	6.5
22-Apr-09	5.2		
06-May-09	5.2		
20-May-09	4.7		
03-Jun-09	4.9		
17-Jun-09	4.8		
01-Jul-09	4.7		
15-Jul-09	4.4		
29-Jul-09	3.9		
12-Aug-09	4.3		
26-Aug-09	4.3		
09-Sep-09	4.3		
23-Sep-09	4.4		
07-Oct-09	4.7		
20-Oct-09	4.7		
04-Nov-09	4.7		
Average	4.74	Average	7.23

Table S2.6. Average shell length (mm) of the zebra and quagga mussels from the rivers Rhine and Meuse

Species	**Size class**	**Rhine (Lexkesveer)**	**Meuse (Middelaar)**
Zebra mussel	Small (S; < 15 mm)	14	9.01
	Medium (M; 15–22 mm)	18.44	16.25
	Large (L; ≥ 22 mm)	-	-
Quagga mussel	Small (S; < 15 mm)	12.07	8.20
	Medium (M; 15–22 mm)	17.21	17.91
	Large (L; ≥ 22 mm)	23.35	22.17

Table S2.7. Total suspended solids (TSS; g/mL) and dissolved metal concentrations measured at two sampling sites (average ± standard deviation; µg/L)

	Rhine (Lexkesveer)	**Meuse (Middelaar)**
TSS	$8 \cdot 10^{-6}$	$6.4 \cdot 10^{-6}$
^{52}Cr	1.226	0.829 (± 0.110)
^{55}Mn	46.44	38.615 (± 0.841)
^{56}Fe	41.83	100.72 (± 5.346)
^{59}Co	0.389	0.637 (± 0.001)
^{60}Ni	2.987	4.552 (± 0.054)
^{63}Cu	3.062	2.773 (± 0.040)
^{66}Zn	28.76	30.365 (± 12.155)
^{68}Zn	28.75	29.870 (± 10.889)
^{82}Se	0.872	0.567 (± 0.030)
^{111}Cd	0.030	0.070 (± 0.019)
^{112}Cd	0.078	0.100 (± 0.008)
^{118}Sn	0.761	0.649 (± 0.116)
^{208}Pb	0.373	0.468 (± 0.049)

Table S2.8. Measured metal concentrations in the zebra and quagga mussels from the rivers Rhine and Meuse (µg/g dry wt)

Site	Species	Size	^{52}Cr	^{55}Mn	^{56}Fe	^{59}Co	^{60}Ni	^{63}Cu	^{66}Zn	^{68}Zn	^{82}Se	^{111}Cd	^{112}Cd	^{118}Sn	^{208}Pb
Rhine	Zebra mussels	M	4.597	200.892	2077.685	1.759	12.679	16.589	124.407	123.759	5.259	1.177	1.220	0.761	7.229
	Quagga mussel	S	4.426	269.991	2396.440	1.828	10.844	17.956	112.298	111.499	5.794	1.117	1.155	0.417	7.485
		S	7.394	201.545	2292.389	1.828	13.059	19.673	121.556	120.906	5.999	1.208	1.178	1.278	7.018
		M	3.979	135.981	2059.926	1.594	10.668	16.135	114.646	113.909	6.158	1.173	1.156	0.735	6.271
		M	5.454	170.552	1821.783	1.748	13.859	18.826	136.561	136.858	6.546	1.294	1.240	0.340	6.605
		M	4.880	183.238	2465.870	1.942	11.906	29.614	120.067	119.767	6.037	1.189	1.213	0.410	7.827
		L	3.644	101.618	1710.600	1.411	10.708	13.345	91.564	90.581	4.477	0.860	0.868	1.074	6.213
		L	4.708	85.661	1925.110	1.438	12.448	13.025	109.907	109.506	5.052	1.021	0.985	0.206	6.030
		L	4.453	108.434	1951.608	1.483	12.128	13.778	109.503	109.502	5.214	1.091	1.023	0.276	6.067
Meuse	Zebra mussel	S	5.847	1098.797	2658.876	7.335	35.945	26.192	247.669	247.768	6.556	5.382	5.058	0.288	7.909
		M	9.518	795.866	2784.673	8.425	46.081	41.662	291.364	290.877	5.902	4.741	4.466	0.481	9.540
	Quagga mussel	S	6.240	983.601	3380.775	6.497	18.603	19.134	183.918	183.423	5.958	4.520	4.398	0.462	8.876
		S	5.340	937.927	3637.716	6.270	18.377	22.187	232.600	233.849	8.028	5.234	5.196	0.263	10.041
		S	5.608	830.896	3564.535	6.073	19.101	21.418	206.117	203.116	7.132	5.082	4.906	0.524	9.497
		M	4.013	302.077	2371.010	3.661	18.237	17.041	151.923	151.328	7.209	5.272	5.080	0.403	6.510
		M	5.271	262.895	2667.366	3.318	19.732	18.260	159.479	160.723	7.587	5.545	5.521	0.409	7.362

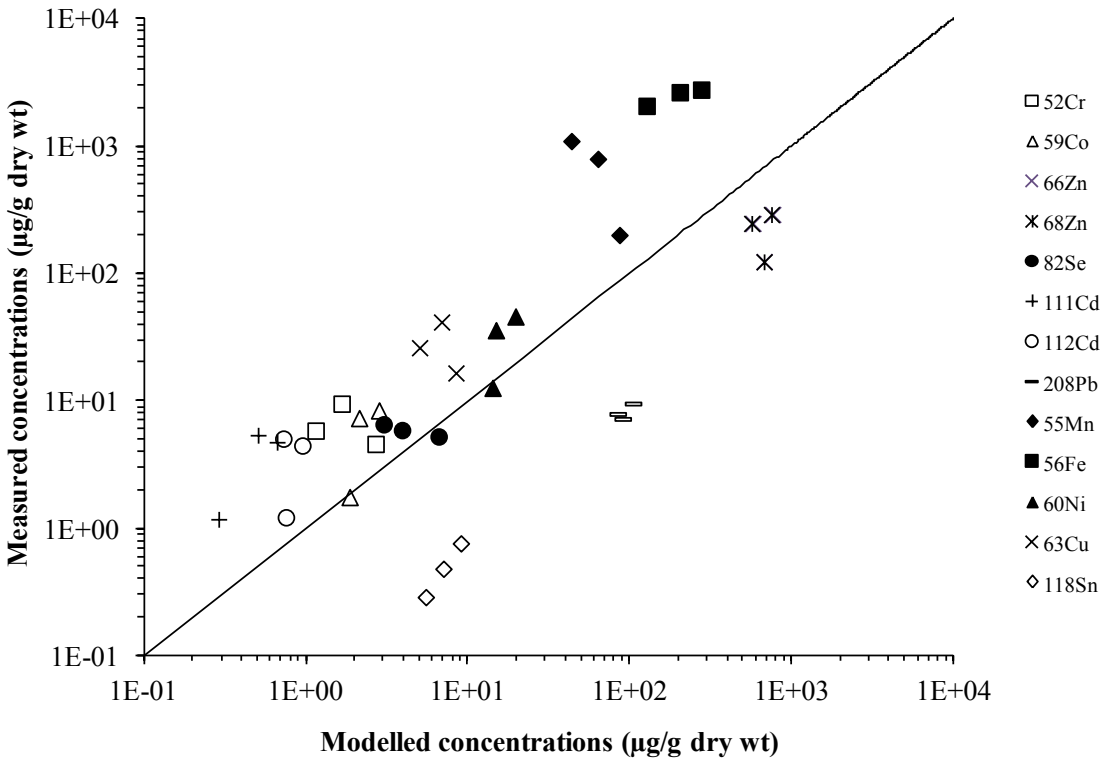

Figure S2.2. Relationships between modelled metal concentrations and measured metal concentrations in zebra mussels

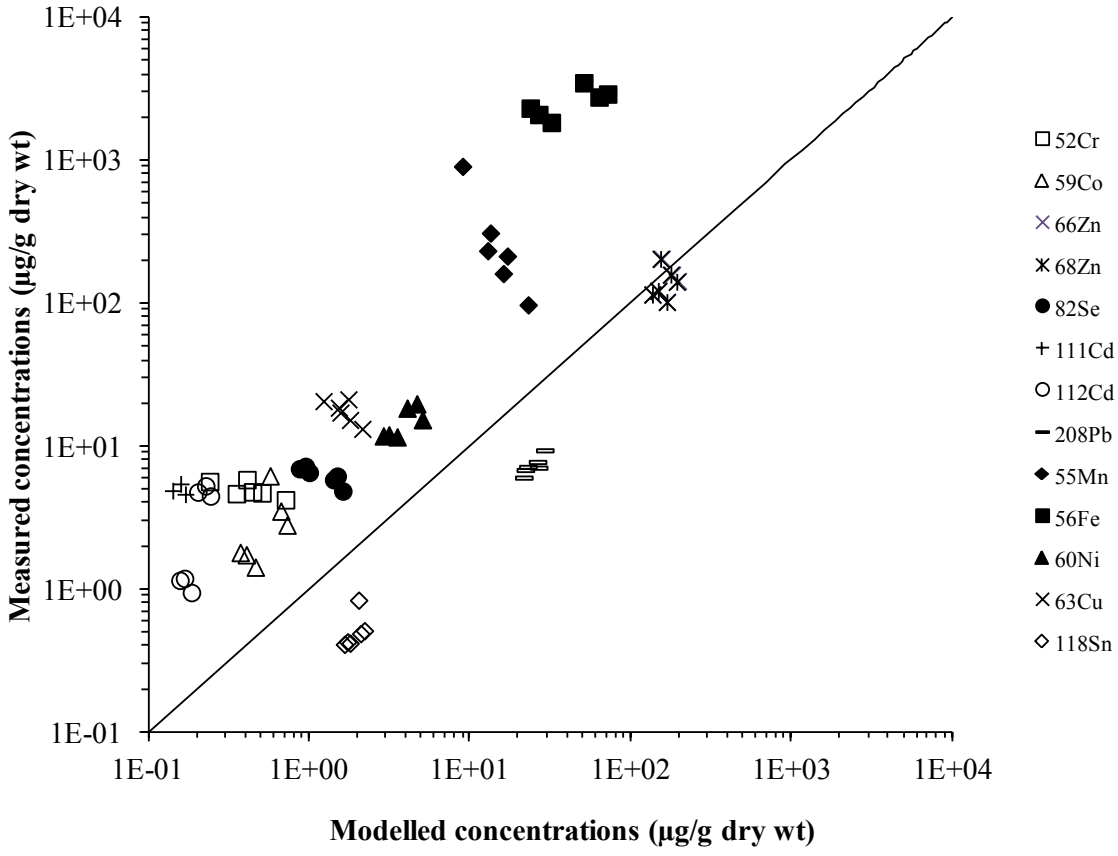

Figure S2.3. Relationships between modelled metal concentrations and measured metal concentrations in quagga mussels

Table S2.9. Relationships between modelled metal concentrations and measured metal concentrations expressed by r^2

r^2	^{52}Cr	^{59}Co	^{66}Zn	^{68}Zn	^{82}Se	^{111}Cd	^{112}Cd	^{208}Pb	^{55}Mn	^{56}Fe	^{60}Ni	^{63}Cu	^{118}Sn
Zebra	0.16	0.71	0.12	0.08	0.93	0.72	0.13	0.82	0.97	0.90	0.72	0.08	0.99
Quagga	0.54	0.25	0.06	0.04	0.81	0.94	0.77	0.73	0.61	0.48	0.56	0.59	0.20

Table S2.10. Modelled metal bio-concentration factors (BCF; L/kg), bio-accumulation factors (BAF; L/kg) of zebra and quagga mussels, and average contribution of uptake from food to the total metal uptake (%)

Metals	BCF			BAF			Average contribution from food (%)
	Zebra mussels	Quagga mussels	Zebra mussels	Zebra mussels	Quagga mussels	Zebra mussels	
^{52}Cr	$1.57 \cdot 10^3$	$3.66 \cdot 10^2$	$3.0 \cdot 10^3$	$1.85 \cdot 10^3$	$4.27 \cdot 10^2$	$1.7 \cdot 10^4$	14.43
^{59}Co	$3.68 \cdot 10^3$	$9.22 \cdot 10^2$		$4.15 \cdot 10^3$	$1.04 \cdot 10^3$		11.20
^{66}Zn	$3.85 \cdot 10^3$	$9.68 \cdot 10^2$		$2.24 \cdot 10^4$	$5.49 \cdot 10^3$		82.33
^{68}Zn	$3.85 \cdot 10^3$	$9.68 \cdot 10^2$		$2.24 \cdot 10^4$	$5.49 \cdot 10^3$		82.33
^{82}Se	$6.46 \cdot 10^3$	$1.66 \cdot 10^3$		$6.55 \cdot 10^3$	$1.68 \cdot 10^3$	$1.6 \cdot 10^4$	1.30
^{111}Cd	$4.99 \cdot 10^3$	$1.27 \cdot 10^3$		$8.75 \cdot 10^3$	$2.20 \cdot 10^3$		42.30
^{112}Cd	$4.99 \cdot 10^3$	$1.27 \cdot 10^3$		$8.75 \cdot 10^3$	$2.20 \cdot 10^3$	$40.0 \cdot 10^4$	42.30
^{208}Pb	$2.12 \cdot 10^5$	$5.81 \cdot 10^4$	$0.15 \cdot 10^3$	$2.14 \cdot 10^5$	$5.86 \cdot 10^4$		0.79
^{55}Mn	$1.53 \cdot 10^3$	$3.54 \cdot 10^2$		$1.53 \cdot 10^3$	$3.54 \cdot 10^2$		
^{56}Fe	$2.58 \cdot 10^3$	$6.32 \cdot 10^2$		$2.58 \cdot 10^3$	$6.32 \cdot 10^2$		
^{60}Ni	$4.09 \cdot 10^3$	$1.03 \cdot 10^3$		$4.09 \cdot 10^3$	$1.03 \cdot 10^3$		
^{63}Cu	$2.33 \cdot 10^3$	$5.67 \cdot 10^2$	$3.4 \cdot 10^3$	$2.33 \cdot 10^3$	$5.67 \cdot 10^2$		
^{118}Sn	$1.03 \cdot 10^4$	$2.70 \cdot 10^3$		$1.03 \cdot 10^4$	$2.70 \cdot 10^3$		
Study	Present study	Present study	Chevreuil et al. (1996)	Present study	Present study	Roditi et al. (2000)	Present study

(Data from other studies are for Cd and Zn in general)

3

Chapter 3

Predicting effects of cations on copper toxicity to lettuce (*Lactuca sativa*) by the Biotic Ligand Model

T.T. Yen Le, Willie J.G.M. Peijnenburg, A. Jan Hendriks, Martina G. Vijver

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Abstract—A biotic ligand model (BLM) was developed to estimate Cu^{2+} toxicity to lettuce (*Lactuca sativa*) in terms of root elongation after 4 d of exposure. Effects of H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity were examined. The addition of these cations resulted in a 50-fold difference in the copper median effective activity ($EC50_{Cu}$). However, these variations could not be interpreted entirely as a function of the concentrations of these cations alone. In particular, only the relationship between $EC50_{Cu}$ and the activity of protons was found to be significant in the whole range of pH examined from 5.0 to 7.0. The addition of K^+, Na^+, Ca^{2+}, and Mg^{2+} at concentrations up to 20 mmol/L resulted in a 16-fold difference in $EC50_{Cu}$ values. This difference was significant, as indicated by nonoverlapping standard deviations of the negative logarithm of $EC50_{Cu}$ ($pEC50_{Cu}$) obtained with (7.37 ± 0.22) and without (6.76 ± 0.22) additions of K^+, Na^+, Ca^{2+}, and Mg^{2+}. The variations were not statistically significantly related to concentrations of these cations; therefore, only protons can be integrated in the BLM predicting Cu^{2+} toxicity to lettuce *Lactuca sativa* with the important parameters: $\log K_{HBL}$ = 6.27, $\log K_{CuBL}$ = 7.40, and $f50_{Cu}$ = 0.36 at pH = 7. The lack of significant relationships between $EC50_{Cu}$ and concentrations of the cations was not in line with the main assumption of the BLM about the competition between cations for binding sites. Environ. Toxicol. Chem. 2012;31:355–359.

Keywords — Biotic ligand model Copper Binding constant Plants Toxicity

3.1. Introduction

The biotic ligand model (BLM), combining metal speciation and interactions of metals at toxic sites, is gaining increased interest (Steenbergen et al., 2005; Lock et al., 2006). It has been incorporated into developing water quality criteria by the U.S. Environmental Protection Agency and proposed for use in European Union risk assessment. The BLM was originally developed to estimate toxicity to aquatic organisms, with the main assumption that metal toxicity results from the binding of free metal ions or other reactive metal species to physiologically active or transport sites at the organism–water interface (Steenbergen et al., 2005; Lock et al., 2006). That is, the concentration of metal–biotic ligand complexes is expected to determine the extent of toxic effect.

Antunes et al. (2006) noted significant difficulties in developing and applying the BLM to plants. They included determination of free ion concentrations in soil solutions and of ligand concentrations, incorporation of nonequilibrium dissociation into the equilibrium BLM, and metal estimation as well as speciation in plant roots. However, recent findings show that application of the BLM to terrestrial organisms is theoretically and empirically possible. In particular, the assumption that the free metal ion is the main reactive form may be met in terrestrial ecosystems; previous experimental findings show that responses of plants and some other soil organisms to metals can be explained by variations in this metal species in the water phase of soil (Spark, 1995). Moreover, toxicity mechanisms of aquatic and terrestrial organisms are assumed to be similar (Steenbergen et al., 2005; Peijnenburg et al., 2007). The influence of solution chemistry on metal bioavailability and toxicity depends on the properties of metals and competing components in the water phase as well as root characteristics (Wu and Hendershot, 2009). In the original BLM developed for fish, the gill was considered to be the biotic ligand. Analogous to the gill, metal binding sites to roots of the apoplasm are biotic ligands in terrestrial studies (Antunes et al., 2006). Recently, BLMs for plants such as barley have been developed (Thakali et al., 2006a,b). These studies indicated that the BLM is a promising method for estimating metal toxicity to terrestrial plants (Lock et al., 2007b).

According to the BLM assumption, cations (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, and H^{+}) are expected to compete with Cu^{2+} for binding sites, thus reducing Cu^{2+} toxicity to organisms (Di Toro et al., 2001). This ameliorative effect has been observed in studies on barley, beans, wheat, and lettuce (Lock et al., 2007a; Maksymiec and Baszynski, 1998; Parker et al., 1998; Cheng and Allen, 2001).

Copper exposure at high concentrations deters plants from accessing water and nutrients, because root elongation and branching are sensitive to Cu (Wong and Bradshaw, 1982; Sheldon and Menzies, 2005). Metal accumulation in roots and in leaves of lettuce has been reported with higher amounts of metals retained in roots and changes in the tendency of accumulation at higher exposure concentrations (Garate et al., 1993). The present study aimed to estimate Cu^{2+} toxicity to lettuce (*Lactuca sativa*) in hydroponic solutions by developing a BLM, taking into account the effects of H^{+}, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}.

3.2. Methods

3.2.1. Test species, effect endpoints, experimental design

Lactuca sativa was selected as the test species because of its hyperaccumulating potential for metals such as Cd and Zn (Garate et al., 1993). It was also recommended by the Organisation for Economic Cooperation and Development as a suitable test organism (OECD, 2006). In the present study, Cu^{2+} toxicity to this species was assessed in terms of root elongation.

The impact of various cations on Cu^{2+} toxicity was systematically assessed by varying concentrations of one cation at a time, whereas concentrations of other cations were kept constant. Different sets of copper toxicity tests were conducted, covering ranges of pH from 5 to 7, and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} concentrations ranging from 0 to 20 mmol/L for each cation (Table S3.1, Supplementary information). At each set, Cu^{2+} activities varied, whereas concentrations of other components in the experimental solutions were the same.

3.2.2. Preparation of test solutions

Steiner solution was used as the test medium (Table S3.2, Supplementary information) (Steiner, 1961). The pH set included the same medium with pH in the range of 5 to 7, whereas for other sets, pH of the media was adjusted to 7 using 3-[N-morpholino] propane sulfonic acid at 0.75 g/L and NaOH (Lock et al., 2007a). The 3-[N-morpholino] propane sulfonic acid buffer was used because it does not form complexes with metals (Kandegedara and Rorabacher, 1999). This buffer was recommended by the U.S. Environmental Protection Agency, because it does not affect the toxicity of effluents and sediment pore waters (US EPA, 1991). Moreover, according to De Schamphelaere et al. (2004), metal toxicity to *Daphnia magna* and *Pseudokirchneriella subcapitata* was not influenced by 3-[N-morpholino] propane sulfonic acid buffering at 0.75 g/L. In the range of 5 to 6, pH adjustment was performed using 2-[Nmorpholino] ethane sulfonic acid buffering at $0.75 \cdot 10^{-3}$ g/L and NaOH. The 2-[N-morpholino] ethane sulfonic acid is a good biological buffer with midrange p*K*a, maximum water solubility, chemical and enzymatic stability, and minimal salt effects (Good et al., 1966).

The free Cu^{2+} activity and pH of the solutions were checked and adjusted daily. The free Cu^{2+} activity was controlled by adding $Cu(NO_3)_2$ to the Steiner nutrient solution, and pH was adjusted by the addition of HNO_3 or KOH. All cations were added as nitrate salts.

3.2.3. Toxicity assays

Before commencing the test, seeds of *Lactuca sativa* were germinated for 4 d at 15 ^{o}C in the Steiner solution during a normal light cycle of 16: 8-h light: dark. During the toxicity tests with the nutrient solutions, four germinated plants were fixed in a parafilm strap that floated on the surface of a glass beaker with the roots immersed in the medium. For each medium, a toxicity test consisting of different treatments (control and various free Cu^{2+} activities) was carried out. The root growth was calculated by comparing root lengths at the first day and after 4 d of exposure, and then used to determine relative root elongation (RRE; %) according to Equation 3.1:

$$\mathrm{RRE} = \frac{\mathrm{RG_s}}{\mathrm{RG_c}} \times 100\% \tag{3.1}$$

where RG_s (mm) is the average root growth of four plants in sample solutions and RG_c (mm) is the average root growth of four plants in the control solution.

3.2.4. Chemical measurements

Free Cu^{2+} activities and pH of the solutions were measured by using hydrogen and copper ion–selective electrodes, respectively (Metrohm Switzerland). The copper ion–selective electrode was calibrated using a set of solutions with pH = 3, $NaNO_3$ 5 mol/L, and $Cu(NO_3)_2$ at the concentration range from 10^{-3} to 10^{-7} mol/L. Total concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were calculated as the total of the concentrations added and the concentrations of the Steiner solution.

3.2.5. Data treatment and statistics

Median effective activity of Cu^{2+} ($EC50_{Cu}$) was determined by fitting a sigmoid curve to the relationships between pCu (i.e., negative of logarithm of free Cu^{2+} activity) and the normalized root growth response using Graphpad Prism software.

3.2.6. Brief mathematical description of the BLM

In this research, the effects of H^+, Na^+, Ca^{2+}, Mg^{2+}, and K^+ on Cu^{2+} toxicity were investigated. These cations can form complexes with biotic ligands at a ratio expressed by the stability constants. At equilibrium, for example, the stability constant for Cu^{2+} binding to biotic ligands K_{CuBL} (L/mol) can be expressed as a function of concentrations of cation–biotic ligand complexes [CuBL] (mol/L) and unoccupied biotic ligand sites [BL] (mol/L)

$$K_{\mathrm{CuBL}} = \frac{[\mathrm{CuBL}]}{\{\mathrm{Cu}^{2+}\} \times [\mathrm{BL}]} \tag{3.2}$$

where $\{Cu^{2+}\}$ is the free Cu^{2+} activity (mol/L).

According to the BLM concept, Cu^{2+} toxicity is determined by the fraction of the total number of biotic ligand sites occupied by Cu (f_{Cu}), with the assumption that the complexation capacity is independent of water quality characteristics (De Schamphelaere and Janssen, 2002).

$$f_{\mathrm{Cu}} = \frac{[\mathrm{CuBL}]}{[\mathrm{BL}]_{\mathrm{T}}} = \frac{K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\}}{1 + K_{\mathrm{HBL}} \times \{\mathrm{H}^{+}\} + K_{\mathrm{NaBL}} \times [\mathrm{Na}^{+}] + K_{\mathrm{CaBL}} \times [\mathrm{Ca}^{2+}] + K_{\mathrm{KBL}} \times [\mathrm{K}^{+}] + K_{\mathrm{MgBL}} \times [\mathrm{Mg}^{2+}] + K_{\mathrm{CuBL}} \times \{\mathrm{Cu}^{2+}\}} \tag{3.3}$$

In the BLM approach, for each species this fraction is assumed to be constant at 50% effect ($f50_{Cu}$), for example, independent of the water quality properties (Meyer et al., 1999). Equation 3.3 can be rewritten as the following equation:

$$f50_{Cu} = \frac{K_{CuBL} \times EC50_{Cu}}{1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}] + K_{CuBL} \times EC50_{Cu}} \quad (3.4)$$

where $EC50_{Cu}$ is the free Cu^{2+} activity resulting in 50% growth reduction in lettuce roots after 4 d of exposure. This value can be derived from Equation 3.4, as follows:

$$EC50_{Cu} = \frac{f50_{Cu}}{(1 - f50_{Cu}) \times K_{CuBL}} \times \left(1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}]\right) \quad (3.5)$$

According to the BLM concept, a linear relationship between $EC50_{Cu}$ and the activity of one cation is expected when activities of other cations are constant. Therefore, K_{HBL}, K_{NaBL}, K_{CaBL}, K_{KBL}, and K_{MgBL} could be calculated from the slope and intercept of the regression line between $EC50_{Cu}$ and concentrations of H^+, Na^+, Ca^{2+}, K^+, and Mg^{2+}, respectively (Meyer et al., 1999). In addition, K_{CuBL} was calculated based on the best fit of the logit of the root response versus f_{Cu} for varying K_{CuBL}. The value of $f50_{Cu}$ was determined based on the logistic relationship between f_{Cu} and the root elongation at the obtained K_{CuBL}. A detailed description of the derivation of BLM is presented in S3.3, Supplementary information.

3.3. Results

3.3.1. Effects of copper on root morphology and growth

In addition to effects on the root growth of lettuce, copper modified both root branching and color. In particular, at lower Cu^{2+} activities, roots were white and side roots were more developed. At higher activities, the lower part or the whole root was black or brown, whereas almost all lateral roots either disappeared or were shortened.

3.3.2. Influence of cations and protons on Cu^{2+} toxicity

Copper toxicity was expressed as the exposure concentration reflecting 50% inhibition of root elongation (i.e., $EC50_{Cu}$) and expressed in terms of Cu^{2+} activities. The negative logarithm of $EC50_{Cu}$ ($pEC50_{Cu}$) for *Lactuca sativa* after 4 d of exposure was found to be in the range of 5.81 to 7.52 at varying pH and free concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} (Figs. 3.1 and 3.2). Values of $pEC50_{Cu}$ decreased significantly with an increase in H^+ activity ($p = 0.002$; $r^2 = 0.996$) (Fig. 3.1). In particular, decreases in pH from 7 to 5 resulted in a 52-fold increase in the 4-d $EC50_{Cu}$: that is, at low pH, Cu^{2+} is less toxic to lettuce. Additions of Na^+, K^+, Ca^{2+}, and Mg^{2+} to the Steiner solution at concentrations ranging from 0 to 20 mmol/L resulted in a 16-fold difference in $EC50_{Cu}$. These additions had significant effects on Cu^{2+} toxicity as shown by nonoverlapping standard deviations of $pEC50_{Cu}$ found with (7.37 ± 0.22) and without (6.76 ± 0.22) additions of Na^+, K^+, Ca^{2+}, and Mg^{2+}. However, no consistent or statistically significant linear correlations were found between the total concentrations of Na^+ ($r^2 = 0.26$; $p = 0.24$), K^+ ($r^2 = 0.18$; $p = 0.34$), Ca^{2+} ($r^2 = 0.22$; $p = 0.29$), and Mg^{2+} ($r^2 = 0.51$; $p = 0.05$), with $pEC50_{Cu}$ in the concentration range investigated (0–20 mmol/L) (Fig. 3.2). A downward trend in Cu^{2+} toxicity was observed because of the additions of these cations at the lowest concentrations (Na^+: 5 mmol/L; K^+: 10 mmol/L; Ca^{2+}: 1.3 mmol/L;

Mg^{2+}: 15 mmol/L); however, further reduction was not shown at higher concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+}. The wide confidence intervals of $pEC50_{Cu}$ found at the highest concentrations of K^+ may reflect high uncertainties at these levels because of the high ratio between K^+ and Na^+, which may negatively affect the plant growth (Chen et al., 2007). In addition, at high concentrations, Mg^{2+} may block the K^+ channel, possibly contributing to wide confidence intervals of $pEC50_{Cu}$ at high Mg^{2+} concentrations (Wu et al., 1991).

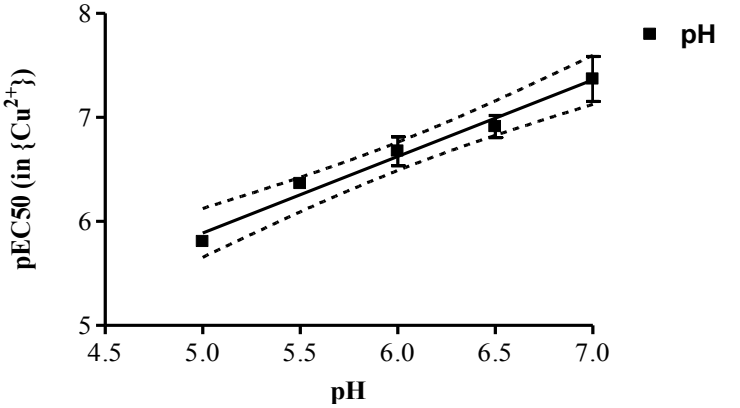

Figure 3.1. The negative logarithm of the median effective activity of Cu^{2+} toxicity ($pEC50_{Cu}$) for *Lactuca sativa* as a function of pH. The solid line represents the linear regression line, and the dotted lines represent the 95% confidence interval. Error bars indicate the standard deviation.

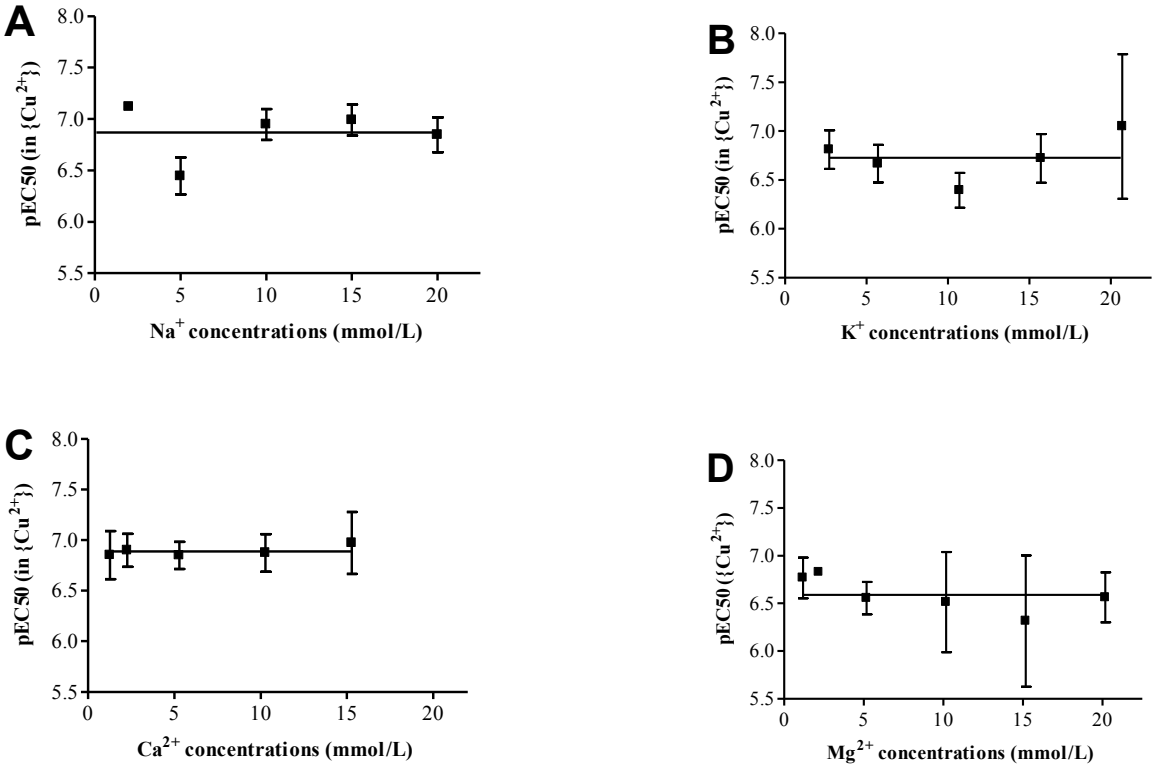

Figure 3.2. The median effective activity of Cu^{2+} ($EC50_{Cu}$) as a function of Na^+ (A), K^+ (B), Ca^{2+} (C), and Mg^{2+} (D). Error bars indicate 95% confidence intervals. Solid lines represent average values.

3.3.3. Estimating BLM parameters

The effects of H^+ were incorporated in the BLM because its ameliorative influence was statistically significant (Eqn. 3.6). Using the slope and the intercept of the linear regression of $EC50_{Cu}$ versus the H^+ activity (Fig. 3.1; Eqn. 3.6), the conditional binding constant of H^+ was calculated as $\log K_{HBL} = 6.27$ (Table 3.1). Moreover, a value of $\log K_{CuBL}$ of 7.40 resulted in the statistically best description of the relationship between the f_{Cu} and the logit of the normalized root responses. Another important BLM parameter is $f50_{Cu}$ of 0.36 at pH = 7 (Table 3.1). This yielded the following equation to predict the pH dependence of Cu^{2+} toxicity to lettuce roots:

$$EC50_{Cu} = 0.149 \times \{H^+\} + 8 \times 10^{-8} \quad (r^2 = 0.9957) \tag{3.6}$$

Because of insignificant relationships between $EC50_{Cu}$ and concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+}, the cations are not incorporated in the BLM equation. This is not in agreement with the theory of the BLM (Di Toro et al., 2001) in which these cations should have a competing effect with Cu^{2+}. There was a 3-fold difference between the $EC50_{Cu}$ value calculated by Equation 3.6 and the value determined according to Equation 3.5 leaving out effects caused by Na^+, K^+, Ca^{2+}, and Mg^{2+}. This difference was also reported by De Schamphelaere and Janssen (2002) and attributed to substantial effects of Na^+, K^+, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity, which were not included in the BLM.

Table 3.1. Binding constants for the biotic ligand model

Studies	Organisms	Conditional binding constants (log)		$f50_{Cu}$
		K_{CuBL}	K_{HBL}	
Thakali et al. (2006a)	Barley (*Hordeum vulgare*)	7.41 ± 0.23	6.48 ± 0.26	
Luo et al. (2008)	Wheat (*Triticum aestivum)*	6.28		0.44
De Schamphelaere and Janssen (2002)	Water flea (*Daphnia magna*)	8.02	5.4	0.33
Present study	Lettuce (*Lactuca sativa)*	7.40	6.27	0.36

3.4. Discussion

The effects of Cu^{2+} on root morphology observed in the present study were consistent with previous studies by Arduini et al. (1995) on pines and by Mahmood et al. (2007) on cereal crops. These effects on the root branching and color as described, as well as on the root elongation, indicate that plant roots are sensitive to Cu^{2+} toxicity.

3.4.1. BLM parameters

The conditional binding constant of Cu^{2+} and H^+ (presented as $\log K_{CuBL}$ and $\log K_{HBL}$) for lettuce, found in the present study, were in the range of values for barley *Hordeum vulgare* (Thakali et al., 2006a) (Table 3.1). However, the value of $\log K_{HBL}$ is higher than the reported number for wheat *Triticum aestivum* (Luo et al., 2008). In addition, conditional binding constants of H^+ for these plants are generally higher than binding constants found for daphnids *Daphnia magna* (De Schamphelaere and Janssen, 2002). Opposite results were found for conditional binding constants of Cu^{2+} (De Schamphelaere and Janssen, 2002).

3.4.2. Cations competition for binding sites

The inhibition of Cu^{2+} toxicity to lettuce by H^+ found in the present study was reported in another study on lettuce by Voigt et al. (2006). However, studies on barley showed various findings (Thakali et al., 2006a; Lock et al., 2007a,b). In the present study, additions of K^+, Na^+, Ca^{2+}, and Mg^{2+} resulted in considerable differences in $EC50_{Cu}$ values; however, these differences could not be explained by statistically significant correlations between $EC50_{Cu}$ and concentrations of these cations. Therefore, only protons could be included in the BLM as competing cations. This was consistent with findings from the study by Thakali et al. (2006a) on barley. Inconsistent and statistically insignificant effects of Ca^{2+} on Cu^{2+} toxicity to lettuce found in the present study were also reported in the study by Voigt et al. (2006). Similar to protons, findings on effects of these cations also diverge. For example, Cu^{2+} toxicity to sugar beet *Beta vulgaris* was not affected by Ca^{2+}, Mg^{2+}, or K^+ (Saleh et al., 1999), whereas Ca^{2+} and Mg^{2+} inhibited Cu^{2+} toxicity to wheat *Triticum aestivum* and barley *Hordeum vulgare* (Lock et al., 2007a; Parker et al., 1998; Wang et al., 2009).

In addition, the lack of a consistent downward trend in $pEC50_{Cu}$ values at high concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} may result from saturation, which may be reached with certain additions of these cations. For example, the half saturation activity of K^+ in wheat root happened at the concentration of 8.8 mmol/L (Gassmann and Schroeder, 1994; Schroeder and Fang, 1991). Mechanisms of metal bindings to biotic ligands, including the concentration of binding sites, should therefore be integrated in the BLM. Toxicity depends not only on the competition for binding sites, but also on the affinities and numbers of binding sites (Wu and Hendershot, 2009). For example, high-affinity ligands are not directly related to effects and can be saturated at low Cu^{2+} activities when the accumulation of Cu still occurs at the low-affinity ligands (Antunes et al., 2007). Based on the binding constant found in the present study, ligands for Cu^{2+} binding in lettuce belong to the high-affinity group. This may be one of the reasons for the insignificant relationships between cation concentrations and Cu^{2+} toxicity in the present study. Characteristics and concentrations of ligands, conversely, are influenced by conditions such as pH or exposure to metal mixtures (Niyogi and Wood, 2004; Boyle and Hale, 2006).

The value of $\log K_{HBL}$ found in the present study was in the range reviewed by Niyogi and Wood (2004) for aquatic organisms. However, the model developed by these authors may be not applicable to plants. Unlike in aquatic organisms, the presence of Na^+-channels has not been reported and Na^+ is dominantly taken up via Ca^{2+} channels or K^+-selective channels at the plasma membrane of root protoplasts (Lunevsky et al., 1993; Moran et al., 1984; Schachtman et al., 1991). This may be related to the differences about the transport sites for Cu^{2+} uptake. Particularly, according to the review by Niyogi and Wood (2004), Cu^{2+} blocked the Na^+ channel. Competitive effects of Na^+ on Cu^{2+} toxicity were expected for aquatic organisms; however, this was not shown in the present study. Therefore, mechanisms of competition for binding sites in plants may be different from those in aquatic organisms.

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SUPPLEMENTARY INFORMATION

Table S3.1. Bioassay set characteristics

pH	Na (mmol/L)	K (mmol/L)	Ca (mmol/L)	Mg (mmol/L)
7	0	0	0	0
6.5	0.1	0.08	0.01	0.02
6	2	2	1	1
5.5	5	5	2	2
5	10	10	5	5
	15	15	10	10
	20	20	15	15
				20

Table S3.2. The ionic composition of the Steiner solution

Cations	Na^+	Mg^{2+}	K^+	Ca^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}
Concentrations (µmol/L)	4.48	182.62	700.32	281.75	1.16	0.88	0.17

S3.3. Detailed description of the Biotic Ligand Model derivation

In this research, effects of H^+, Na^+, Ca^{2+}, Mg^{2+}, and K^+ on Cu^{2+} toxicity were taken into account. These cations can form complexes with biotic ligands (BLs) at a ratio expressed by the stability constants. At equilibrium for example, the stability constant for Cu^{2+} binding to biotic ligands K_{CuBL} (L/mol) can be expressed as

$$K_{CuBL} = \frac{[CuBL]}{\{Cu^{2+}\} \times [BL]} \quad \text{(S3.1)}$$

where $\{Cu^{2+}\}$ is the free Cu^{2+} activity (mol/L).

The concentration of the BL (mol/L) can be described as the total of concentrations of cation-BL complexes (mol/L) and unoccupied BL sites (mol/L).

$$[BL]_T = [BL] + [HBL] + [NaBL] + [CaBL] + [KBL] + [MgBL] + [CuBL] \quad \text{(S3.2)}$$

The concentration of Cu-BL complex in the presence of other cations can be written as

$$[CuBL] = \frac{K_{CuBL} \times \{Cu^{2+}\} \times [BL]_T}{1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}] + K_{CuBL} \times \{Cu^{2+}\}} \quad \text{(S3.3)}$$

According to the BLM concept, copper toxicity is determined by the fraction of the total number of biotic ligand sites occupied by copper f_{Cu} with the assumption that the complexation capacity is independent of the water quality characteristics.

$$f_{Cu} = \frac{[CuBL]}{[BL]_T} = \frac{K_{CuBL} \times \{Cu^{2+}\}}{1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}] + K_{CuBL} \times \{Cu^{2+}\}} \quad \text{(S3.4)}$$

In the BLM approach, it is assumed that for each species this fraction is constant at 50% effect ($f50_{Cu}$), i.e. independent of the water quality properties (De Schamphelaere and Janssen, 2002). Equation S3.4 can be rewritten as:

$$f50_{Cu} = \frac{K_{CuBL} \times EC50_{Cu}}{1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}] + K_{CuBL} \times EC50_{Cu}} \quad \text{(S3.5)}$$

where $EC50_{Cu}$ is the free Cu^{2+} activity resulting in 50% growth reduction in lettuce roots after 4 d of exposure. This value can be derived from Equation S3.5:

$$EC50_{Cu} = \frac{f50_{Cu}}{(1 - f50_{Cu}) \times K_{CuBL}} \times (1 + K_{HBL} \times \{H^+\} + K_{NaBL} \times [Na^+] + K_{CaBL} \times [Ca^{2+}] + K_{KBL} \times [K^+] + K_{MgBL} \times [Mg^{2+}]) \quad \text{(S3.6)}$$

Based on the assumptions of the BLM, there should be a linear relationship between $EC50_{Cu}$ and the activity of one cation when activities of other cations are constant. Therefore, K_{HBL}, K_{NaBL}, K_{CaBL}, K_{KBL}, and K_{MgBL} can be calculated from the slope and intercept of the regression line between $EC50_{Cu}$ and activities of H^+, Na^+, Ca^{2+}, K^+, and Mg^{2+}, respectively (De Schamphelaere and Janssen, 2002). For example, the slope and intercept of the relationships between $EC50_{Cu}$ and free ion activity of H^+ can be written as follows:

$$slope_{H^+} = \frac{f50_{Cu}}{(1 - f50_{Cu}) \times K_{CuBL}} \times K_{HBL} \quad \text{(S3.7)}$$

$$intercept_{H^+} = \frac{f50_{Cu}}{(1 - f50_{Cu}) \times K_{CuBL}} \quad \text{(S3.8)}$$

K_{HBL} can be then calculated from these slope and intercept values.

$$K_{HBL} = \frac{slope_{H^+}}{intercept_{H^+}} \quad \text{(S3.9)}$$

K_{CuBL} and $f50_{Cu}$ are calculated based on the best fit of the root growth response versus f_{Cu} for varying K_{CuBL}.

Chapter 4

Interactions in Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures affect toxicity to lettuce *Lactuca sativa*

T.T. Yen Le, Martina G. Vijver, Thomas B. Kinraide, Willie J.G.M. Peijnenburg, A. Jan Hendriks

Submitted

Abstract—Toxicity of single metals and mixtures to lettuce *Lactuca sativa* in hydroponic solutions was determined based on the concepts of concentration addition (CA) and response addition (RA). On the basis of the conventional models assuming no interaction between mixture components, Ag^{+} was the most toxic, followed by Cu^{2+} and Zn^{2+}. Furthermore, ion-ion interactions were included in quantitatively estimating toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures by linearly expanding the CA and RA models. About 80–92% of the variability in the root growth could be explained by this approach. Estimates by the extended models indicate significant alleviative effects of Zn^{2+} on Cu^{2+} toxicity whereas Cu^{2+} did not significantly affect Zn^{2+} toxicity. According to the extended CA model, Cu^{2+} significantly reduced Ag^{+} toxicity while Ag^{+} enhanced Cu^{2+} toxicity. Similar effects were not found by the extended RA model. These predictions could be explained by reported mechanisms of the uptake and toxicity of these metals.

Keywords — Mixture Toxicity Interaction Plant Metal

4.1. Introduction

Metals are usually present in the environment in mixtures of varying composition. Toxicity of metal mixtures may vary widely while interactions in mixtures may deviate significantly from the biological actions of single metals (Norwood et al., 2003; Otitoloju, 2002; Manzo et al., 2010). Exposure to metal mixtures at concentrations below environmental quality guideline levels for individual components was reported to result in adverse effects, attributed to interactions between the constituents (Cooper et al., 2009). The reliability of toxicity estimations can thus be improved by taking into account interactions in mixtures (Otitoloju, 2002). Such interactions occur at different levels, i.e., in the environment, at the root surface, and within the plant (Kabata-Pendias and Pendias, 1984; Pahlsson, 1989). Interactions outside organisms determine the environmental availability of metals depending on the physicochemical conditions. Subsequently, in the toxicokinetic phase, interactions between different metals influence the uptake of metals by organisms. In the toxicodynamic phase, interactions at ligands within organisms affect their joint toxicity. While metal-metal interactions in the environment have been predicted well by speciation modelling, interactions at the toxicokinetic and toxicodynamic phases are usually excluded in available models for assessment of mixture toxicity, e.g., concentration addition (CA) and response addition (RA) (Bliss, 1939; Hewlett and Plackett, 1979). The conventional concept of these models is based on the assumption that the presence of one substance does not affect the biological action of the others in their mixture. Accordingly, deviations from the ideal behaviour of mixtures, which result from the interactions, cannot be quantified by these models based on the conventional concept. In the assssments using these models, toxicity of mixtures is mainly predicted from toxicological data for single substances only (Manzo et al., 2010; Sharma et al., 1999). However, this approach may not accurately estimate mixture toxicity (Ren et al., 2004). Instead, joint toxicity of multiple chemicals should be assessed by tests on mixtures (Frias-Espericueta et al., 2009).

The present study aimed at modelling toxicity of metal mixtures taking into account potential interactions between their different components. This was achieved by developing mathematical relationships to express the interactions. Specifically, toxicity of single metals, i.e., Cu^{2+}, Zn^{2+}, and Ag^{+}, was assessed by fitting the empirical data to mathematical expressions of the conventional concept of CA and RA assuming no interactions between these ions. Furthermore, toxicity of interactive mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} was

simulated through expansion of the conventional CA and RA models based on assumed linear interactions in the mixtures. Metal toxicity was assessed in terms of inhibition of the root elongation of lettuce *Lactuca sativa*.

4.2. Methods

4.2.1. Test species and toxic endpoint

Metal toxicity was assessed on lettuce, *Lactuca sativa*, in hydroponic exposures, in order to allow for controlled modifications of the test media. Lettuce was selected as test species in view of its high capacity to accumulate metals and the presence of a protocol by the Organisation for Economic Cooperation and Development (McKenna et al., 1993; OECD, 2006; Le et al., 2012). Root elongation was reported to be sensitive to metal exposure and has been widely used as toxic endpoint (Thakali et al., 2006; Lock et al., 2007; Voigt et al., 2006; Kinraide, 1999; Kinraide et al., 2004; Kopittke et al., 2011). Consequently, the root growth was used to evaluate metal toxicity in the present study.

4.2.2. Preparation of the test solutions

Steiner solution was used as test medium (Steiner, 1961). Metal ions Cu^{2+}, Zn^{2+}, and Ag^{+} were added into the Steiner solution in the form of nitrate salts. This form was used because of negligible interference of NO_3^- on the measurement of ion-selective electrodes compared to other anions such as Cl^-. Solution pH was kept at 7.0 using 3-[N-morpholino] propane sulfonic acid at 0.75 g/L and NaOH (Le et al., 2012). Exposure solutions were daily renewed. The ratio between one exposure level expressed as the free metal ion activity and the next higher level was kept below 2 except for the exposure solution at the background level of the Steiner solution.

4.2.3. Chemical measurements and speciation

Free ion activities of H^+, Cu^{2+}, and Ag^+ were measured by using hydrogen, copper, and silver sulfide ion-selective electrodes (Metrohm), respectively. These electrodes were calibrated by measurements at different concentrations of these ions in solution (Le et al., 2012). Additionally, free Zn^{2+} activities in the exposure solutions were determined from total zinc concentrations by the speciation model Windermere Humic-Aqueous Model VI with Steiner solution as the default medium (Tipping, 1998). The chemical composition of the Steiner solution used in the chemical speciation is given in Table S4.1, Supplementary information. The ranges of free ion activities of Cu^{2+}, Zn^{2+}, and Ag^+ in the solutions studied were: Cu^{2+}: 10^{-10}–10^{-6} mol/L; Zn^{2+}: 10^{-6}–10^{-3} mol/L; and Ag^+: 10^{-8}–10^{-7} mol/L.

4.2.4. Toxicity assays

Seeds of *Lactuca sativa* were germinated for 4 d at 15 ^oC in the Steiner solution during a normal light cycle of 16: 8 hours light: dark. The germinated plants were then fixed in a parafilm strap with a surface area of around 30 cm^2. The parafilm strap floated on the surface of a glass beaker (10 cm height and volume: 100 mL) with the roots immersed in the medium. Four plants were put in each beaker. The growth of lettuce (Growth; mm) exposed to a given exposure solution was calculated as the average of the increase of the root length of the 4 plants after 4 d of exposure. The replication by repeating toxicity tests on the same solution was not carried out in the present study. However, this lack of replicates was expected not to undermine the statistical significance of the toxicological data generated because of the experimental design applied in the present study: small gap between the exposure levels as

described above and 4 plants grown in each solution. In total, 238 toxicity tests were carried out, including 122 tests performed without additions of Ag^{+} and 116 tests performed without additions of Zn^{2+} to the Steiner solution.

4.2.5. Mathematical expression of metal toxicity

Toxicity of metals following single exposure

The response of plants in terms of root growth (Growth; mm) after single exposure to metal ion M^{n+} can be expressed in relation to its free ion activity in the solution $\{M^{n+}\}$ (µmol/L) according to the following equation

$$\text{Growth} = \frac{b}{\exp[(c \times \{M^{n+}\})^{d}]} \tag{4.1}$$

where coefficient b (mm) is the growth of lettuce roots in the medium free of the metal ion (i.e., $\{M^{n+}\} = 0$); coefficient c (L/µmol) reflects the metal-specific toxicity strength. Metal toxicity increases with increasing strength coefficient; and coefficient d (dimensionless) describes the slope of the adjacent curve representing toxicity of the metal ion. This exponential equation was found to be the most suitable to describe the root elongation following metal exposure and has been applied in a number of studies investigating metal toxicity to plants (Kinraide and Parker, 1989; Kinraide, 1999; Kinraide et al., 2004; Kopittke et al., 2011).

Toxicity of non-interactive mixtures

If mixture components do not interact with each other, the growth of lettuce roots exposed to the mixture can be written according to the conventional concept of CA and RA assuming no interactions between mixture constituents. The CA model is based on the assumption that different substances in their mixture have the same modes of action (Bliss, 1939). Accordingly, the growth of lettuce roots (Growth; mm) following exposure to a non-interactive mixture of Cu^{2+}, Zn^{2+}, and Ag^{+} can be written as

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\} + c_2 \times \{Zn^{2+}\} + c_3 \times \{Ag^{+}\})^{d}]} \tag{4.2}$$

where b (mm) is the growth of lettuce roots in the medium free of Cu^{2+}, Zn^{2+}, and Ag^{+}; coefficients c_1, c_2, and c_3 (L/µmol) represent the strength of toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in their non-interactive mixtures, respectively; d (dimensionless) is the slope parameter describing toxicity of these metals in non-interactive mixture; and $\{Cu^{2+}\}$, $\{Zn^{2+}\}$, and $\{Ag^{+}\}$ (µmol/L) are the free ion activity of Cu^{2+}, Zn^{2+}, and Ag^{+} in the solution, respectively (Kinraide, 1999).

In the RA model, mixture components are supposed to have different modes of action of toxicity (Hewlett and Plackett, 1979). Therefore, based on the RA concept, the response of lettuce exposed to non-interactive mixtures can be expressed as a multiplicative function of the response of the plants following exposure to each constituent separately. For example, without interactions between Cu^{2+}, Ag^{+}, and Zn^{2+}, the growth of lettuce roots exposed to a mixture of these metal ions can be expressed by the following equation

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\})^{d_1} + (c_2 \times \{Zn^{2+}\})^{d_2} + (c_3 \times \{Ag^{+}\})^{d_3}]} \tag{4.3}$$

where coefficients b (mm), and c_1, c_2, and c_3 (L/µmol) have the same meaning as in Equation 4.2; coefficients d_1, d_2, and d_3 (dimensionless) are slope parameters describing toxicity of

Cu^{2+}, Ag^{+}, and Zn^{2+} in their non-interactive mixtures, respectively; and $\{Cu^{2+}\}$, $\{Zn^{2+}\}$, and $\{Ag^{+}\}$ (µmol/L) are the free ion activity of Cu^{2+}, Ag^{+}, and Zn^{2+} in the exposure solution, respectively (Kinraide, 1999).

Equations 4.2 and 4.3 are conventional expressions of CA and RA assuming that the presence of one metal does not affect the toxicity of the others in their mixtures. Accordingly, toxicity of metals in these non-reactive mixtures is similar to their toxicity following single exposure. Coefficients describing toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually as in Equation 4.1, i.e., strength coefficient c and slope parameter d, were similar to the corresponding coefficients in Equations 4.2 and 4.3. Consequently, toxicity of single metals can be determined from toxicological data of non-interactive mixtures. Specifically, the determination of the strength coefficient and slope parameter describing toxicity of each metal can be done by fitting the toxicological data on mixtures to Equations 4.2 and 4.3 in multiple regression analyses.

Toxicity of interactive mixtures

If metals in mixtures interact with each other, i.e., the presence of one metal affects the toxicity of the others in the mixtures, the interactions can be taken into account in quantifying toxicity of the mixtures by expanding the conventional CA and RA models. In particular, expansion coefficients representing the interactions can be incorporated in the strength coefficients in Equations 4.2 and 4.3 in two different ways as described in S4.A, Supplementary information (Kinraide, 1999; Kinraide et al., 2004). The expansion approach that results in higher statistical significance was selected as the best simulation of the interactions. The comparison of the expansion coefficient with zero determines whether one substance reduces or increases the toxicity of another. In addition, the interactive effect was considered statistically significant if the 95% confidence interval (CI) of the expansion coefficient does not encompass zero. A full description of the equation derivation is presented in S4.A, Supplementary information.

Mixtures of Cu^{2+} and Zn^{2+}. An expansion coefficient c_{12} (L/µmol) representing interactive effects of Zn^{2+} on Cu^{2+} toxicity can be integrated into the strength coefficient of Cu^{2+} toxicity. Similarly, another expansion coefficient c_{21} (L/µmol) might be incorporated into the strength coefficient of Zn^{2+} toxicity to reflect effects of Cu^{2+} on Zn^{2+} toxicity. According to the CA model and based on the assumption of linear interactions, the response of lettuce exposed to Cu^{2+}–Zn^{2+} mixtures expressed as the root growth (Growth; mm) could be fitted well to Equation 4.4 as all coefficients in this equation estimated by the regression analysis were statistically significant:

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}}{1 + c_{12} \times \{Zn^{2+}\}} + c_2 \times \{Zn^{2+}\}\right)^{d}\right]} \tag{4.4}$$

where b (mm) is the growth of lettuce roots in the medium free of Cu^{2+} and Zn^{2+}; c_1 (L/µmol) is the strength coefficient of Cu^{2+} toxicity in the medium free of Zn^{2+}; c_2 (L/µmol) is the strength coefficient of Zn^{2+} toxicity in the medium free of Cu^{2+}; c_{12} (L/µmol) is the expansion coefficient representing effects of Zn^{2+} on the toxicity of Cu^{2+}; d (dimensionless) reflects the slope of the adjacent curve; and $\{Cu^{2+}\}$ and $\{Zn^{2+}\}$ (µmol/L) are the free ion activity of Cu^{2+} and Zn^{2+} in the solution, respectively.

Based on the assumption of linear interactions, in the RA model, the growth of lettuce roots (Growth; mm) in response to exposure to mixtures of Cu^{2+} and Zn^{2+} follows Equation

4.5 as coefficients in this equation estimated by the regression analysis were statistically significant:

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}}{1 + c_{12} \times \{Zn^{2+}\}}\right)^{d_1} + \left(c_2 \times \{Zn^{2+}\}\right)^{d_2}\right]} \quad (4.5)$$

where coefficients b, c_1, c_2, and c_{12} have the same meaning as in Equation 4.4; coefficients d_1 and d_2 (dimensionless) reflect the slope of the adjacent curve describing toxicity of Cu^{2+} and Zn^{2+} in their mixtures, respectively; and $\{Cu^{2+}\}$ and $\{Zn^{2+}\}$ (µmol/L) are the free ion activity of Cu^{2+} and Zn^{2+} in the solution, respectively.

Mixtures of Cu^{2+} and Ag^{+}. Interactive effects of Ag^{+} on Cu^{2+} toxicity can be represented by an expansion coefficient c_{13} (L/µmol) that is incorporated into the strength coefficient of Cu^{2+} toxicity. A similar expansion coefficient c_{31} (L/µmol) reflecting effects of Cu^{2+} on Ag^{+} toxicity can be integrated into the strength coefficient of Ag^{+} toxicity. The CA model can be extended as Equation 4.6 to express joint toxicity of Cu^{2+} and Ag^{+}, taking into account effects of their interactions, because coefficients in this equation estimated by the regression analysis were statistically significant:

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}}{1 + c_{13} \times \{Ag^{+}\}} + c_3 \times \left(1 + c_{31} \times \{Cu^{2+}\}\right) \times \{Ag^{+}\}\right)^{d}\right]} \quad (4.6)$$

where coefficient b (mm) is the growth of lettuce roots in the medium free of Cu^{2+} and Ag^{+}; c_1 (L/µmol) is the strength coefficient of Cu^{2+} toxicity in the medium free of Ag^{+}; c_3 (L/µmol) is the strength coefficient of Ag^{+} toxicity in the medium free of Cu^{2+}; c_{13} (L/µmol) is the expansion coefficient representing effects of Ag^{+} on the toxicity of Cu^{2+}; c_{31} (L/µmol) is the expansion coefficient describing effects of Cu^{2+} on the toxicity of Ag^{+}; coefficient d (dimensionless) reflects the slope of the adjacent curve describing toxicity of Cu^{2+} and Ag^{+} in their mixtures; and $\{Cu^{2+}\}$ and $\{Ag^{+}\}$ (µmol/L) are the free ion activity of Cu^{2+} and Ag^{+} in the solution, respectively.

By contrast, no expansion coefficient was found to be statistically significant to represent interactions between these metal ions according to the extended RA model.

4.2.6. Statistical analyses

Coefficients in Equations 4.2–4.6 were determined by multiple regression analyses using the SYSTAT software. Coefficients are considered statistically significant if their 95% CI is statistically deviating from zero, i.e., not encompassing zero. The strength of the significance increases with increasing absolute value of the ratio between the estimate of the coefficient and the asymptotic standard error, i.e., parameter/ASE in the regression results. All individual toxicity data generated in the present study were used to assess toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} in non-interactive mixtures or toxicity of these single metals as the presence of one metal does not affect the biological actions of the others in the mixtures. Data from 122 tests without additions of Ag^{+} were used to assess toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} as Ag^{+} was not present in the solutions. Moreover, toxicity of interactive mixtures of Cu^{2+} and Ag^{+} was evaluated using results from 118 tests with no Zn^{2+} added to the Steiner solution assuming negligible effects caused by Zn^{2+} at the background concentration in the default medium. Furthermore, Akaike's information criterion (AIC) was calculated to compare different models developed for estimating toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures

(Burnham and Anderson, 2002). While the r^2 value describes the potential of the model in explaining the variability in the toxic effect, the AIC value indicates the most suitable model, i.e., the lower AIC value the more suitable the model.

4.3. Results

4.3.1. Toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually

Differences between the strength of single metal toxicity predicted by the non-interactive mixture models based on the concepts of CA and of RA were small (Table 4.1). Estimates of all coefficients and statistical parameters are given in Tables S4.2 and S4.3 (Supplementary information). There was no statistically significant difference between the estimates by the CA and RA models in predicting the strength coefficients of Cu^{2+} and Zn^{2+} toxicity as shown by an overlap between the 95% CIs of the strength coefficients of their toxicities (Table 4.1). An opposite observation was found in predicting toxicity of Ag^{+} (Table 4.1). The difference between the strength coefficients of Ag^{+} toxicity of the CA and RA models was small, but significant as shown by their non-overlapping 95% CIs. The assessment based on both models indicates that Zn^{2+} was far less toxic than Cu^{2+} and Ag^{+} while there were negligible differences between toxicities of Cu^{2+} and of Ag^{+} (Table 4.1). These findings suggest that the CA and RA models did not yield substantial differences in the estimates of toxicity of these single metals, based on the assumption of no interactions between them.

Table 4.1. Estimates of strength coefficients of toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually found in the present study for *Lactuca sativa* according to the conventional concept of concentration addition (CA) and response addition (RA) models and in the study of Kopittke et al. (2011) for *Vigna unguiculata* following exposure to single metals. 95% confidence intervals (CI) are provided.

Source	Species	Model	Strength coefficient c_i (L/µmol)		
			Cu^{2+}	Zn^{2+}	Ag^{+}
			c_1 (95% CI)	c_2 (95% CI)	c_3 (95% CI)
Present study	*Lactuca sativa*	CA	2.92 (2.54-3.30)	$6.15 \cdot 10^{-3}$ ($5.29 \cdot 10^{-3}$-$7.01 \cdot 10^{-3}$)	2.93 (2.52-3.34)
		RA	3.13 (2.71-3.55)	$6.31 \cdot 10^{-3}$ ($5.41 \cdot 10^{-3}$-$7.21 \cdot 10^{-3}$)	3.79 (3.50-4.08)
Kopittke et al. (2011)	*Vigna unguiculata*	Single-metal exposure	2.00	$4.26 \cdot 10^{-2}$	25.9

4.3.2. Toxicity of interactive binary mixtures

Mixtures of Cu^{2+} and Zn^{2+}. Based on the r^2 value, the extended CA (Eqn. 4.4) and RA (Eqn. 4.5) models estimated the toxicity of Cu^{2+}–Zn^{2+} mixtures at different exposure levels

equally well, i.e., approximately 92% of the variability in the root growth following exposure to different mixtures of Cu^{2+} and Zn^{2+} could be explained by the models (r^2 = 0.92; Fig. 4.1).

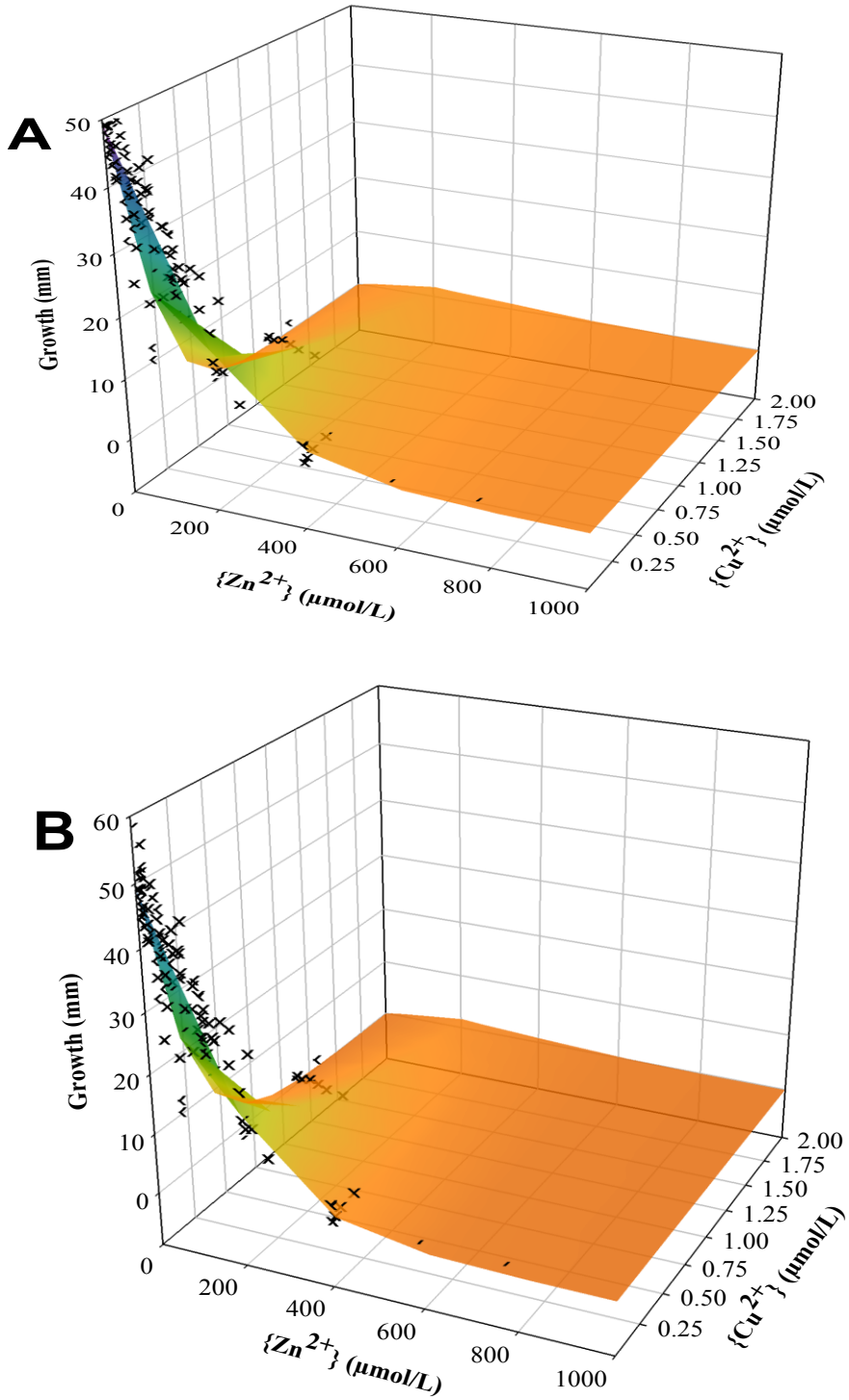

Figure 4.1. The response of lettuce expressed as the root growth (Growth; mm) as a function of the free ion activity of Cu^{2+} ({Cu^{2+}}; µmol/L) and the free ion activity of Zn^{2+} ({Zn^{2+}}; µmol/L) in the solution according to the extended concentration addition (CA) model (A) and the extended response addition (RA) model (B): the surface describing the estimations based on the regression analysis by fitting experimental data to Equations 4.4 (extended CA model) and 4.5 (extended RA model) and dotted points representing the experimental data.

Table 4.2. Estimates of the coefficients in Equation 4.4 and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), determined by the regression analysis representing toxicity of interactive Cu^{2+}–Zn^{2+} mixtures as well as toxicological interactions between Cu^{2+} and Zn^{2+} according to the extended concentration addition model (n = 122; r^2 = 0.92)

Parameter	Definition	Estimate	ASE	Parameter/ ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.46	1.14	43.42	47.20	51.72
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	3.88	0.40	9.65	3.09	4.68
c_{12} (L/µmol)	Effects of Zn^{2+} on Cu^{2+} toxicity	$25.28 \cdot 10^{-3}$	$8.23 \cdot 10^{-3}$	3.07	$8.98 \cdot 10^{-3}$	$41.57 \cdot 10^{-3}$
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$6.53 \cdot 10^{-3}$	$0.36 \cdot 10^{-3}$	18.29	$5.82 \cdot 10^{-3}$	$7.24 \cdot 10^{-3}$
d (dimensionless)	Slope	1.26	0.10	12.07	1.05	1.46

Table 4.3. Estimates of the coefficients in Equation 4.5 and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), determined by the regression analysis representing toxicity of interactive Cu^{2+}–Zn^{2+} mixtures as well as toxicological interactions between Cu^{2+} and Zn^{2+} according to the extended response addition model (n = 122; r^2 = 0.92)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.28	1.10	44.78	47.10	51.46
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	3.54	0.40	8.76	2.74	4.33
c_{12} (L/µmol)	Effects of Zn^{2+} on Cu^{2+} toxicity	$14.31 \cdot 10^{-3}$	6.14	2.33	$2.14 \cdot 10^{-3}$	$26.47 \cdot 10^{-3}$
d_1 (dimensionless)	Slope of Cu^{2+} toxicity curve	1.08	0.12	9.11	0.84	1.31
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$6.68 \cdot 10^{-3}$	0.36	18.57	$5.97 \cdot 10^{-3}$	$7.39 \cdot 10^{-3}$
d_2 (dimensionless)	Slope of Zn^{2+} toxicity curve	1.36	0.13	10.47	1.10	1.62

However, the growth of lettuce roots exposed to mixtures of Cu^{2+} and Zn^{2+} at their low activities in solution was frequently underestimated. According to both extended CA and extended RA models, Zn^{2+} significantly reduced toxicity of Cu^{2+} as the 95% CI of the expansion coefficient c_{12} were statistically significantly deviating from zero (Tables 4.2 and 4.3). By contrast, Cu^{2+} did not have significant effects on Zn^{2+} toxicity, i.e., no statistically significant value of the expansion coefficient c_{21} was found to represent these impacts.

Mixtures of Cu^{2+} and Ag^{+}. Approximately 80% of the variability in the growth of lettuce roots exposed to mixtures of Cu^{2+} and Ag^{+} at different free ion activities could be explained by the mathematical relationship expressed by Equation 4.6 (n = 116; r^2 = 0.80; Fig. 4.2). The 95% CIs of the expansion coefficients representing effects of Ag^{+} on Cu^{2+} toxicity (c_{13}) and effects of Cu^{2+} on Ag^{+} toxicity (c_{31}) deviated significantly from zero (Table 4.4). This indicates that Cu^{2+} and Ag^{+} interacted with each other, significantly affecting their toxicity to lettuce. Particularly, Ag^{+} significantly enhanced Cu^{2+} toxicity ($c_{13} < 0$) while Cu^{2+} significantly reduced Ag^{+} toxicity ($c_{31} < 0$) (See S4.A, Supplementary information).

Table 4.4. Estimates of the coefficients in Equation 4.6 and statistic parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), determined by the regression analysis representing toxicity of interactive Cu^{2+}–Ag^{+} mixtures as well as toxicological interactions between Cu^{2+} and Ag^{+} according to the extended concentration addition model (n = 116; r^2 = 0.80)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	50.23	1.77	28.41	46.72	53.73
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	4.12	0.45	9.22	3.23	5.01
c_{13} (L/µmol)	Effects of Ag^{+} on Cu^{2+} toxicity	-2.14	0.45	-4.78	-3.03	-1.26
c_3 (L/µmol)	Strength of Ag^{+} toxicity	3.53	0.26	13.71	3.02	4.04
c_{31} (L/µmol)	Effects of Cu^{2+} on Ag^{+} toxicity	-8.16	2.38	-3.43	-12.88	-3.44
d (dimensionless)	Slope	1.76	0.25	6.96	1.26	2.27

4.3.3. Comparison of the different models

Generally, the interactive models were better than the non-interactive models in estimating mixture toxicity, especially for mixtures of Cu^{2+} and Zn^{2+} (Table 4.5). This result indicates that interactions between different ions should be taken into account in modelling their joint toxicity and the incorporation will improve the estimations. Additionally, based on the assumption that mixture components do not interact with each other, the concept of RA was

better than the CA concept in predicting toxicity of the Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures (Table 4.5). By contrast, based on an assumption of interactive mixtures, negligible difference was found between the AIC values for the CA and RA models in estimating toxicity of Cu^{2+}–Zn^{2+} mixtures.

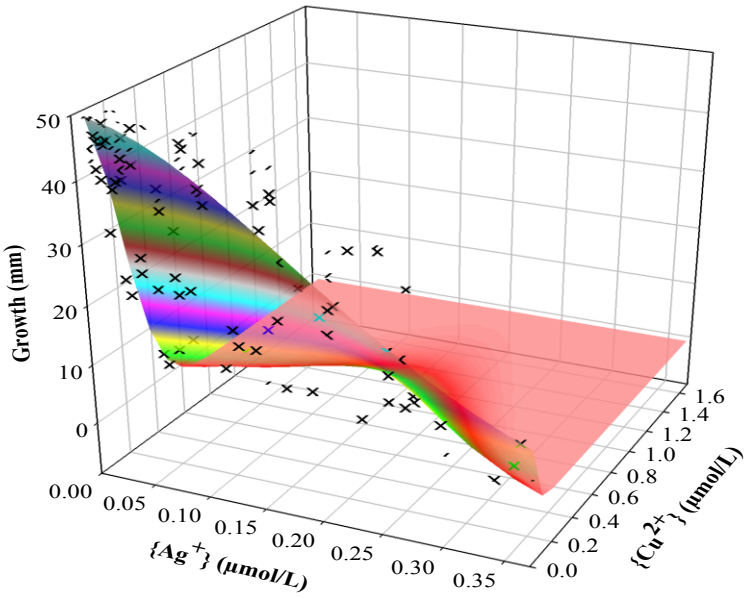

Figure 4.2. The response of lettuce expressed as the root growth (Growth; mm) as a function of the free ion activity of Cu^{2+} ({Cu^{2+}}; µmol/L) and the free ion activity of Ag^{+} ({Ag^{+}}; µmol/L) in the solution according to the extended concentration addition model: the surface describing the estimations based on the regression analysis by fitting experimental data to Equation 4.6 and dotted points representing the experimental data.

Table 4.5. Akaike's information criterion calculated for non-interactive and interactive models based on the concepts of concentration addition (CA) and response addition (RA) for predicting toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures

Mixtures	Non-interactive models		Interactive models	
	CA	RA	CA	RA
Cu^{2+}–Zn^{2+}	155	149	144	145
Cu^{2+}–Ag^{+}	192	183	190	

4.4. Discussion

4.4.1. Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually

In the present study, toxicity to lettuce *Lactuca sativa* decreased in the sequence of Ag^{+} > Cu^{2+} > Zn^{2+}, similar to the order reported by Kopittke et al. (2011) for cowpea *Vigna unguiculata* (Table 4.1). An inconsiderable difference was found in toxic potency of Cu^{2+} to

the two plant species (Table 4.1). Yet, toxic potency of Ag^+ and Zn^{2+} to *Vigna unguiculata* was about one order of magnitude higher than that to *Lactuca sativa* (Table 4.1).

4.4.2. Toxicity of interactive mixtures of Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+}

Metal-metal interactions are complicated as single metals, e.g., Cu^{2+}, Ag^+, and Zn^{2+}, separately may have toxic effects on plants via a number of mechanisms. These processes may account for the interactions in Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+} mixtures predicted in the present study.

In general, modes of action of Cu^{2+} include increasing the membrane potential and subsequently affecting the membrane permeability, and blocking ion channels (Demidchik et al., 1997; Kiss and Osipenko, 1994; Salama et al., 1992; Gilly and Amstrong, 1982). Noticeably, Cu^{2+} does not affect the conductance of the K^+-channel (Demidchik et al., 1997). Cu^{2+} can competitively replace other cations, e.g., Zn^{2+} and Ca^{2+}, at their binding sites in plant cell protein and lipid compounds, disrupting the metabolism (Mierle and Stokes, 1976; Watkins and Ferguson, 1982; Lidon and Henriques, 1993). According to Coskun et al. (2012), Ag^+ inhibited K^+ influx by two different mechanisms: directly as a K^+-channel blocker at lower concentrations and indirectly via membrane destruction, e.g., increased permeability, at higher concentrations. This is consistent with the observation by Hendrix and Higinbotham (1974) that in plants, Ag^+ was able to substitute K^+ in membranes, thus inhibiting the uptake of other cations by roots. Similar to Cu^{2+}, elevated concentrations of Zn^{2+} rapidly result in changes in the membrane potential, i.e., depolarisation, of the root cell (Kenderesova et al., 2012). In addition, both deficiency and excess of Zn^{2+} may increase membrane permeability (Michael and Krishnaswamy, 2011; Chen et al., 2009; Kaya and Higgs, 2000). Furthermore, the induction and expression of proteins, e.g., ZIP, at the plasma membrane involved in the transport of metals like Cu^{2+} and Zn^{2+} are increased under Zn^{2+} deficiency and inhibited under Zn^{2+} sufficiency (Grotz and Guerinot, 2006; Maser et al., 2001; Eckhardt et al., 2001; Ramesh et al., 2003; Ishimaru et al., 2005; Eide et al., 1996; Desbrosses-Fonrouge et al., 2005).

Based on the modes of action of Cu^{2+}, Ag^+, and Zn^{2+} as mentioned above, some interactions are expected to occur following exposure to their mixtures and might explain the predictions obtained in the present study by the extended CA and RA models. If Cu^{2+} and Ag^+ act in the same ways, i.e., reducing the membrane potential or blocking the K^+-channel, they may interact with each other, determining their toxicities. Particularly, either of these two mechanisms leads to the inhibition of the transport and subsequent toxicity of Cu^{2+} and Ag^+ by the other. As such, Cu^{2+} reduces Ag^+ toxicity. Other mechanisms additionally contribute to the alleviative effects of Cu^{2+} on Ag^+ toxicity. In particular, Cu^{2+}-induced compounds can detoxify Ag^+ and this mechanism was found to contribute to the alleviative effects of Cu^{2+} on Ag^+ toxicity (Howe and Merchant, 1992). In addition, the decline in the induction of non-selective uptake mechanisms, e.g., polypeptides, that results from the elevated exposure level of Cu^{2+} further reduces Ag^+ uptake and toxicity (Howe and Merchant, 1992). Additionally, Ag^+ leads to damage to the cell membrane, increasing Cu^{2+} uptake and subsequently enhancing Cu^{2+} toxicity. These interactions potentially account for the interactions predicted in the present study according to the CA model, i.e., Cu^{2+} reduced Ag^+ toxicity while Ag^+ enhanced Cu^{2+} toxicity. Considering the possibility that Cu^{2+} and Ag^+ have different modes of action (RA model), e.g., Ag^+ blocks the K^+-channel while Cu^{2+} does not affect the conductance of the K^+-channel, it would be expected that Cu^{2+} and Ag^+ do not affect toxicity of the other as predicted in the present study.

The prediction of alleviative effects of Zn^{2+} on Cu^{2+} toxicity to lettuce *Lactuca sativa* in the present study is consistent with reported results for cress *Lepidium sativum*, duckweed *Lemna minor*, and pigeon pea *Cajanus cajan* in other studies (Montvydiene and

Marciulioniene, 2007; Ince et al., 1999; Dirilgen et al., 1994; Sresty and Rao, 1999). This common inhibition can be explained by interactions between these metals as follows. Possessing similar physical properties as noted by Weast (1976), Cu^{2+} and Zn^{2+} share main modes of actions and transport mechanisms as described above and reported previously (Arguello, 2003; Rensing et al., 1999; Axelsen and Palmgren, 1998). Consequently, their uptake can be inhibited by each other due to their direct interactions at the plasma membrane (Bowen, 1969; Giordano et al., 1974; Kausar et al., 1976; Hawf and Schmid, 1967; Chaudhry and Loneragan, 1972). As such, elevated Zn^{2+} may reduce Cu^{2+} transport, alleviating Cu^{2+} toxicity. Moreover, at high levels of Zn^{2+}, the decline in the synthesis and expression of transporter proteins as reviewed above reduces Cu^{2+} uptake further. Zn^{2+} can be replaced by Cu^{2+} due to higher affinity of Cu^{2+} for exchangeable sites on the root cell walls compared to Zn^{2+} (Nishizono et al., 1987; Ernst et al., 1992; Franco et al., 2002). The inhibition of Zn^{2+} toxicity is additionally caused by changes in the structure of transporter proteins, which are induced by binding of Cu^{2+} to amino acids in the proteins (Bal et al., 1993; Freedman et al., 1982; Lopez-Millan et al., 2004; Orfei et al., 2003; Ooi et al., 1996; Petris et al., 2003; Stephens et al., 2011). However, under Zn^{2+} deficiency, the enhanced induction of proteins associated with the transport of Zn^{2+} as mentioned above increases Zn^{2+} uptake, compensating for the inhibition of Zn^{2+} uptake caused by Cu^{2+}. These mechanisms potentially contribute to the insignificant effects of Cu^{2+} on Zn^{2+} toxicity as well as alleviative effects of Zn^{2+} on Cu^{2+} toxicity predicted in the present study. Another mode of action that contributes to the interactions between Cu^{2+} and Zn^{2+} is their effects on decreasing the membrane potential, which subsequently reduce uptake of these metals. As a result of this change in the surface potential, Zn^{2+} may alleviate Cu^{2+} toxicity as found in the present study. The effects of these cations on the membrane potential vary, depending on their concentrations, affinity, and mode of action (Kenderesova et al., 2012). Cu^{2+} is more effective than Zn^{2+} in decreasing negative charge (Irving and Williams, 1948; Bowen, 1966; Isermann, 1979). However, the Cu^{2+} activities in the solution tested in the present study were some orders of magnitude lower than the Zn^{2+} activities, contributing to insignificant effects of Cu^{2+} on the membrane potential as well as on Zn^{2+} uptake toxicity as reported in the present study.

4.4.3. Modelling metal-metal interactions by mathematical equations

The results of the present study demonstrate that the equations used predict interactions between different metals ions and their joint toxicity well, explaining around 80–92% of the variability observed. The underestimation of the root growth of lettuce exposed to mixtures of Cu^{2+} and Zn^{2+} at their low activities in the exposure solution might be related to their essentiality. The different interactions in mixtures of Cu^{2+} and Ag^{+} predicted by the extended CA and RA models indicate that the conclusion about interactions is strongly influenced by the mathematical relation used, consistent with the observation of Hernandez and Blazer (2006). Linearity as applied in the present study has been widely used to express interactions between different chemicals (Kinraide et al., 2004; Preacher et al., 2006). Linear relationships provide a simple description of the data from the perspective that the contribution of each predictor is summarised in a single coefficient (Hastie and Tibshirani, 1990). However, the use of linearity as in the present study to interpret interactions between different metals may lead to particular uncertainties. For example, linear relationships imply an increase in toxic effects with increasing exposure levels that does not hold under conditions of deficiency of essential metals. In addition, the dependence of the interactions between different metal ions on their doses as demonstrated above is not included in the linear relationship. Besides the linearity, interactions between differrent substances may follow other patterns and accordingly be expressed by other mathematical relations (Hamm et al., 2005). The use of linearity only while excluding other relationships does not necessarily reflect the actual interaction between

variables (Lubinski and Humphresys, 1990; Cohen, 1978; Birnbaum, 1973; Busemeyer and Jones, 1983). Particularly, the statistical significance of the interaction found by the linear regression may be mainly due to an overlap with unchecked, but significant, nonlinear relations (Cortina, 1993; Lubinski and Humphresys, 1990).

In short, interactions among metals should be investigated at different plant cell compartments separately because of a variety of involved mechanisms affecting both toxicokinetics and toxicodynamics. The type of interactive effects on metal toxicity, e.g., alleviative or enhancing, depends on the comparison of these different mechanism. Moreover, the method of using mathematical relationships as applied in the present study shows good predictive power in incorporating metal-metal interactions in quantifying toxicity of metal mixtures. Additionally, the regression analyses reveals a full dose-response curve, describing toxicity as a function of the free ion activity of all mixture components, instead of providing only one single value of the concentration or activity at a certain response level, e.g., 50%. Furthermore, the mathematical extension of the CA and RA models provides quantitative estimates of toxicity of interactive mixtures while the application of conventional concept of the models only offer qualitative estimations (higher or lower than additive effects).

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SUPPLEMENTARY INFORMATION

S4.1. Ionic composition of the Steiner solution used for chemical speciation

Metals	Na^+	Mg^{2+}	K^+	Ca^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}
Concentration (mol/L)	$1.02\cdot 10^{-6}$	$1.83\cdot 10^{-3}$	$6.98\cdot 10^{-3}$	$2.82\cdot 10^{-3}$	$1.16\cdot 10^{-5}$	$4.96\cdot 10^{-5}$	$1.72\cdot 10^{-6}$

S4.A. Derivation of mathematical equations describing toxicity of interactive and non-interactive metal mixtures

Toxicity of non-interactive mixtures

The response of plants expressed as the root growth (Growth; mm) after exposure to single metal M can be expressed in relation to its free ion activity in the solution $\{M^{n+}\}$ (μmol/L) according to the following equation (Kinraide and Parker, 1989; Kopittke et al., 2011)

$$\text{Growth} = \frac{b}{\exp[(c \times \{M^{n+}\})^d]} \tag{S4.1}$$

where coefficient b (mm) is the growth of lettuce in the medium free of the metal ion (i.e., $\{M^{n+}\} = 0$); coefficient c (L/μmol) reflects the metal-specific strength of toxicity. Its value increases with increasing strength of metal toxicity. When $\{M^{n+}\}=c^{-1}$, Growth = 36.8% b; and coefficient d (dimensionless) is the slope parameter that reflects sigmoidality when its values is greater than 1.

If mixture components do not interact to each other, the growth of plants exposed to the mixture can be written according to the conventional concept of concentration addition (CA) and response addition (RA) models assuming no interactions between the mixture constituents. According to the CA model, mixture components have the same modes of action of toxicity (Bliss, 1939). In other words, the presence of one substance can be considered as a simple dilution of others. Therefore, according to the CA concept, response of lettuce exposed to non-interactive mixtures in terms of root growth can be written as follows (Kinraide, 1999):

$$\text{Growth} = \frac{b}{\exp[(\sum(c \times \{M^{n+}\}))^d]} \tag{S4.2}$$

For instance, the growth of lettuce roots (Growth; mm) following exposure to a non-interactive mixture of Cu^{2+}, Zn^{2+}, and Ag^+ is determined by the following equation according to the CA model:

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\} + c_2 \times \{Zn^{2+}\} + c_3 \times \{Ag^+\})^d]} \tag{S4.3}$$

where b (mm) is the growth of lettuce roots in the medium free of Cu^{2+}, Zn^{2+}, and Ag^+; coefficients c_1, c_2, and c_3 (L/μmol) represent the strength of toxicity of Cu^{2+}, Zn^{2+}, and Ag^+ individually as well as in non-interactive mixtures, respectively; d (dimensionless) is the slope parameter describing toxicity of these metals individually as well as in non-interactive mixtures; and $\{Cu^{2+}\}$, $\{Zn^{2+}\}$, and $\{Ag^+\}$ (μmol/L) are the free ion activity of Cu^{2+}, Zn^{2+}, and Ag^+ in the solution, respectively.

In the RA model, mixture components are supposed to have different modes of action of toxicity (Hewlett and Plackett, 1979). Therefore, based on the RA concept, the response of lettuce exposed to non-interactive mixtures can be expressed as a multiplicative function of the response of the plants following exposure to each constituent separately (Eqn. S4.4) (Kinraide, 1999):

$$\text{Growth} = \frac{b}{\prod \exp[(c_i \times \{M^{n+}\})^{d_i}]} = \frac{b}{\exp[\sum(c_i \times \{M^{n+}\})^{d_i}]} \tag{S4.4}$$

For example, when Cu^{2+}, Ag^+, and Zn^{2+} do not affect toxicity of one another, the growth of lettuce exposed to mixtures of these three metal ions can be written as a multiplication of the responses of lettuce following exposure to these metal ions individually (Eqn. S4.5):

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\})^{d_1} + (c_2 \times \{Zn^{2+}\})^{d_2} + (c_3 \times \{Ag^+\})^{d_3}]} \tag{S4.5}$$

where coefficient b (mm) is the growth of lettuce roots in the medium free of Cu^{2+}, Ag^+, and Zn^{2+}; coefficients c_1, c_2, and c_3 (L/μmol) represent strength of toxicity of Cu^{2+}, Ag^+, and Zn^{2+} individually as well as in non-

interactive mixtures, respectively; d_1, d_2, and d_3 (dimensionless) are slope parameters describing toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually as well as in their non-interactive mixtures; and $\{Cu^{2+}\}$, $\{Zn^{2+}\}$, and $\{Ag^{+}\}$ (μmol/L) are the free ion activity of Cu^{2+}, Ag^{+}, and Zn^{2+} in the exposure solution, respectively.

Equations S4.2-S4.5 are based on the conventional concepts of the CA and RA models assuming no interactions between mixture components, i.e., the presence of one metal does not affect the toxicity of another. In other words, toxicity of metals following exposure to these non-interactive mixtures is similar to the toxicity of these metals individually.

Toxicity of interactive mixtures

When metals in the mixtures are interactive, the interactions can be taken into account in determining the mixture toxicity by expanding the conventional CA and RA models. In particular, strength coefficients c_1, c_2, and c_3 in Equations S4.3 and S4.5 are linearly extended to include expansion coefficients. For example, based on an assumed linear interaction between Cu^{2+} and Zn^{2+}, the expanded strength coefficient describing the Cu^{2+} toxicity in interactive mixtures with Zn^{2+} (c_1^*) can be expanded as in Equation S4.6 or S4.7 (Kinraide et al., 2004):

$$c_1^* = c_1 \times (1 + c_{12} \times \{Zn^{2+}\}) \quad \text{(S4.6)}$$

or

$$c_1^* = \frac{c_1}{1 + c_{12} \times \{Zn^{2+}\}} \quad \text{(S4.7)}$$

where c_{12} (L/μmol) is the expansion coefficient, representing interactive effects of Zn^{2+} on Cu^{2+} toxicity. In Equation S4.6, Zn^{2+} enhances toxicity of Cu^{2+} if c_{12} is positive while in Equation S4.7, Zn^{2+} increases Cu^{2+} toxicity if c_{12} is negative. By contrast, alleviative effects of Zn^{2+} on Cu^{2+} toxicity occur when c_{12} in Equation S4.6 is negative or when c_{12} in Equation S4.7 is positive. The interactive effects are found to be statistically significant when the 95% confidence interval (CI) of c_{12} does not encompass zero. Similar equations can be written to integrate ion-ion interactions in modelling toxicity of Zn^{2+} in mixtures with Cu^{2+}. If interactions only affect toxicity of one component of the mixture, the strength coefficient of only this substance is extended. All the coefficients were determined by multiple nonlinear regression analyses using the SYSTAT software.

Table S4.2. Estimates of the coefficients in Equation 4.2 and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), determined by the regression analysis representing toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in non-interactive mixtures according to the concentration addition model (n = 238; r^2 = 0.83)

Coefficient	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	50.02	1.23	40.87	47.78	52.62
c_1 (L/μmol)	Strength of Cu^{2+} toxicity	2.92	0.19	15.23	2.54	3.30
c_2 (L/μmol)	Strength of Zn^{2+} toxicity	$6.15 \cdot 10^{-3}$	$0.44 \cdot 10^{-3}$	14.12	$5.29 \cdot 10^{-3}$	$7.01 \cdot 10^{-3}$
c_3 (L/μmol)	Strength of Ag^{+} toxicity	2.93	0.21	13.95	2.52	3.34
d (dimensionless)	Slope	1.30	0.11	11.43	1.07	1.52

Table S4.3. Estimates of the coefficients in Equation 4.3 and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), determined by the regression analysis representing toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in non-interactive mixtures according to the response addition model (n = 238; r^2 = 0.86)

Coefficient	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.12	0.92	53.39	47.31	50.93
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	3.13	0.21	14.75	2.71	3.55
d_1 (dimensionless)	Slope of Cu^{2+} toxicity curve	1.02	0.11	10.56	0.83	1.21
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$6.31 \cdot 10^{-3}$	$0.46 \cdot 10^{-3}$	13.80	$5.41 \cdot 10^{-3}$	$7.21 \cdot 10^{-3}$
d_2 (dimensionless)	Slope of Zn^{2+} toxicity curve	1.45	0.17	8.49	1.11	1.79
c_3 (L/µmol)	Strength of Ag^{+} toxicity	3.79	0.15	25.99	3.50	4.08
d_3 (dimensionless)	Slope of Ag^{+} toxicity curve	2.89	0.38	7.58	2.14	3.65

Chapter 5

Modeling toxicity of binary metal mixtures (Cu^{2+}–Ag^{+}, Cu^{2+}–Zn^{2+}) to lettuce, *Lactuca sativa*, with the Biotic Ligand Model

T.T. Yen Le, Martina G. Vijver, A. Jan Hendriks, Willie J.G.M. Peijnenburg

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Abstract—The Biotic Ligand Model (BLM) was applied to predict metal toxicity to lettuce *Lactuca sativa*. Cu^{2+} had the lowest median effective activity ($EA50_M$), compared to Ag^+ and Zn^{2+} ($EA50_{Cu}$ = 2.60· 10^{-8} mol/L; $EA50_{Ag}$ = 1.34· 10^{-7} mol/L; $EA50_{Zn}$ = 1.06· 10^{-4} mol/L). At the 50% response level, the fraction of the total number of biotic ligands occupied by ions ($f50_M$) was lowest for Ag^+ among the metals ($f50_{Ag}$ = 0.22; $f50_{Cu}$ = 0.36; $f50_{Zn}$ = 0.42). Cu^{2+} had the highest affinity for biotic ligands compared to Ag^+ and Zn^{2+} as shown by stability constants of the cation-biotic ligand binding, expressed as $\log K_{MBL}$ ($\log K_{CuBL}$ = 7.40; $\log K_{AgBL}$ = 6.39; $\log K_{ZnBL}$ = 4.00). Furthermore, the BLM was combined with the toxic equivalency factor approach in predicting toxicity of mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+. The fraction of biotic ligands occupied by ions was used to determine the relative toxic potency of metals and the toxic equivalency quotient (TEQ) of mixtures. This approach allowed including interactions in estimating mixture toxicity and showed good predictive power (r^2 = 0.64–0.84). The TEQ at the 50% response level (TEQ50; Cu^{2+} equivalents) for Cu^{2+}–Zn^{2+} mixtures were significantly lower than the value for Cu^{2+}–Ag^+ mixtures. Joint toxicity depended on both TEQ and specific composition of the mixture. The present study supports using the accumulation of metal ions at the biotic ligands as a predictor of toxicity of single metals and mixtures. Environ. Toxicol. Chem. DOI 10.1002/etc.2039.

Keywords — Metal mixtures Plants Biotic Ligands Toxic Equivalency Factor Stability Constant

5.1. Introduction

The Biotic Ligand Model (BLM) is usually applied to predict toxicity of single metals, taking into account effects of common cations, e.g., H^+, Ca^{2+}, and Mg^{2+}. According to the BLM concept, ions compete with each other for transport sites at the biotic ligands and this competition acts as a mechanism for ion-ion interactions (Niyogi and Wood, 2004; Di Toro et al., 2001). This assumption is based on physiological findings, which indicate that toxic cations, e.g., Cu^{2+} and Ag^+, may inhibit the uptake of Na^+ or Ca^{2+} for specific binding sites at the fish gill, leading to adverse effects (Morgan et al., 1997; Bury et al., 1999; Wood et al., 1996). Furthermore, the assumption potentially allows taking into account interactions between different metal ions in assessment of mixture toxicity (Niyogi and Wood, 2004; Norwood et al., 2003; Borgmann et al., 2008). In particular, it is possible to predict how different metals interact with one another if their stability constants are known. If two metals compete for binding to the same site of toxic action, the total amount of the metals bound to the site would be a key property, determining mixture toxicity. Alternatively, if competitive binding does not occur following exposure to metal mixtures, bioavailability of each component estimated by the BLM can be a reliable predictor of mixture toxicity through the effects addition model.

In the present study, the assumption of competitive binding was applied to metal mixtures, i.e., metals following exposure to their mixtures may compete for transport sites at the biotic ligands. This assumption is supported by the observation that the uptake of metals usually involves transporter proteins (Simkiss and Taylor, 1995). Furthermore, based on the functions of the transporters, the physiological mechanism of metal binding can be classified into three categories (Niyogi and Wood, 2003). According to this classification, Cu^{2+} and Zn^{2+} may bind to the same transporters, which are responsible for the uptake of divalent cations, while the uptake of Ag^+ is related to the participation of transporters for monovalent cations. Consequently, mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+ were chosen in the present study as an

attempt to model toxicity of both competitive and non-competitive mixtures with the assumption that Cu^{2+} and Zn^{2+} compete for binding sites at the biotic ligands whereas this competition does not occur between Cu^{2+} and Ag^{+}.

According to the BLM concepts, toxic effects result from binding of metal ions to biotic ligands. In other words, the extent of toxic effects is determined by the fraction of the biotic ligands occupied by metal ions in the total number of biotic ligands. This fraction was used as a basic unit in the toxic unit approach for estimating toxicity of metal mixtures to duckweed *Lemna paucicostata* by Hatano and Shoji (2008) and to bacteria *Vibrio fishcheri* by Jho et al. (2011). This modelling approach was found to result in higher predictive potential than the free ion activity model and the total metal concentration model. In other words, the accumulation of metal ions at the biotic ligands was a better indicator of toxicity of metal mixtures compared to metal concentrations or activities in the solution. As both the BLM and the free metal ion activity model are based on the assumption that free ions are the main reactive species of metals determining metal toxicity, these authors further suggested that the advantage of the BLM over the other models was attributed to the integration of competitive binding of metal ions to biotic ligands.

The toxic equivalency factor (TEF) approach has been widely used in assessment of mixture toxicity. In this approach, a substance is used as a reference compound (TEF = 1) to which others are related (Birnbaum and DeVito, 1995). The TEF of each mixture component reflects its toxic potency relative to the reference compound. This approach is usually performed based on concentrations of substances in mixtures. Particularly, the toxic equivalency quotient (TEQ) of mixtures is calculated as the sum of the equivalency concentration of each component which is a product of the concentration and the TEF of the compound. Therefore, the exposure level can be expressed by a single concentration of the reference substance, which represents the overall toxicity of the mixture assuming no interactions between different components. However, the concentration of metals in the solution might not be a reliable predictor of their toxicity as chemical properties of the exposure medium, such as chelators and pH, affect binding of the metals with biotic and abiotic ligands, influencing metal uptake (Cheng and Allen, 2001).

The present study aimed to predict toxicity of binary metal mixtures (Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+}) to lettuce, *Lactuca sativa*, by combining the BLM and the TEF approach for the first time. In particular, the accumulation of metals at the biotic ligands, which determines toxicity of the metals following single exposure according to the BLM principle, was used to determine the TEF of the metals in mixtures. The accumulation of metals at the biotic ligands is, in turn, influenced by interactions between the metals and other competing cations. As such, interactions between different metal ions in mixtures at the biotic ligands can be integrated in estimating toxicity of metal mixtures, overcoming disadvantages of the conventional TEF approach based on metal concentrations.

5.2. Methods

5.2.1. Toxicity assays

Metal toxicity was assessed in terms of relative root elongation (RRE; %) in hydroponic experiments using Steiner solution as the test medium (Le et al., 2012). Cu^{2+}, Zn^{2+}, and Ag^{+} were added to the Steiner solution as nitrate salts while the concentrations of other cations in the solution were kept at the background level of the default medium. The solution pH was kept at 7 by using the 3-[N-morpholino] propane sulfonic acid buffering (Le et al., 2012).

5.2.2. Metal measurements and speciation

Free Zn^{2+} activities in solutions were derived from the total Zn^{2+} concentrations in the exposure solution, which were the sum of the Zn^{2+} concentration in Steiner solution and the Zn^{2+} concentration added. The determination of free Zn^{2+} activities was performed by the speciation model Windermere Humic-Aqeous Model VI with Steiner solution as the default medium (Tipping, 1998). The chemical composition of the Steiner solution used for chemical speciation is given in Table S5.1, Supplementary information. In addition, free ion activities of H^+, Cu^{2+}, and Ag^+ were measured by using Hydrogen, Copper, and Silver sulfide ion-selective electrodes (Metrohm, Switzerland) which had been calibrated at different concentrations of these cations in the solution (Le et al., 2012). The ranges of free ion activities of Cu^{2+}, Zn^{2+}, and Ag^+ in the solutions tested in the present study were: Cu^{2+}: 10^{-10}–10^{-6} mol/L; Zn^{2+}: 10^{-6}–10^{-3} mol/L; and Ag^+: 10^{-8}–10^{-7} mol/L.

5.2.3. Toxicity of single metals

Free metal ions were considered the main reactive species, determining toxicity of the metals. As reviewed in a previous study, different results were reported on the effects of common cations, e.g., Na^+, K^+, Ca^{2+}, and Mg^{2+}, on toxicity of Cu^{2+} (Le et al., 2012). This difference may be related to the species and the exposure levels investigated. The present study and the study of Le et al. (2012) were carried out on the same plant species and with the same test medium. As a result of these similarities, the findings reported in the study of Le et al. (2012) were applied in the present research. According to these authors, Cu^{2+} toxicity to lettuce *Lactuca sativa* was significantly inhibited by protons (Le et al., 2012). The study also indicated that effects of Na^+, Ca^{2+}, Mg^{2+}, and K^+ on Cu^{2+} toxicity to this plant species could not be quantified by the BLM as the pattern of these impacts was inconsistent at the concentration range studied. Therefore, in the present study, we assumed that H^+ competes with toxic cations, i.e., Cu^{2+}, Ag^+, and Zn^{2+}, for binding sites at the biotic ligand while keeping concentrations of all other cations at the background level of the default medium. In other words, the accumulation of these toxic ions, which determines their toxicities according to the BLM principle, is influenced by binding of H^+ with biotic ligands. Accordingly, the faction of the total number of biotic ligands that is occupied by metal ion M^{n+}, i.e., Cu^{2+}, Ag^+, or Zn^{2+}, (f_M) is determined as follows:

$$f_M = \frac{[MBL]}{[BL]_T} = \frac{K_{MBL} \times \{M^{n+}\}}{1 + K_{HBL} \times \{H^+\} + K_{MBL} \times \{M^{n+}\}} \quad (5.1)$$

where $[BL]_T$ (mol/L) is the total number of biotic ligands; K_{HBL} and K_{MBL} (L/mol) are stability constants of binding of H^+ and M^{n+} to biotic ligands, respectively; and $\{H^+\}$ and $\{M^{n+}\}$ (mol/L) are free ion activities of H^+ and M^{n+} in the solution, respectively.

The free ion activity of metal ion M^{n+} in the solution that results in a 50% reduction in the growth of lettuce roots is termed as the median effective activity $EA50_M$. A detailed description of the derivation of a BLM for single metals, e.g., determination of the stability constant K_{MBL} and the fraction of the total number of biotic ligands occupied by metal ions at the 50% response level $f50_M$, were presented in a previous study (Le et al., 2012). Specifically, $f50_M$ was determined by fitting a sigmoid curve to the relationships between f_M and the RRE using Graphpad Prism software according to the following equation:

$$RRE = \frac{100}{1 + 10^{(f50_M - f_M) \times \beta}} \quad (5.2)$$

5.2.4. Toxicity of binary metal mixtures (Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+})

Toxicity of mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} was modelled from the toxicological data for the single metals, i.e., K_{MBL} and $f50_M$, by combining the BLM and the TEF approach. Particularly, the TEF of metals in mixtures as well as the TEQ of mixtures were determined based on the fraction of the total number of biotic ligands occupied by metal ions. Cu^{2+} was considered the reference metal ion to which toxicity of Zn^{2+} and Ag^{+} was related. This selection was used because of the presence of Cu^{2+} in both mixtures studied and high concern about its environmental effects and subsequent large availability of toxicological data. Accordingly, the TEF of metals in mixtures (TEF_M; M denotes Cu^{2+}, Zn^{2+}, or Ag^{+}) was determined according to the following equation:

$$\mathrm{TEF_M} = \frac{f50_{\mathrm{Cu}}}{f50_{\mathrm{M}}} \tag{5.3}$$

where $f50_M$ and $f50_{Cu}$ are the fractions of the biotic ligands occupied by M^{n+} and Cu^{2+} in the total number of biotic ligands at the 50% response level following exposure to these metal ions individually, respectively (Le et al., 2012). TEF_M represents the comparative toxic potency of metal ion M^{n+} in mixtures. Furthermore, the TEQ of mixtures (Cu^{2+} equivalents), which reflects the overall toxicity of the mixture, was calculated from the TEF_M of mixture components and the fraction of the total number of biotic ligands occupied by metal ions in the mixture f_M according to Equation 5.4:

$$\mathrm{TEQ} = \sum\left(f_{\mathrm{M}} \times \mathrm{TEF_M}\right) \tag{5.4}$$

Equations 5.3 and 5.4 were derived from the common expression of the TEF approach based on substance concentrations (Birnbaum and DeVito, 1995). According to the BLM, f_M is determined based on assumed independence of the complexation capacity of ions on water quality characteristics (Le et al., 2012; De Schamphelaere and Janssen, 2002). The calculation of f_M following exposure to metal mixtures was based on the stability constant K_{MBL} determined from the toxicological data following exposure to single metals as presented below. Toxic effects, in terms of root growth represented by RRE (%), were expressed in relation to the TEQ (Cu^{2+} equivalents) according to the following equation:

$$\mathrm{RRE} = \frac{100}{1+10^{(\mathrm{TEQ50}-\mathrm{TEQ})\times\beta}} \tag{5.5}$$

where TEQ50 (Cu^{2+} equivalents) is the TEQ of the mixture at the 50% response level and β (dimensionless) is the slope parameter. Estimates of these coefficients and statistic parameters, e.g., 95% confidence interval (CI), were determined by fitting the empirical data on toxicity of metal mixtures to Equation 5.5 using the GraphPad Prism software.

Mixtures of Cu^{2+} and Zn^{2+}

Taking into account competition between Cu^{2+} and Zn^{2+} and between Cu^{2+}/Zn^{2+} with H^{+}, the fraction of the total number of biotic ligands occupied by Cu^{2+} and Zn^{2+} (f_{Cu} and f_{Zn}, respectively) can be determined as a function of their stability constants and their free ion activities in the solution as follows:

$$f_{\mathrm{Cu}} = \frac{K_{\mathrm{CuBL}} \times \{\mathrm{Cu^{2+}}\}}{1 + K_{\mathrm{ZnBL}} \times \{\mathrm{Zn^{2+}}\} + K_{\mathrm{CuBL}} \times \{\mathrm{Cu^{2+}}\} + K_{\mathrm{HBL}} \times \{\mathrm{H^{+}}\}} \tag{5.6}$$

$$f_{\mathrm{Zn}} = \frac{K_{\mathrm{ZnBL}} \times \{\mathrm{Zn^{2+}}\}}{1 + K_{\mathrm{ZnBL}} \times \{\mathrm{Zn^{2+}}\} + K_{\mathrm{CuBL}} \times \{\mathrm{Cu^{2+}}\} + K_{\mathrm{HBL}} \times \{\mathrm{H^{+}}\}} \tag{5.7}$$

where K_{HBL}, K_{CuBL}, and K_{ZnBL} (L/mol) are stability constants of binding of H^+, Cu^{2+}, and Zn^{2+} to biotic ligands, respectively, and were determined by toxicological data for single metals; and $\{H^+\}$, $\{Cu^{2+}\}$, and $\{Zn^{2+}\}$ (mol/L) are free ion activities of H^+, Cu^{2+}, and Zn^{2+} in the exposure solution, respectively.

Mixtures of Cu^{2+} and Ag^+

As it was assumed that Cu^{2+} and Ag^+ bind to different transporters, binding of these metal ions to transport sites at the biotic ligands is only influenced by H^+ (Eqns. 5.8 and 5.9):

$$f_{Cu} = \frac{K_{CuBL} \times \{Cu^{2+}\}}{1 + K_{CuBL} \times \{Cu^{2+}\} + K_{HBL} \times \{H^+\}} \quad (5.8)$$

$$f_{Ag} = \frac{K_{AgBL} \times \{Ag^+\}}{1 + K_{AgBL} \times \{Ag^+\} + K_{HBL} \times \{H^+\}} \quad (5.9)$$

where K_{HBL}, K_{CuBL}, and K_{AgBL} (L/mol) are stability constants of binding of H^+, Cu^{2+}, and Ag^+ to biotic ligands, respectively, and were estimated by experimental data on toxicity of single metals; and $\{H^+\}$, $\{Cu^{2+}\}$, and $\{Ag^+\}$ (mol/L) are free ion activities of H^+, Cu^{2+}, and Ag^+ in the exposure solution, respectively.

5.3. Results

5.3.1. Toxicity of Cu^{2+}, Ag^+, and Zn^{2+} individually

Generally, Zn^{2+} had the lowest affinity for binding sites at the biotic ligands as shown by its lowest stability constant as compared to Cu^{2+} and Ag^+ (i.e., $\log K_{ZnBL} < \log K_{AgBL} < \log K_{CuBL}$; Table 5.1). This indicates that the formation of complexes of the biotic ligands with Zn^{2+} occurred at a lower extent than the formation of complexes with Cu^{2+} or Ag^+. At the same time, the highest fraction of the total number of biotic ligands occupied by Zn^{2+} was required to result in a 50% inhibition of the root growth (Table 5.1 and Fig. 5.1A), i.e., at the same concentration, the Zn^{2+}-biotic ligand complex resulted in the lowest toxic effects in comparison with the complexes of Cu^{2+} and Zn^{2+}. Consequently, Zn^{2+} had the highest value of the median effective activity among the three metal ions studied (Table 5.1 and Fig. 5.1B). By contrast, Cu^{2+} had the highest affinity for binding sites at the biotic ligands and the lowest median effective activity compared to Zn^{2+} and Ag^+ (Table 5.1 and Fig. 5.1B). Additionally, the fraction of the total number of biotic ligands occupied by Ag^+ to inhibit the root growth by 50% was lower than the corresponding fraction occupied by Cu^{2+} or Zn^{2+} (i.e., $f50_{Ag} < f50_{Cu} < f50_{Zn}$; Table 5.1 and Fig. 5.1A). In other words, the Ag^+-biotic ligand complex led to the highest toxic effects compared to the complexes of Cu^{2+} and Zn^{2+}, contributing to the highest slope of the curve describing the relationship between the RRE and f_M for Ag^+ (Fig. 5.1A and Table S5.2, Supplementary information).

5.3.2. Toxicity of binary metal mixtures (Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+)

Generally, the combination of the BLM and the TEF approach using the TEF values for Cu^{2+}, Ag^+, and Zn^{2+} calculated from $f50_M$ (Table 5.2) performed equally well in estimating the toxicity of Cu^{2+}–Zn^{2+} and of Cu^{2+}–Ag^+ mixtures as indicated by a negligible difference between the values of r^2 (Fig. 5.2 and Table 5.2). Approximately, 70% of the variability in the toxicity of mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+ could be explained by the TEQ based on the fraction of the total number of biotic ligands occupied by metal ions (r^2 = 0.65–0.69).

Table 5.1. Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually to plants as expressed by the median effective activity ($EA50_M$; mol/L) and BLM parameters, i.e., the fraction of the total number of biotic ligands occupied by metal ions at the 50% response level ($f50_M$) and the stability constant ($\log K_{BML}$; L/mol) as found in the present study and in other literature reports. 95% confidence interval (CI) is shown. Toxic equivalency factor (TEF_M; Cu^{2+} equivalents) is determined according to Equation 5.3 based on the determined values of $f50_M$.

Metals	*Vigna unguiculata*	*Lactuca sativa*				*Hordeum vulgare*				
	$EA50_M$ (mol/L)	**$EA50_M$ (mol/L; 95% CI)**	***f*50_M (95% CI)**	**$\log K_{MBL}$**	**TEF_M**	***f*50_M**	**$\log K_{MBL}$**	**$\log K_{MBL}$**	***f*50_M**	**$\log K_{MBL}$**
Cu^{2+}	$2.90 \cdot 10^{-7}$	$2.60 \cdot 10^{-8}$ ($1.87 \cdot 10^{-8}$-$3.61 \cdot 10^{-8}$)	0.36 (0.29-0.43)	7.40	1			7.4±0.2	0.44	6.28
Ag^{+}	$2.4 \cdot 10^{-8}$	$1.34 \cdot 10^{-7}$ ($1.19 \cdot 10^{-7}$-$1.50 \cdot 10^{-7}$)	0.22 (0.20-0.24)	6.39	1.64					
Zn^{2+}	$1.6 \cdot 10^{-5}$	$1.06 \cdot 10^{-4}$ ($9.11 \cdot 10^{-5}$-$1.24 \cdot 10^{-4}$)	0.42 (0.38-0.44)	4.00	0.86	0.38	4.06			
Source	Kopittke et al. (2011)	Present study				Wang et al. (2010)		Thakali et al. (2006)	Luo et al. (2008)	

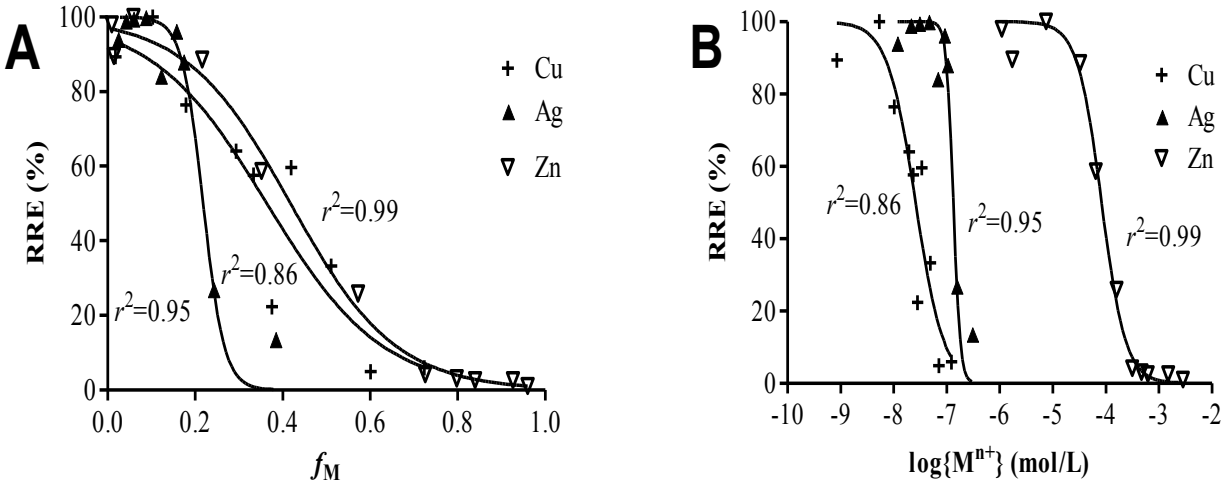

Figure 5.1. Dose-response curves describing toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually are expressed by the relationship between the relative root elongation (RRE; %) and the fraction of the total biotic ligands occupied by metal ions (f_M) (A) and the free metal ion activity in the solution (log{M^{n+}}; mol/L) (B)

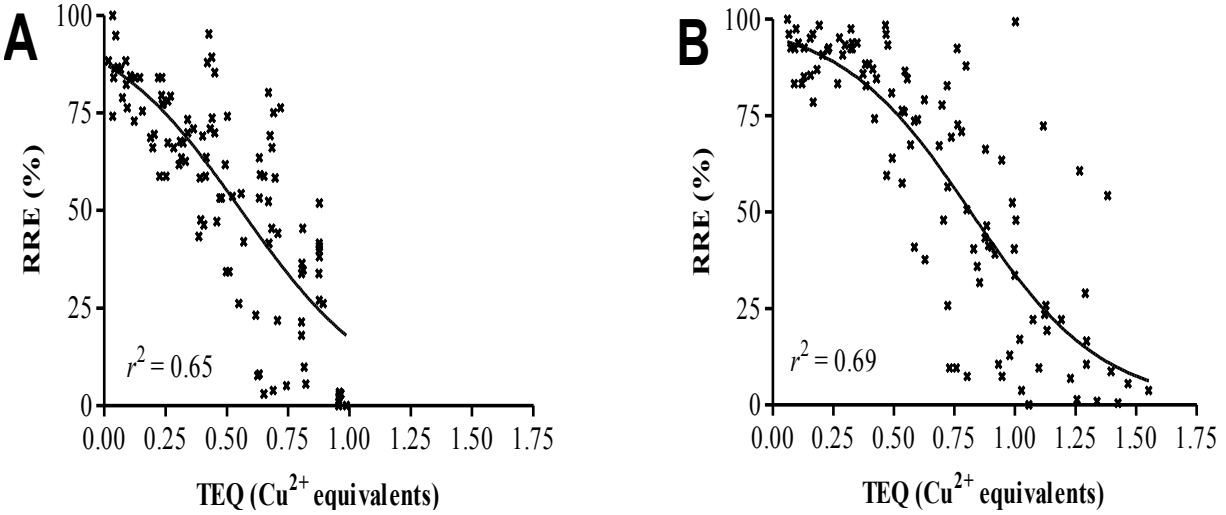

Figure 5.2. Toxic effects of the mixtures of Cu^{2+}–Zn^{2+} (A) and of Cu^{2+}–Ag^{+} (B) are expressed as the relative root elongation (RRE; %). The RRE is plotted as a function of the toxic equivalency quotient (TEQ; Cu^{2+} equivalents) according to Equation 5.5

Moreover, the TEQ50 (Cu^{2+} equivalents) of the Cu^{2+}–Zn^{2+} mixture was lower than the corresponding value for the Cu^{2+}–Ag^{+} mixture (Fig. 5.2 and Table 5.2). This difference is significant since the 95% CIs of the TEQ50 for the two mixtures were statistically significantly deviating from each other (Table 5.2). These results indicate that mixtures of Cu^{2+} and Zn^{2+} were significantly more toxic than mixtures of Cu^{2+} and Ag^{+} based on Cu^{2+} equivalents. Additionally, no significant difference was found between the slopes of the dose-response curves describing the toxicity of the mixtures of Cu^{2+}–Zn^{2+} and of Cu^{2+}–Ag^{+} (Fig. 5.2 and Table 5.2). This similarity in the slopes for the two mixtures was explained by the similar meaning of the two curves, i.e., representing the changes in the RRE with the changes in Cu^{2+} equivalents. This explanation is supported by the overlapping 95% CIs of these slopes with the slope of the curve describing the relationship between f_{Cu} and RRE (Table 5.2 and Table S5.2, Supplementary information).

Table 5.2. Estimations of coefficients, i.e., toxic equivalency quotient at the 50% response level TEQ50 (Cu^{2+} equivalents) and slope parameter β (dimensionless), in Equation 5.5 and statistical parameters representing the toxicity of mixtures of Cu^{2+}–Zn^{2+} and of Cu^{2+}–Ag^{+}. 95% confidence intervals (CI) are shown.

Parameters	Cu^{2+}–Zn^{2+}	Cu^{2+}–Ag^{+}	Cu^{2+}–Ag^{+} $\{Cu^{2+}\} > 2 \cdot 10^{-8}$ mol/L	Cu^{2+}–Ag^{+} $\{Cu^{2+}\} < 2 \cdot 10^{-8}$ mol/L
TEQ50	0.56	0.82	0.88	0.61
(95% CI)	(0.51-0.60)	(0.76-0.87)	(0.82-0.93)	(0.58-0.64)
β	-1.534	-1.599	-1.896	-3.701
(95% CI)	(-1.82 to -1.25)	(-1.93 to -1.27)	(-2.46 to -1.33)	(-4.56 to -2.84)
n	111	107	62	45
r^2	0.65	0.69	0.64	0.84

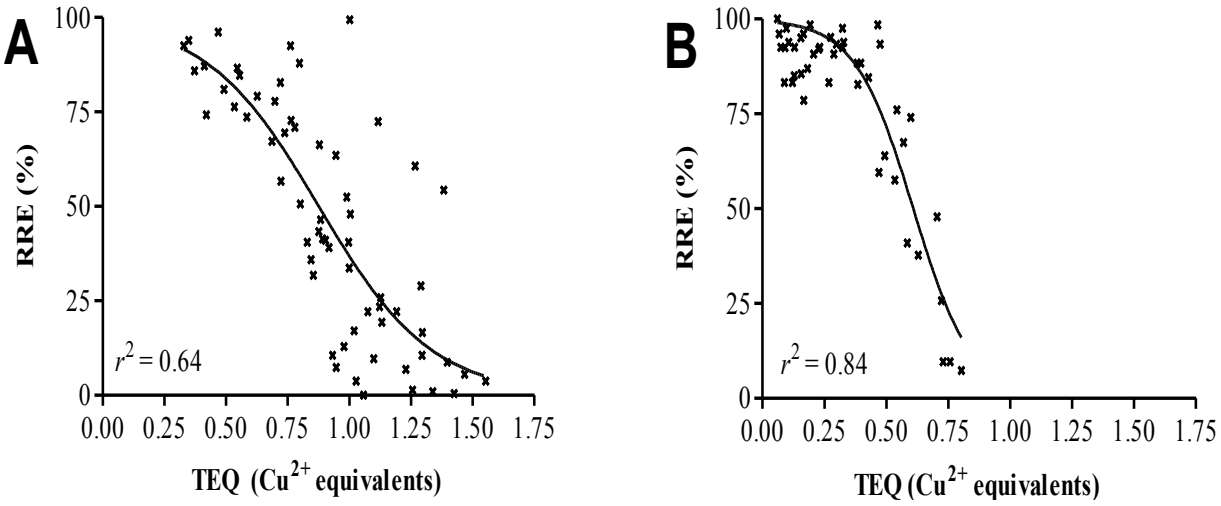

Figure 5.3. The response of lettuce roots exposed to mixtures of Cu^{2+} and Ag^{+} expressed as the relative root elongation (RRE; %) is plotted as a function of the toxic equivalency quotient of mixtures (TEQ; Cu^{2+} equivalent) at two levels of the free ion activity of Cu^{2+} in the exposure solution: $\{Cu^{2+}\} > 2 \cdot 10^{-8}$ (mol/L) (A) and $\{Cu^{2+}\} < 2 \cdot 10^{-8}$ (mol/L) (B)

Noticeably, substantial deviations were found in measurements from predictions for a number of mixtures of Cu^{2+} and Ag^{+} (Fig. 5.2B). These deviated data points correspond to mixtures in which the free Cu^{2+} activities in these mixtures are all below $2 \cdot 10^{-8}$ (mol/L). Additionally, a shift in the trend of toxic effects over this exposure level of Cu^{2+} was shown in the dose-response curve describing toxicity of Cu^{2+} following single exposure (Fig. 5.1B). Particularly, at the free Cu^{2+} activities below $2 \cdot 10^{-8}$ (mol/L), toxic effects of Cu^{2+} did not increase with an increase in the exposure level of Cu^{2+}. These results may be related to the fact that copper is an essential element and apparently, $2 \cdot 10^{-8}$ (mol/L) is the lower level of the optimal activity range, i.e., at which the growth of lettuce roots is not inhibited. Therefore, we divided the exposure solutions containing Cu^{2+}–Ag^{+} mixtures into two categories on the basis

of the free ion activity of Cu^{2+} in the solution: $\{Cu^{2+}\} > 2 \cdot 10^{-8}$ (mol/L) (Fig. 5.3A) and $\{Cu^{2+}\} < 2 \cdot 10^{-8}$ (mol/L) (Fig. 5.3B). A similar cut-off value was not found for the mixtures of Cu^{2+} and Zn^{2+}.

The above method of classifying the mixtures of Cu^{2+} and Ag^{+} based on the cut-off value of $2 \cdot 10^{-8}$ (mol/L) led to substantial improvement in estimating toxicity of the Cu^{2+}–Ag^{+} mixtures at low free ion activities of Cu^{2+} in the exposure solution ($r^2 = 0.84$; Fig. 5.3B and Table 5.2). Moreover, a statistically significant difference was found between the estimated toxicities of the two classified mixture groups as shown by non-overlapping 95% CIs of the TEQ50 (Table 5.2). This indicates that the toxicity of Cu^{2+}–Ag^{+} mixtures did not depend on their TEQ only, but also on the exact amount of their components (Fig. 5.3 and Table 5.2). With the same TEQ below approximately 0.8, mixtures with lower free ion activities of Cu^{2+} in the solution (i.e., $\{Cu^{2+}\} < 2 \cdot 10^{-8}$ mol/L) were statistically significantly more toxic than mixtures with higher $\{Cu^{2+}\}$. This is additionally indicated by a statistically significantly steeper dose-response curve describing toxicity of the mixture with $\{Cu^{2+}\} < 2 \cdot 10^{-8}$ mol/L compared to the curve with $\{Cu^{2+}\} > 2 \cdot 10^{-8}$ mol/L (Figs. 5.3A and 5.3B and Table 5.2). This is in support of the above suggestion that the value of $2 \cdot 10^{-8}$ mol/L may be the lower level of the optimal activity range.

5.4. Discussion

5.4.1. Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually

The results found in the present study indicate that the BLM parameters are better indicators of the intrinsic toxicity of single metals than $EA50_M$. The BLM parameters provide a mechanistic explanation for the difference in comparison of toxicity of Cu^{2+} and Ag^{+} based on $EA50_M$ and $f50_M$ as shown in the *Results* section. In particular, as the Ag^{+}-biotic ligand complex resulted in higher effects than the Cu^{2+}-biotic ligand complex, the lower affinity of Ag^{+} for binding sites at the biotic ligands accounts for the higher median effective activity of Ag^{+} compared to the corresponding value for Cu^{2+}. In other words, the inclusion of interactions between ions and biotic ligands at the environment-organism interface contributes to the different orders of toxicity of Cu^{2+} and Ag^{+} based on $EA50_M$ and on $f50_M$. These observations strongly indicate that the BLM parameters provide better insight into mechanisms of metal binding and toxicity compared to one single value of $EA50_M$.

Based on the median effective activity $EA50_M$, substantial differences were found between the sensitivity of cowpea *Vigna unguiculata* reported by Kopittke et al. (2011) and of lettuce *Lactuca sativa* found in the present study (Table 5.1). On the basis of the estimations of $EA50_M$, *Lactuca sativa* had higher tolerance to Zn^{2+} and Ag^{+}, but was more sensitive to Cu^{2+} than *Vigna unguiculata*. Moreover, the BLM parameters reflecting the toxicity of Cu^{2+} and Zn^{2+} individually obtained in the present study on lettuce *Lactuca sativa* were, in general, in the ranges reported in other studies on barley *Hordeum vulgare* (Wang et al., 2010; Thakali et al., 2006; Luo et al., 2008) (Table 5.1). This indicates inconsiderable differences in the sensitivity of *Lactuca sativa* and *Hordeum vulgare* to Cu^{2+} and Zn^{2+} based on the BLM parameters.

5.4.2. Toxicity of binary metal mixtures (Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+})

The present study shows strong dependence of mixture toxicity on the composition and proportion of the metal mixture, i.e., the TEQ of the mixture and the specific amount of Cu^{2+} in the mixture. This observation was previously reported by Sharma et al. (1999) and Hamm et al. (2006). This dependence is potentially attributed to physiological processes, which are

highly specific, depending on the exposure level. For example, for essential metals like Cu^{2+}, their presence above certain exposure levels is vital and beneficial for plant growth. By contrast, exposure to extremely low or extremely high concentrations of these elements is toxic to the growth of plants. This explanation potentially accounts for the observations on Cu^{2+} toxicity found in the present study, e.g., higher toxic effects caused by the mixtures with lower activities of Cu^{2+} among mixtures with the same TEQ below 0.8. Similarly, physiological responses of plants exposed to Zn^{2+} highly vary depending on the exposure level, ranging from changes in the plant cell vacuolization or in membrane permeability to damages to enzyme systems, respiration, or to photosynthetic apparatus (Luo and Rimmer, 1995; Kabata-Pendias and Pendias, 1984).

5.4.3. The integration of ion-ion interactions in estimating metal toxicity

In the present study, 64–84% of the variability in the toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures could be explained by TEQ (r^2 = 0.64–0.84). Together with the study of Hatano and Shoji (2008) and Jho et al. (2011), findings in the present study support the assumption that the fraction of the biotic ligands bound to metal ions in mixtures in the total number of biotic ligands might be indicative of toxicity of metal mixtures. As presented in the present study, the incorporation of the BLM into the extended TEF approach allows integrating interactions between different metal ions in estimating their joint toxicity based on particular assumptions about metal binding. However, an exact understanding of metal binding is usually lacking, causing difficulties in applying the BLM to predict toxicity of metal mixtures. Diverse binding of metals to a variety of biotic sites further complicates the issue. Moreover, in the BLM, ion-ion interactions are interpreted in terms of competition for binding sites at the biotic ligands. However, previous studies indicate that effects of the interactions on bioaccumulation and toxicity of single metals and mixtures could not be completely interpreted in terms of competitive binding to biotic ligands (Le et al., 2012; Thakali et al., 2006; Norwood et al., 2007). Apart from competition for binding sites, joint toxicity of multiple metals is influenced by a number of other mechanisms, e.g., the production of metal-binding proteins like metallothionein, changes in the permeability of the plasma membrane induced by exposure to metal mixtures, and interactions between essential and non-essential metals (Manzo et al., 2010; Pavicic et al., 1994; MacFarlane and Burchett, 2002; Belyaeva et al., 2004; Llamas et al., 2000; Viarengo, 1985; George, 1990). These mechanisms are not taken into account in estimating toxicity of metal mixtures by the BLM approach, potentially contributing to deviations of predictions from measurements.

In summary, the present study is in support of the BLM principle that the fraction of the total number of biotic ligands occupied by metal ions is a key indicator, determining metal toxicity. The BLM parameters provide a better understanding of metal binding and intrinsic toxicity of single metals. More importantly, the present study indicates the potential applicability of the BLM principle to metal mixtures. This was shown by a good predictive power of the combination of the BLM and the TEF approach, using the TEQ based on the fraction of the total number of biotic ligands bound to metal ions in estimating toxicity of metal mixtures. This modelling approach additionally allows integrating assumed ion-ion interactions in predicting joint toxicity of multiple metals, which are usually excluded in models estimating mixture toxicity. Furthermore, this method of integrating the fraction of biotic ligands occupied by metal ions to the TEF approach is applicable to mixtures of more than two substances, consisting of metals binding to the same or different sites at the biotic ligands.

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SUPPLEMENTARY INFORMATION

Table S5.1. Chemical composition of the Steiner solution used for chemical speciation

Metals	Na^{+}	Mg^{2+}	K^{+}	Ca^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}
Concentration (mol/L)	$1.02 \cdot 10^{-6}$	$1.83 \cdot 10^{-3}$	$6.98 \cdot 10^{-3}$	$2.82 \cdot 10^{-3}$	$1.16 \cdot 10^{-5}$	$4.96 \cdot 10^{-5}$	$1.72 \cdot 10^{-6}$

Table S5.2. The slopes (β; dimensionless) of the curves describing the relationships between the fraction of the total number of biotic ligands occupied by Cu^{2+}, Zn^{2+}, and Ag^{+} (f_{Cu}, f_{Zn}, and f_{Ag}, respectively) and the growth of lettuce roots expressed by the relative root elongation (RRE; %). 95% confidence intervals (CI) are given.

Metals	β	95% CI
Cu^{2+}	-3.307	-5.256 to -1.359
Zn^{2+}	-3.636	-4.354 to -2.918
Ag^{+}	-17.94	-27.60 to -8.284

Chapter 6

Integrating ion-ion interactions in modelling rhizotoxicity of metal mixtures to lettuce *Lactuca sativa* based on the electrostatic approach

T.T. Yen Le, Peng Wang, Martina G. Vijver, Thomas B. Kinraide, A. Jan Hendriks, Willie J.G.M. Peijnenburg

Submitted

Abstract—Effects of ion-ion interactions on metal toxicity to lettuce *Lactuca sativa* were studied based on the electrical potential at the plasma membrane (PM) surface (ψ_0). Ions interact with each other at the proximate outside of the membrane, affecting their activities at the PM surface ($\{M^{n+}\}_0$) (surface interactions). Additions of Na^+, K^+, Ca^{2+}, and Mg^{2+} reduced $\{Cu^{2+}\}_0$ substantially. Additions of Zn^{2+} also reduced $\{Cu^{2+}\}_0$, but Cu^{2+} and Ag^+ at the exposure levels tested had negligible effects on the activity of each other at the PM surface. Subsequently, interactions occurred between the free ions adsorbed, affecting metal toxicity (internal interactions). Metal toxicity was expressed by the strength coefficient and based on $\{M^{n+}\}_0$. Estimates of strength coefficients indicated a decrease of toxicity in the order of $Ag^+ > Cu^{2+} > Zn^{2+}$. Furthermore, the strength coefficient was expanded to assess internal interactions. Na^+, K^+, Ca^{2+}, and Mg^{2+} adsorbed to the membrane had significant and dose-dependent effects on Cu^{2+} toxicity in terms of osmolarity. Internal interactions between Cu^{2+} and Zn^{2+} and between Cu^{2+} and Ag^+ were modelled by expanding the strength coefficient of concentration addition (CA) and response addition (RA) models. These extended models consistently indicated that Zn^{2+} significantly reduced Cu^{2+} toxicity. According to the extended CA model, Ag^+ significantly increased Cu^{2+} toxicity while Cu^{2+} significantly reduced Ag^+ toxicity. In contrast, the RA model predicted insignificant effects of adsorbed Cu^{2+} and Ag^+ on the toxicity of each other. These results were interpreted using ψ_0, demonstrating its role in modelling multiple ion interactions and metal toxicity.

Keywords — Electrical potential Membrane surface Toxicity Metal mixtures Model

6.1. Introduction

Besides chemical speciation in the environment, interactions of ions with organisms are important in controlling metal bioavailability (Worms et al., 2006; Fairbrother et al., 2007). The interactions are therefore of high concern and increasingly integrated in estimating bioavailability and toxicity of metals. Interactions between ions and biotic ligands at the organism-water interface, for example, were integrated in predicting metal bioavailability in the Biotic Ligand Model (De Schamphelaere and Janssen, 2002).

Similar to interactions between ions and organisms at the biological surface, interactions between different ions influence metal bioavailability and toxicity. Biological actions of metal ions in mixtures might deviate from their actions singly (Manzo et al., 2010). For example, exposure to metal mixtures, e.g., Cu, Pb, and Zn, at concentrations below their individual environmental quality guideline levels resulted in adverse effects due to the interactions among these metals (Cooper et al., 2009). However, ion-ion interactions are usually excluded from the current models for mixtures. For example, in the concepts of concentration addition (CA) and response addition (RA or response multiplication), it was assumed that the presence of one substance does not have effects on the biological action of others (Loewe and Muischnek, 1926; Bliss, 1939; Kinraide, 1999). Consequently, deviations from ideal behaviour of mixtures (i.e., additivity) cannot be quantified by these models without modification of the conventional concepts.

The electrostatic approach has been applied in assessment of metal bioavailability and toxicity, taking into account plant-ion interactions at the cell membrane surface (Kinraide, 2006). The basis of this method is the importance of the electrical potential at the plasma membrane (PM) surface (denoted as ψ_0) in the uptake and transport of ions. ψ_0 is induced by the intrinsically negative charge at the PM surface and influences the interactions between ions and plants (Kinraide et al., 2004; Hassler et al., 2004; Kinraide, 2001, 2006; Yermiyahu

and Kinraide, 2005; Kinraide and Wang, 2010; Wang et al., 2011). The significance of ψ_0 is usually assessed through their dual roles in metal toxicity: effects on the ion distribution between the PM surface and the bathing medium (BM) and on the electrical driving force for ion transport through the PM (Kinraide et al., 2001; Wang et al., 2011). The ability to compute ψ_0 potentially enables investigation of ion-ion interactions by determining changes in the activity of ions at the PM surface with varying ionic composition of the BM. This method has been recently used to assess effects of common cations (e.g., Na^+, K^+, Ca^{2+}, and Mg^{2+}) on metal toxicity (Wang et al., 2008, 2011; Kopittke et al., 2011b). Yet, the approach has not been applied to mixtures of toxic metal ions.

Interactions between Cu^{2+} and Ag^+ have not been explicitly investigated previously. Although some studies demonstrated effects of Cu^{2+} and Zn^{2+} on the toxicity of each other to plants, a mechanistic delineation of the interactions at different biological surfaces is lacking (Otitoloju, 2002; Montvydiene and Marciulioniene, 2007; Ince et al., 1999; Dirilgen et al., 1994; Sresty and Rao, 1999; Luo and Rimmer, 1995). The present study aimed at integrating ψ_0 into the assessment of ion-ion interactions and metal rhizotoxicity to the root growth of lettuce, *Lactuca sativa*. The interactions were incorporated in estimating Cu^{2+} toxicity in the presence of common cations, i.e., Na^+, K^+, Ca^{2+}, and Mg^{2+}, and in predicting toxicity of mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+. Interactions are distinguished as surface interactions (1) and internal interactions (2) (Fig. 6.1). Surface interactions occur between ions at the proximate outside of the membrane, affecting the activity of ions at the PM surface. These interactions are directly responsive to ψ_0, i.e., the ionic composition of the BM influences the activity of ions at the PM surface through changes in ψ_0. Internal interactions occur between free ions adsorbed and affect metal toxicity. The role of ψ_0 in these interactions was indirectly expressed by the use of free metal ion activity at the PM surface as a key factor determining metal toxicity. The surface interactions were explicitly delineated by determining changes in ψ_0 while the internal interactions following exposure to metal mixtures were predicted by mathematically expanding models simulating concepts of CA and RA.

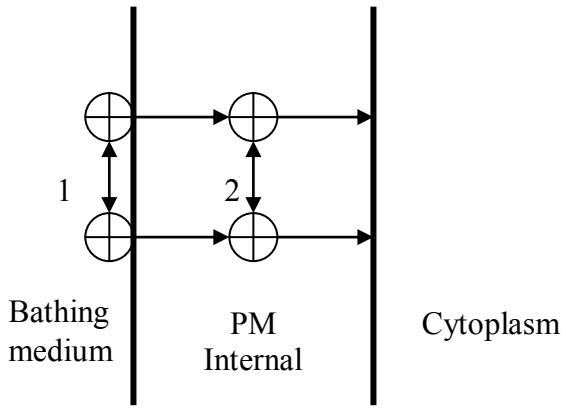

Figure 6.1. Interactions between free ions (denoted by the circles with a plus sign) at the proximate outside of the membrane (surface interactions) and between free ions adsorbed (internal interactions). PM stands for plasma membrane.

6.2. Methods

6.2.1. Test species and toxic endpoint

Toxic effects of metals were assessed on lettuce, *Lactuca sativa*, in terms of the inhibition of the root elongation after 4 d of exposure. This plant species was chosen because of its high capacity to accumulate metals (Garate et al., 1993; McKenna et al., 1993). Additionally,

Lactuca sativa was recommended by the Organisation for Economic Cooperation and Development (OECD, 2006).

6.2.2. Preparation of the test solutions

Steiner solution was used as the test medium (Steiner, 1961). The solution pH was stabilised by MOPS (3-[N-morpholino] propane sulfonic acid) buffering at 0.75 g/L and NaOH (Le et al., 2012). The MOPS buffer was used because of its negligible effects on biotic and non-biotic factors. Specifically, this chemical does not form complexes with metals (Kandegedara and Rorabacher, 1999). It does not affect the toxicity of effluents and sediment pore waters and, consequently, was recommended by the U.S. Environmental Protection Agency (US EPA, 1991). Moreover, at the concentration of 0.75 g/L, the MOPS-buffering does not influence metal toxicity to *Daphnia magna* and *Pseudokirchneriella subcapitata* (De Schamphelaere et al., 2004). Metal ions investigated (Cu^{2+}, Ag^{+}, and Zn^{2+}) were added into the Steiner solution as nitrate salts.

In copper and silver toxicity tests, pH and Cu^{2+}/Ag^{+} activities in the exposure solutions were checked daily and adjusted by adding HNO_3/KOH and $Cu(NO_3)_2$/$AgNO_3$ during the exposure period. In addition, in zinc toxicity tests, solutions were renewed daily as Zn^{2+} activities were not measured empirically in the present study. The same approach was applied for mixture toxicity tests to avoid interferences of different metal ions on the measurements of the free metal ion activity by the ion-selective electrodes.

6.2.3. Metal measurements and speciation in the hydroponic solution

Free ion activities of H^{+}, Cu^{2+}, and Ag^{+} were measured with ion-selective electrodes (Metrohm). Copper and silver sulfide ion-selective electrodes were calibrated by using a set of measurements at different concentrations of Cu^{2+} and Ag^{+} in solution (Le et al., 2012). Free Zn^{2+} activities were computed from the total Zn^{2+} concentrations in the solution by using the Windermere Humic Aqueous model (WHAM) VI with Steiner solution as the default medium (Tipping, 1998). A disadvantage of the WHAM VI is the exclusion of Ag^{+} in this version. A survey was performed to investigate effects of Ag^{+} at the activity range studied on the activities of other cationic constituents of the Steiner solution by using the Chemical Equilibria in Aquatic Systems (CHEAQS) model (Verweij, 2004). The results from this survey indicated a negligible influence of Ag^{+} on activities of other cations. Consequently, activities of cations in solution (except for Cu^{2+} and Ag^{+}) were specified by the WHAM VI model although Ag^{+} is excluded in this speciation model. Ionic composition of the Steiner solution used for the chemical speciation is given in Table S6.1, Supplementary information.

6.2.4. Toxicity assays

Seeds of *Lactuca sativa* were germinated in the Steiner solution for 4 d under a normal light cycle of 16: 8 hours light: dark at 15 ^{o}C, which is in the range of the average temperature in the Netherlands. Germinated plants were fixed in parafilm straps that floated on the surface of a glass beaker with the roots immersed in the medium for 4 d. For each beaker, 4 plants were put in. The root growth (Growth) of lettuce exposed to each solution was determined as the average of the increases in the root length of the 4 plants grown in the solution after 4 d of exposure compared to the initial length. In total, 180 toxicity tests were carried out to investigate effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity. Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} were added to the Steiner solutions up to the concentrations of 10, 20, 10, and 20 mmol/L, respectively. With each combination of these cations, 8–10 toxicity tests were carried out at varying free ion activities of Cu^{2+} in solution. Additionally, 238 toxicity tests were performed in the assessment of joint toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+}, including 122 tests without

additions of Ag^+ and 116 tests without additions of Zn^{2+} to the Steiner solution. The free ion activities of Cu^{2+}, Zn^{2+}, and Ag^+ in solution studied varied in the ranges: Cu^{2+}: 10^{-10}–10^{-6} mol/L; Zn^{2+}: 10^{-6}–10^{-3} mol/L; and Ag^+: 10^{-8}–10^{-7} mol/L. These ranges were selected from preliminary toxicity tests carried out at different activities of these cations in solution varying from the background level of the Steiner solution to the extremely toxic level.

6.2.5. Metal ion activity at the plasma membrane surface

ψ_0 was calculated from free ion activities of all cations in the solution by using the model developed by Kinraide and Wang (2010). The calculated surface potential was then used to determine free ion activities of metals at the PM surface according to the Nernst Equation (Eqn. 6.1):

$$\{M^{n+}\}_0 = \{M^{n+}\}_b \times \exp\left[-\frac{F \times n \times \psi_0}{R \times T}\right] \tag{6.1}$$

where $\{M^{n+}\}_0$ and $\{M^{n+}\}_b$ (mol/L) are free ion activities of metal ion M^{n+} at the PM surface and in the BM, respectively; n (dimensionless) is the charge of metal ion M^{n+}; F (J/mV) is the Faraday constant (F = 96.485); R (J/mol/K) is the universal gas constant (R = 8.314); and T (K) is the experimental temperature (288 K in the present study).

6.2.6. Derivation of mathematical equations describing metal toxicity

Toxicity of single metals was found to follow the Weibull Equation (Kinraide and Parker, 1989). Accordingly, the response of lettuce exposed to single metals expressed by the root growth (Growth; mm) can be related to $\{M^{n+}\}_b$ (Eqn. 6.2) or $\{M^{n+}\}_0$ (µmol/L) (Eqn. 6.3) as:

$$\text{Growth} = \frac{b}{\exp[(c \times \{M^{n+}\}_b)^d]} \tag{6.2}$$

$$\text{Growth} = \frac{b}{\exp[(c \times \{M^{n+}\}_0)^d]} \tag{6.3}$$

where coefficient b (mm) is the growth of lettuce roots when the metal ion is not present in the solution (i.e., $\{M^{n+}\}_b = 0$) or at the PM surface (i.e., $\{M^{n+}\}_0 = 0$); coefficient c (L/µmol) reflects the metal-specific strength of toxicity. Its value increases with increasing strength of metal toxicity; and coefficient d (dimensionless) is a shape parameter; when coefficient d is greater than 1, the curves are sigmoidal (see illustrations in Kinraide et al., 2004).

Toxicity of Cu^{2+} in the presence of Na^+, K^+, Ca^{2+}, and Mg^{2+}

Common cations, e.g., Na^+, K^+, Ca^{2+}, and Mg^{2+}, were assumed to act as osmoticants, i.e., intoxication resulted from the reduction in water potential (Kinraide, 1999). Consequently, in the present study, their effects on Cu^{2+} toxicity were evaluated by a common term "osmolarity". Osmotic effects of these major cations on Cu^{2+} toxicity were evaluated by incorporating expansion coefficients into the strength coefficient in Equation 6.3. Osmolarity was calculated based on the osmotic coefficients and the total concentrations of salts in the solution (Robinson and Stoke, 2002). Furthermore, the relationship between root elongation and osmolarity followed a sigmoidal curve (Kinraide, 1999). Therefore, toxicity of Cu^{2+} expressed by the root growth (Growth; mm) can be written as a function of the surface activity of Cu^{2+} ($\{Cu^{2+}\}_0$; µmol/L) and osmolarity (Os; µmol/L) as follows:

$$\text{Growth} = \frac{b}{\exp\left[\left(c_1 \times \left(1 + c_{10} \times \text{Os} + c_{20} \times \text{Os}^2\right) \times \{Cu^{2+}\}_0\right)^d\right]} \tag{6.4}$$

where coefficients c_{10} and c_{20} represent osmotic effects on Cu^{2+} toxicity. The effects were considered statistically significant if the estimate of the 95% confidence interval (CI) of these strength coefficients determined by the regression analysis did not encompass zero.

Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in non-interactive mixtures

If mixture components do not interact with each other, the growth of lettuce roots exposed to the mixture can be written according to the conventional concepts of CA and RA assuming no interactions between the mixture constituents. According to the CA, mixture substances are supposed to act by the same mechanism (Bliss, 1939). Subsequently, the growth of lettuce roots (Growth; mm), following exposure to a non-interactive mixture of Cu^{2+}, Zn^{2+}, and Ag^{+}, can be determined based on the free ion activity of these metals at the PM surface according to the following equation

$$\text{Growth} = \frac{b}{\exp\left[\left(c_1 \times \{Cu^{2+}\}_0 + c_2 \times \{Zn^{2+}\}_0 + c_3 \times \{Ag^{+}\}_0\right)^d\right]} \tag{6.5}$$

where c_1, c_2, and c_3 (L/µmol) are the strength coefficients of toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in their non-reactive mixtures, respectively; d (dimensionless) is the shape coefficient of the dose-response curve describing toxicity of these metal ions according to the CA model (Kinraide, 1999).

The RA model is based on the assumption that mixture components have different modes of action of toxicity (Loewe and Muischnek, 1926). Therefore, the response of organisms exposed to non-interactive mixtures can be expressed as a multiplicative function of the response of the organisms following exposure to each constituent separately (Kinraide, 1999). Accordingly, the root growth of lettuce (Growth; mm) in response to the exposure to a non-interactive mixture of Cu^{2+}, Zn^{2+}, and Ag^{+} can be written as Equation 6.6 according to the concept of RA:

$$\text{Growth} = \frac{b}{\exp\left[\left(c_1 \times \{Cu^{2+}\}_0\right)^{d_1} + \left(c_2 \times \{Zn^{2+}\}_0\right)^{d_2} + \left(c_3 \times \{Ag^{+}\}_0\right)^{d_3}\right]} \tag{6.6}$$

where c_1, c_1, and c_3 (L/µmol) are the strength coefficients of toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in their non-reactive mixtures, respectively; and d_1, d_2, and d_3 (dimensionless) are the shape coefficients of the dose-response curves describing toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} according to the RA model, respectively (Kinraide, 1999).

Equations 6.5 and 6.6 are based on the conventional concept of CA and RA, i.e., the presence of one metal at the membrane surface does not affect the toxicity of another. In other words, toxicity of metals following exposure to the non-interactive mixtures of Cu^{2+}, Zn^{2+}, and Ag^{+} is similar to the toxicity of these metals following single exposure. As a result, the strength coefficient and the slope parameter describing toxicity of single metals as in Equation 6.3 can be determined by the regression analysis using toxicological data on metal mixtures.

Toxicity of interactive mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+}

If metals adsorbed following exposure to mixtures interact with each other, i.e., the presence of one metal affects the toxicity of another metal, the interactions can be taken into account in estimating the joint toxicity by expanding the conventional CA and RA models. In particular, expansion coefficients that describe interactive effects can be integrated into the strength coefficients in Equations 6.5 and 6.6 (S6.A, Supplementary information). The strength coefficient can be extended in two different ways and accordingly expressed by two different Equations S6.6 and S6.7 in S6.A, Supplementary information. The comparison of

the expansion coefficient with zero indicates whether one substance reduces or increases the toxicity of the others. In addition, the interactive effect was considered statistically significant when the 95% CI of the expansion coefficient does not encompass zero. Of the two expansion equations available (Equations S6.6 and S6.7 in S6.A, Supplementary information), the one with higher statistical significance was used to simulate the interactions.

Mixtures of Cu^{2+} and Zn^{2+}. Coefficient c_{12} (L/μmol) that describes effects of Zn^{2+} on Cu^{2+} toxicity can be incorporated into the strength coefficient of Cu^{2+} toxicity. Similarly, coefficient c_{21} (L/μmol) can be integrated into the strength coefficient of Zn^{2+} toxicity to represent effects of Cu^{2+} on Zn^{2+} toxicity. Toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} expressed by the root growth (Growth; mm) can be written as Equations 6.7 and 6.8 according to the extended CA and RA models, respectively, as coefficients in these equations are statistically significant (See *Results*):

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}_0}{1 + c_{12} \times \{Zn^{2+}\}_0} + \frac{c_2 \times \{Zn^{2+}\}_0}{1 + c_{21} \times \{Cu^{2+}\}_0}\right)^d\right]} \quad (6.7)$$

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}_0}{1 + c_{12} \times \{Zn^{2+}\}_0}\right)^{d_1} + \left(c_2 \times \{Zn^{2+}\}_0\right)^{d_2}\right]} \quad (6.8)$$

where c_1 (L/μmol) is the strength coefficient of Cu^{2+} toxicity in the solution without Zn^{2+}; c_2 (L/μmol) is the strength coefficient of Zn^{2+} toxicity in the solution free of Cu^{2+}; d (dimensionless) represents the slope of the curve describing toxicity of Cu^{2+} and Zn^{2+} in their interactive mixtures according to the extended CA model; and d_1 and d_2 (dimensionless) are slope parameters representing toxicity of Cu^{2+} and Zn^{2+}, respectively, in their interactive mixture on the basis of the extended RA model.

Mixtures of Cu^{2+} and Ag^{+}. Toxicity of Cu^{2+} following exposure to its mixtures with Ag^{+} can be expressed by including coefficient c_{13} (L/μmol) that describes effects of Ag^{+} on Cu^{2+} toxicity in the strength coefficient of Cu^{2+} toxicity. Similarly, coefficient c_{31} (L/μmol) can be incorporated into the strength coefficient of Ag^{+} toxicity to represent effects of Cu^{2+} on Ag^{+} toxicity. Based on the extended CA model, the growth of lettuce roots (Growth; mm) following exposure to interactive mixtures of Cu^{2+} and Ag^{+} can be written by Equation 6.9 as coefficients in this equation were estimated to be statistically significant (See *Results*):

$$\text{Growth} = \frac{b}{\exp\left[\left(\frac{c_1 \times \{Cu^{2+}\}_0}{1 + c_{13} \times \{Ag^{+}\}_0} + c_3 \times \left(1 + c_{31} \times \{Cu^{2+}\}_0\right) \times \{Ag^{+}\}_0\right)^d\right]} \quad (6.9)$$

where c_1 (L/μmol) is the strength coefficient of Cu^{2+} toxicity in the medium free of Ag^{+}; c_3 (L/μmol) is the strength coefficient of Ag^{+} toxicity in the solution without Cu^{2+}; and d (dimensionless) is the slope parameter describing toxicity of Cu^{2+} and Ag^{+} to lettuce exposed to their interactive mixtures according to the extended CA model.

By contrast, expansion coefficients could not be integrated into the strength coefficient of Cu^{2+} and Ag^{+} toxicity based on the extended RA model as no expansion coefficient was found to be statistically significant. A full description of the derivation of Equations 6.5–6.9 is presented in S6.A, Supplementary information.

6.2.7. Regression analyses

Coefficients in all above equations were determined by multiple nonlinear regression analyses using the SYSTAT software. A coefficient is considered significant if its 95% CI statistically significantly deviates from zero, i.e., not encompassing zero (S6.A, Supplementary information). Additionally, the strength of the significance increases with an increase in the absolute value of the ratio between the estimate of the parameter and the asymptotic standard error, i.e., parameter/ASE in the regression result. Toxicity data generated from all tests of mixture toxicity were used to assess toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} in non-interactive mixtures as the presence of one metal does not affect the biological actions of the other metals in the mixtures. Toxicological data from 122 tests without additions of Ag^{+} were used to assess toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} as Ag^{+} was not present in the solutions. Moreover, toxicity of the interactive Cu^{2+}–Ag^{+} mixtures was evaluated using 116 tests without additions of Zn^{2+} assuming negligible effects of Zn^{2+} at the background concentration in the default medium. Furthermore, Akaike's information criterion (AIC) was calculated to compare different models developed for estimating toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures (Burnham and Anderson, 2002). The r^2 value indicates the potential of the model in explaining the variability in the response of lettuce exposed to metal mixtures whereas the AIC value represents the suitability of the model. The model with the lowest value of AIC is the most appropriate model.

6.3. Results

6.3.1. Surface interactions affect the activity of ions at the plasma membrane surface

Interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}

Additions of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} at the concentrations in solution tested increased ψ_0 from –18 to –4.0 mV (Fig. S6.1, Supplementary information). This variation in ψ_0 then resulted in a decrease of about half of an order of magnitude of $\{Cu^{2+}\}_0$ at a given exposure level expressed by $\{Cu^{2+}\}_b$ (Fig. S6.1, Supplementary information). Moreover, a statistically significant relationship was found between $\{Cu^{2+}\}_b$ (µmol/L) and $\{Cu^{2+}\}_0$ (µmol/L) (Fig. S6.1, Supplementary information; $p < 0.0001$; $F = 2910$; $n = 180$; $r^2 = 0.94$). Furthermore, in the presence of varying common cation concentrations, $\{Cu^{2+}\}_0$ ($n = 180$; $r^2 = 0.79$) was a better indicator of Cu^{2+} toxicity compared to $\{Cu^{2+}\}_b$ ($n = 180$; $r^2 = 0.65$) according to nonlinear regression with the Weibull Equation (Table S6.2, Supplementary information).

Interactions in mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+}

At the exposure levels studied, surface interactions also occur between metals following exposure to their mixtures, influencing their activities at the PM surface. The effects were metal-specific and dose-dependent. Additions of Zn^{2+} substantially reduced $\{Cu^{2+}\}_0$ as shown by a difference of around half of an order of magnitude of $\{Cu^{2+}\}_0$ at a given $\{Cu^{2+}\}_b$ with varying concentrations of Zn^{2+} added (Fig. S6.2A, Supplementary information). In contrast, Cu^{2+} did not substantially affect $\{Zn^{2+}\}_0$ as indicated by negligible variations in $\{Zn^{2+}\}_0$ at a given $\{Zn^{2+}\}_b$ in mixtures with different $\{Cu^{2+}\}_b$ (Fig. S6.2B, Supplementary information). This difference was attributed to a larger decrease in the negativity of ψ_0 induced by the addition of Zn^{2+} than the reduction caused by the addition of Cu^{2+} at the exposure levels tested (Fig. S6.2A, Supplementary information). $\{Zn^{2+}\}_0$ decreased with an increase in $\{Zn^{2+}\}_b$ at the lowest exposure levels in the solution from 1 to 10 µmol/L as the increase in $\{Zn^{2+}\}_b$ was compromised by a reduction in the negativity of ψ_0 induced by Cu^{2+} added (Fig. S6.2B, Supplementary information). Similar $\{Zn^{2+}\}_0$ was found for the treatment with the lowest (1

μmol/L) and highest (100 μmol/L) activity of Zn^{2+} in the solution (Fig. 6.2B, Supplementary information). These results demonstrate the significance of ψ_0 in determining $\{Zn^{2+}\}_0$, even with small variations in ψ_0. A similar important role of ψ_0 in determining $\{Cu^{2+}\}_0$ following exposure to Cu^{2+}–Ag^+ mixtures was observed (Fig. S6.3A, Supplementary information). Moreover, the presence of Cu^{2+} and Ag^+ in solution in the toxicity tests in the present study only resulted in small variations in the negativity of ψ_0 (Fig. S6.3, Supplementary information). Consequently, there were only negligible variations in $\{Cu^{2+}\}_0$ and $\{Ag^+\}_0$ with varying additions of Ag^+ or Cu^{2+} into the solution (Fig. S6.3, Supplementary information).

6.3.2. Internal interactions affect metal toxicity

Interactions between Cu^{2+} and Na^+, K^+, Ca^{2+}, and Mg^{2+}

Osmolarity had statistically significant effects on Cu^{2+} toxicity as expansion coefficients c_{10} and c_{20} in Equation 6.4 deviated statistically significantly from zero (Table 6.1). Approximately 72% of the variability in the growth of lettuce roots can be explained by Equation 6.4 (Fig. 6.2 and Fig. S6.4, Supplementary information; n = 180; r^2 = 0.72). Moreover, the opposite signs of these coefficients (c_{10} is negative and c_{20} is positive) indicate dependence of these effects (alleviation or enhancement) on the value of osmolarity or on the concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} in solution in other words. The osmotic effects of adsorbed Na^+, K^+, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity were negligible as the strength coefficient of Cu^{2+} toxicity in the medium free of these cations (i.e., c_1 in Equation 6.4) was 5 and 10 orders of magnitude higher than expansion coefficients c_{10} and c_{20}, respectively (Table 6.1). This accounts for a lack of improvement in predicting Cu^{2+} toxicity from incorporating the internal interactions between Cu^{2+} and Na^+, K^+, Ca^{2+}, and Mg^{2+} (r^2 = 0.72; Table 6.1) compared to the exclusion of these interactions (r^2 = 0.79; Table S6.2, Supplementary information).

Table 6.1. Estimates of coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (CI), in Equation 6.4 integrating effects of Na^+, K^+, Ca^{2+}, and Mg^{2+} adsorbed in terms of osmolarity in estimating Cu^{2+} toxicity (n = 180; r^2 = 0.72)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	42.42	2.50	16.95	37.48	47.36
c_1 (L/μmol)	Strength of Cu^{2+} toxicity	4.57	0.96	4.79	2.69	6.46
c_{10} (L/μmol)	Linear expansion coefficient	$-0.39 \cdot 10^{-4}$	$0.02 \cdot 10^{-4}$	-18.76	$-0.44 \cdot 10^{-4}$	$-0.35 \cdot 10^{-4}$
c_{20} (L/μmol)	Sigmoidal expansion coefficient	$0.44 \cdot 10^{-9}$	$0.04 \cdot 10^{-9}$	11.94	$0.36 \cdot 10^{-9}$	$0.51 \cdot 10^{-9}$
d (dimensionless)	Slope	0.66	0.10	6.67	0.47	0.86

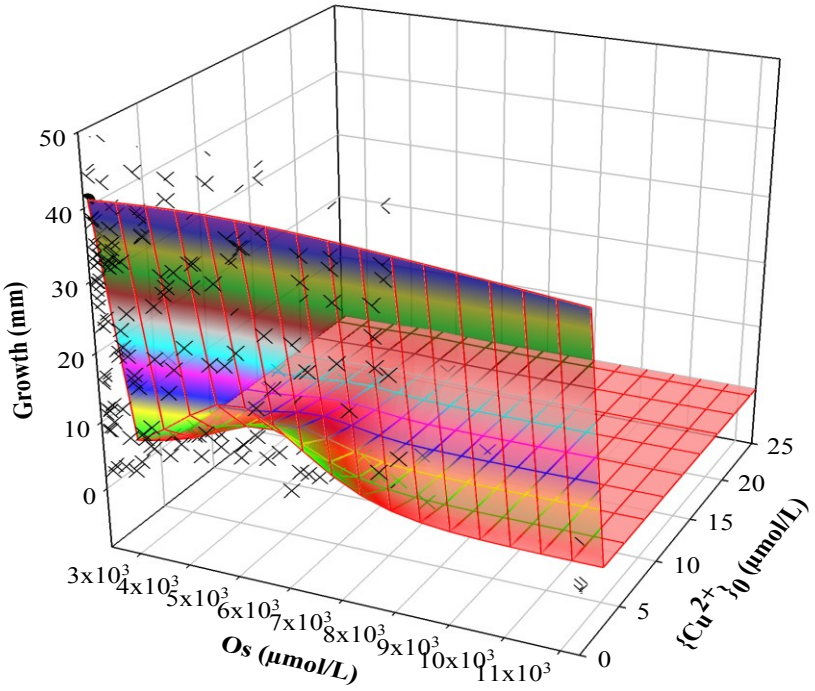

Figure 6.2. The root growth of lettuce (Growth; mm) exposed to Cu^{2+} in the presence of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} is plotted as a function of the free Cu^{2+} activity at the PM surface ($\{Cu^{2+}\}_0$; µmol/L) and the osmolarity (Os; µmol/L): the surface represents the estimations based on the regression analysis according to Equation 6.2 and dotted points represent the experimental data.

Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in non-interactive mixtures

The conventional concepts of CA and RA models, which assumes no interactions in mixtures, as expressed by Equations 6.5 and 6.6, respectively, performed equally well in estimating toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} in non-interactive mixtures with r^2 values of 0.83 and 0.86, respectively (Tables S6.3 and S6.4, Supplementary information). Based on the strength coefficients estimated by both CA and RA models, Ag^{+} and Cu^{2+} were far more rhizotoxic than Zn^{2+} while Ag^{+} was slightly more toxic than Cu^{2+} (Table 6.2). Estimates of the strength coefficient of toxicity of Cu^{2+} and Zn^{2+} predicted by the CA model were not statistically significantly different from those predicted by the RA model, i.e., the 95% CI of the strength coefficients estimated by these models overlapped (Table 6.2). In addition, the strength coefficient of toxicity of Ag^{+} predicted by the RA model was slightly higher than the estimation by the CA model (Table 6.2).

Interactions in mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+}

According to the extended CA model, adsorbed Cu^{2+} and Zn^{2+} had significant effects on the toxicity of each other as the 95% CIs of the expansion coefficients c_{12} and c_{21} did not encompass zero (Table 6.3). Particularly, Zn^{2+} significantly reduced Cu^{2+} toxicity ($c_{12} > 0$) and Cu^{2+} significantly increased Zn^{2+} toxicity ($c_{21} < 0$). Based on the extended RA model, Zn^{2+} significantly reduced Cu^{2+} toxicity as c_{12} deviated significantly from zero while Cu^{2+} did not have significant effects on Zn^{2+} toxicity as no statistically significant value of this coefficient was found (Table 6.4). Moreover, the predictive powers of the extended CA and RA models were similar in estimating toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} (Figs. 6.3A, 6.3B and Fig. S6.5, Supplementary information; $n = 122$; $r^2 = 0.92$).

Table 6.2. Estimates of coefficients describing strength of toxicity of Cu^{2+} (c_1; L/µmol), Zn^{2+} (c_2; L/µmol), and Ag^{+} (c_3; L/µmol) individually found in the present study for *Lactuca sativa* according to the conventional concept of concentration addition (CA) and response addition (RA) models and in the study of Kopittke et al. (2011a) for *Vigna unguiculata* following exposure to single metals. 95% confidence intervals (CI) are provided.

Source	Species	Model	Strength coefficient (L/µmol)		
			Cu^{2+} (c_1, 95% CI)	Zn^{2+} (c_2, 95% CI)	Ag^{+} (c_3, 95% CI)
Present study	*Lactuca sativa*	CA	0.67 (0.59-0.76)	$1.66 \cdot 10^{-3}$ ($1.45 \cdot 10^{-3}$-$1.87 \cdot 10^{-3}$)	1.43 (1.24-1.63)
		RA	0.73 (0.63-0.83)	$1.75 \cdot 10^{-3}$ ($1.54 \cdot 10^{-3}$-$1.95 \cdot 10^{-3}$)	1.81 (1.68-1.95)
Kopittke et al. (2011a)	*Vigna unguiculata*	Single-metal exposure	0.154	$3.68 \cdot 10^{-3}$	6.71

Table 6.3. Estimates of the coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), in Equation 6.7 estimating toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} according to the extended concentration addition model (n = 122; r^2 = 0.92)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.40	1.15	42.90	47.12	51.68
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	1.02	0.13	8.21	0.78	1.27
c_{12} (L/µmol)	Effects of Zn^{2+} on Cu^{2+} toxicity	0.01	0.01	2.56	0.003	0.02
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$1.75 \cdot 10^{-3}$	$0.09 \cdot 10^{-3}$	20.43	$1.58 \cdot 10^{-3}$	$1.92 \cdot 10^{-3}$
c_{21} (L/µmol)	Effects of Cu^{2+} on Zn^{2+} toxicity	-0.17	0.05	-3.25	-0.27	-0.07
d (dimensionless)	Slope	1.33	0.12	11.51	1.10	1.55

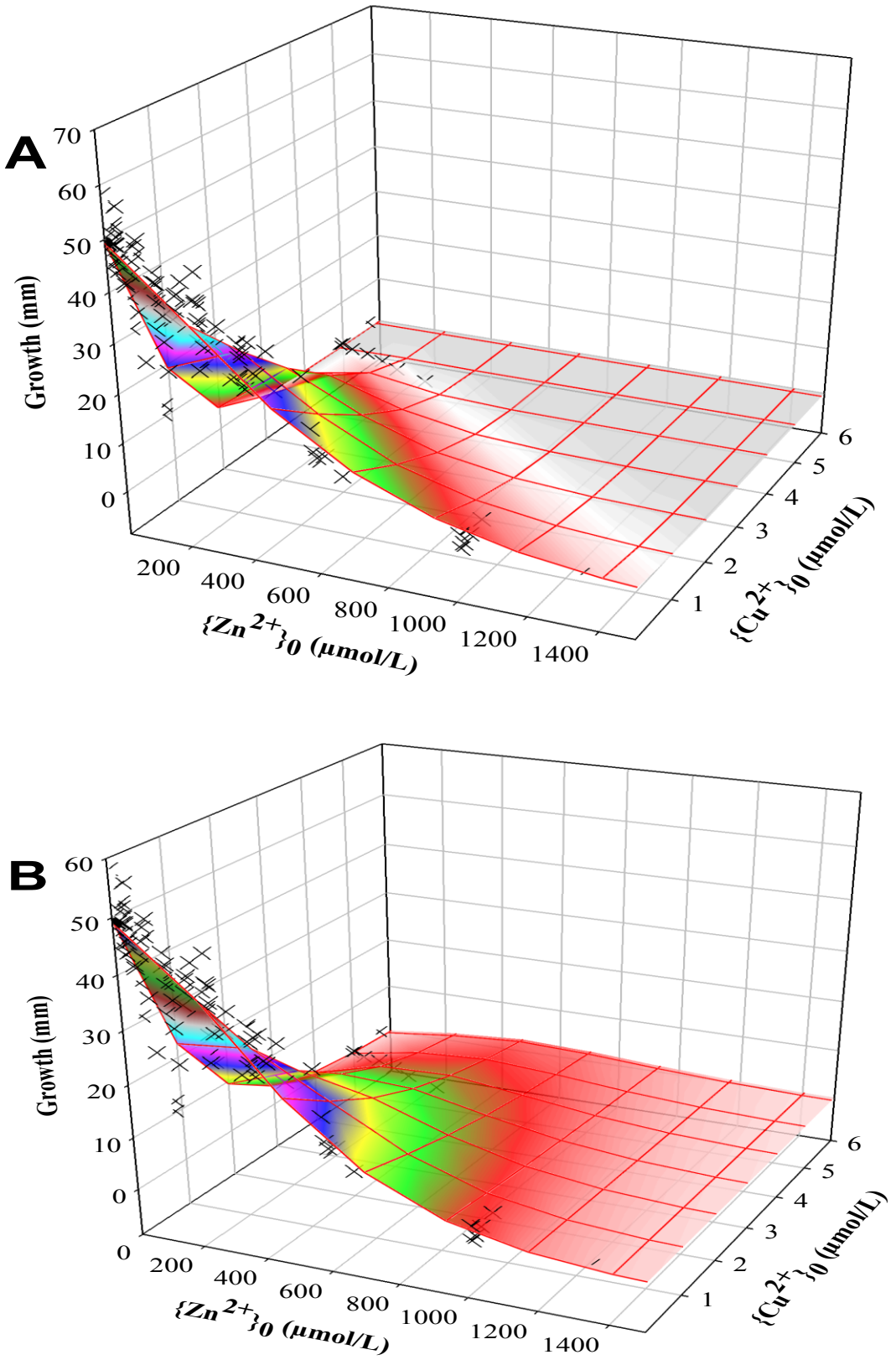

Figure 6.3. The root growth of lettuce (Growth; mm) as a function of the free ion activity of Cu^{2+} ($\{Cu^{2+}\}_0$; µmol/L) and the free ion activity of Zn^{2+} ($\{Zn^{2+}\}_0$; µmol/L) at the plasma membrane surface according to the extended concentration addition (CA) model (A) and the extended response addition (RA) (B) model: the surfaces represent the estimations based on the regression analysis and dotted points represent the experimental data.

Table 6.4. Estimates of the coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), in Equation 6.8 estimating toxicity of interactive mixtures of Cu^{2+} and Zn^{2+} according to the extended response addition model (n = 122; r^2 = 0.92)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	48.99	1.07	45.80	46.87	51.11
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	0.81	0.09	8.67	0.62	0.99
c_{12} (L/µmol)	Effects of Zn^{2+} on Cu^{2+} toxicity	$2.89 \cdot 10^{-3}$	$1.40 \cdot 10^{-3}$	2.07	$0.12 \cdot 10^{-3}$	$5.65 \cdot 10^{-3}$
d_1 (dimensionless)	Slope of Cu^{2+} toxicity curve	1.07	0.12	9.06	0.84	1.31
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$1.82 \cdot 10^{-3}$	$0.08 \cdot 10^{-3}$	21.58	$1.65 \cdot 10^{-3}$	$1.98 \cdot 10^{-3}$
d_2 (dimensionless)	Slope of Zn^{2+} toxicity curve	1.57	0.15	10.68	1.28	1.86

In the assessment based on the extended CA model, the statistically significant deviation of expansion coefficients c_{13} (L/µmol) and c_{31} (L/µmol) from zero as shown in Table 6.5 indicates significant effects of Cu^{2+} and Ag^{+} adsorbed on the toxicity of each other. Specifically, Ag^{+} significantly increased Cu^{2+} toxicity while Cu^{2+} had significant alleviative effects on Ag^{+} toxicity. Moreover, about 80% of the variability in the growth of lettuce roots (Growth; mm) following adsorption of Cu^{2+} and Ag^{+} to the membrane surface could be explained by Equation 6.6 in relation to $\{Cu^{2+}\}_0$ (µmol/L) and $\{Ag^{+}\}_0$ (µmol/L) (Fig. 6.4 and Fig. S6.6, Supplementary information; n = 116; r^2 = 0.80). By contrast, according to the extended RA model, Cu^{2+} and Ag^{+} adsorbed to the membrane surface did not have significant effects on the toxicity of each other as no statistically significant value of the expansion coefficients was found.

6.3.3. Comparison of the different models

Both the concepts of CA and RA were integrated into the interactive and non-interactive models in the present study. In other words, these concepts were combined with the assumptions that biological actions of one substance affect or do not affect the biological actions of the other.

Table 6.5. Estimates of the coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), in Equation 6.9 describing toxicity of interactive mixtures of Cu^{2+} and Ag^{+} according to the extended concentration addition model (n = 116; r^2 = 0.80)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	50.22	1.77	28.42	46.72	53.73
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	0.95	0.10	9.22	0.74	1.15
c_{13} (L/µmol)	Effects of Ag^{+} on Cu^{2+} toxicity	-1.03	0.22	-4.79	-1.45	-0.60
c_3 (L/µmol)	Strength of Ag^{+} toxicity	1.69	0.12	13.71	1.45	1.93
c_{31} (L/µmol)	Effects of Cu^{2+} on Ag^{+} toxicity	-1.87	0.55	-3.43	-2.96	-0.79
d (dimensionless)	Slope	1.76	0.25	6.96	1.26	2.27

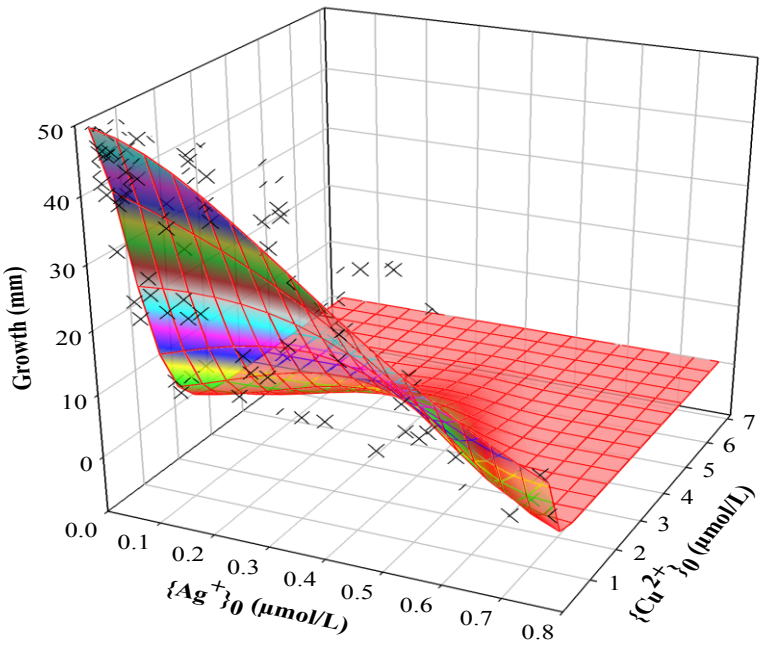

Figure 6.4. The root growth of lettuce (Growth; mm) as a function of the free ion activity of Cu^{2+} ($\{Cu^{2+}\}_0$; µmol/L) and the free ion activity of Ag^{+} ($\{Ag^{+}\}_0$; µmol/L) at the plasma membrane surface according to the extended concentration addition model: the surface represents the estimations based on the regression analysis and dotted points represent the experimental data

Table 6.6. The value of Akaike's information criterion calculated for non-interactive and interactive models based on the concepts of concentration addition (CA) and response addition (RA) for predicting toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures to lettuce *Lactuca sativa*

Mixtures	Non-interactive models		Interactive models	
	CA	RA	CA	RA
Cu^{2+}–Zn^{2+}	156	148	144	143
Cu^{2+}–Ag^{+}	191	182	190	

Based on the value of AIC calculated, generally the RA model was better than the CA model while the interactive models were more suitable than the non-interactive models for estimating toxicity of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} mixtures (Table 6.6). However, the differences in the AIC value of different models were not substantial. The lower AIC value for the interactive model compared to the non-interactive model, especially in predicting joint toxicity of Cu^{2+} and Zn^{2+}, clearly demonstrate that ion-ion interactions should be integrated in estimating mixture toxicity and the integration of these interactions would improve the predictions.

6.4. Discussion

6.4.1. Toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} individually

The order of strength of toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} found in the present study, i.e., Ag^{+} > Cu^{2+} > Zn^{2+}, was consistent with the findings of Kopittke et al. (2011a) (Table 6.2). While *Vigna unguiculata* was more sensitive to Cu^{2+} than *Lactuca sativa*, *Lactuca sativa* had lower tolerance to exposure to Zn^{2+} and Ag^{+} than *Vigna unguiculata* (Table 6.2). Moreover, based on the assumption of no interactions among Cu^{2+}, Zn^{2+}, and Ag^{+}, the CA and RA models resulted in negligible differences in estimating toxicity of these metals. In other words, if metals do not interact with each other, their (dis)similar actions do not have substantial effects on their strength of toxicity in mixtures.

6.4.2. Ion-ion interactions and metal toxicity

According to Kabata-Pendias and Pendias (1984), interactions occur at the PM surface and within organisms as well. This observation is confirmed by results in the present study. Surface interactions influence the free ion activity at the PM surface, thus affecting metal uptake. Therefore, the free metal ion activity at the PM surface is a better indicator of metal uptake than the free metal ion activity in the solution (Kinraide, 2001; Wang et al., 2011; Kopittke et al., 2011b). Internal interactions directly determine metal toxicity. Surface interactions are directly quantified through ψ_0 while ψ_0 may contribute to explain the internal interactions predicted in the present study. Moreover, ψ_0 provides an alternative for site-specific competition for binding sites in interpreting ion-ion interactions (see below). Both surface and internal interactions contribute to explain metal uptake and toxicity as presented below.

Interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}

The higher predictive power of $\{Cu^{2+}\}_0$ over $\{Cu^{2+}\}_b$ in estimating Cu^{2+} toxicity in the presence of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, as reported in the present study, emphasizes the significance of integrating the surface interactions in estimating metal toxicity. The variation in ψ_0 induced by additions of these common cations as calculated in the present study is substantially lower than the level reported in previous studies (Kinraide et al., 2004; Wang et al., 2011). This was due to high background concentrations of cations in the Steiner solution, which led to an electrical potential of around –20 mV at the PM of lettuce exposed to the default medium. Additionally, the small variation in ψ_0 accounts for the significant relationship presented above between $\{Cu^{2+}\}_b$ and $\{Cu^{2+}\}_0$ when Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} were added. The reduction in $\{Cu^{2+}\}_0$ following the decrease in the negativity of ψ_0 potentially contributes to the decreasing toxicity of Cu^{2+} with additions of the common cations reported in a previous study (Le et al., 2012). This observation is consistent with the results in a recent study investigating toxicities of Cu and Pb to cowpea (*Vigna unguiculata*) (Kopittke et al., 2011b). Particularly, the effects of the common cations, e.g., Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, on $\{Cu^{2+}\}_0$ were attributed to the decrease in ψ_0 with the additions of these common cations (Kinraide, 1998; Wang et al., 2008). However, effects of the common cations on Cu^{2+} toxicity cannot be completely explained based on the reduction in $\{Cu^{2+}\}_0$ with additions of the common cations while excluding the internal interactions. Specifically, if effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity are determined only by the decreasing $\{Cu^{2+}\}_0$, a consistent decline in Cu^{2+} toxicity with increasing concentrations of these common cations would be expected, contrasting with the results reported by Le et al. (2012). Moreover, the inconsistent effects of the common cations on Cu^{2+} toxicity found by these authors cannot completely be explained in terms of competitive binding (Le et al., 2012). In contrast, the interactive effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity can be interpreted taking into account internal interactions between Cu^{2+} and these common cations. Particularly, the dependence of the type of internal effects (i.e., increasing or decreasing) on the concentration of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} potentially accounts for the inconsistent trend of Cu^{2+} toxicity with varying concentrations of the major cations reported. As such, the modelling approach applied in the present study provides an alternative for competition for binding sites in explaining ion-ion interactions. Cu^{2+} toxicity in the presence of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} is therefore influenced by both surface and internal interactions between Cu^{2+} and these common cations. As presented in *Results*, surface interactions significantly reduced $\{Cu^{2+}\}_0$ while effects of internal interactions on the strength of Cu^{2+} toxicity were negligible. Therefore, generally, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} had alleviative effects on Cu^{2+} toxicity as found in the previous study (Le et al., 2012).

Interactions in metal mixtures

Toxicity of metal mixtures is influenced by both surface and internal interactions between mixture components. The relative importance of these interactions is metal-specific and dose-dependent. Following exposure to Cu^{2+}–Zn^{2+} mixtures, Zn^{2+} substantially reduced $\{Cu^{2+}\}_0$. After exposure to mixtures of Cu^{2+} and Ag^{+}, these metals had considerable internal effects on the toxicity of each other as shown by small differences between strength coefficients and expansion coefficients (Table 6.5). Therefore, both surface and internal interactions should be incorporated in estimating toxicity of metal mixtures.

In terms of electrical reactions, the surface interactions between Cu^{2+} and Zn^{2+} as well as between Cu^{2+} and Ag^{+} observed in the present study could be interpreted based on their binding constants in combination with the exposure levels tested. For example, the substantial decrease in $\{Cu^{2+}\}_0$ as well as in the negativity of ψ_0 induced by Zn^{2+} is ascribed to substantially higher exposure levels of Zn^{2+} in the test solutions compared to the exposure

concentrations of Cu^{2+} together with similar affinity constants of these metal ions to the membrane surface as reported by Kinraide and Wang (2010). This impact declines with decreasing differences between their activities in the solution. Moreover, the presence of Cu^{2+} at activities some orders of magnitude lower than the levels of Zn^{2+} did not lead to substantial changes in ψ_0. Furthermore, these results indicate a strong dependence of the surface interactions between different metals on the exposure level tested. Additionally, these dose-dependent interactions potentially account for different effects of Cu^{2+} on Zn^{2+} uptake reported in previous studies at different exposure levels studied, which cannot be explained by competition for binding sites (Beckett and Davis, 1978; Luo and Rimmer, 1995; Sanders et al., 1987; Tani and Barrington, 2005; Kabta-Pendia and Pendias, 1984).

Moreover, ψ_0 may provide insight into the potential mechanisms in which different metals interact with each other within organisms. Specifically, alleviative effects of Zn^{2+} adsorbed on Cu^{2+} toxicity are potentially related to the reduction in the negativity of ψ_0 induced by the additions of Zn^{2+}. Particularly, the decline in the negativity of ψ_0 results in depolarisation, and subsequent decreases in internal metal transport and metal toxicity (Wang et al., 2011). In contrast, the lack of interactive effects of Cu^{2+} on Zn^{2+} toxicity are due to negligible variations in ψ_0 with additions of Cu^{2+} at the exposure levels tested in the present study. A similar explanation is applicable to explain the internal interactions between Cu^{2+} and Ag^{+}. These results demonstrate that ψ_0 should be taken into account in modelling ion-ion interactions.

In summary, the electrostatic modelling approach as presented in the present study allows explicitly delineating surface interactions on the one hand and predicting potential internal interactions on the other hand. Metal toxicity occurs as a result of both surface and internal interactions. Consequently, these interactions should be included in predicting joint toxicity of multiple metals. Furthermore, these interactions could be interpreted in terms of the electrical potential at the PM surface. In other words, the surface potential provides a mechanistic understanding of metal-metal interactions and should be included in assessment of metal toxicity and ion-ion interactions, consistent with findings by Kinraide (1998, 2006).

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SUPPLEMENTARY INFORMATION

Table S6.1. Ionic composition of the Steiner solution used for chemical speciation

Metals	Na^+	Mg^{2+}	K^+	Ca^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}
Concentration (mol/L)	$1.02 \cdot 10^{-6}$	$1.83 \cdot 10^{-3}$	$6.98 \cdot 10^{-3}$	$2.82 \cdot 10^{-3}$	$1.16 \cdot 10^{-5}$	$4.96 \cdot 10^{-5}$	$1.72 \cdot 10^{-6}$

S6.A. Derivation of mathematical equations describing toxicity of interactive and non-interactive metal mixtures

Toxicity of non-interactive mixtures

The response of plants expressed as the root growth (Growth; mm) after exposure to metal ion M^{n+} singly can be expressed in relation to its free ion activity at the PM surface $\{M^{n+}\}_0$ (μmol/L) according to the following equation (Kinraide and Parker, 1989)

$$\text{Growth} = \frac{b}{\exp[(c \times \{M^{n+}\}_0)^d]} \tag{S6.1}$$

where coefficient b (mm) is the growth of lettuce in the medium free of the metal ion (i.e., $\{M^{n+}\}_0 = 0$); coefficient c (L/μmol) reflects the metal-specific strength of toxicity. Its value increases with increasing strength of metal toxicity; and coefficient d (dimensionless) is the slope parameter.

If mixture components do not interact to each other, the growth of plants exposed to the mixture can be written according to the conventional concept of concentration addition (CA) and response addition (RA) models assuming no interactions between the mixture constituents. According to the CA model, mixture components have the same modes of action of toxicity (Kinraide, 1999). In other words, the presence of one substance can be considered as a simple dilution of others. Therefore, according to the CA concept, response of lettuce exposed to non-interactive mixtures in terms of root growth can be written as follows (Kinraide, 1999):

$$\text{Growth} = \frac{b}{\exp[(\sum(c \times \{M^{n+}\}_0))^d]} \tag{S6.2}$$

For instance, the growth of lettuce roots (Growth; mm) following exposure to a non-interactive mixture of Cu^{2+}, Zn^{2+}, and Ag^+ is determined by the following equation according to the CA model:

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\}_0 + c_2 \times \{Zn^{2+}\}_0 + c_3 \times \{Ag^+\}_0)^d]} \tag{S6.3}$$

where b (mm) is the growth of lettuce roots in the medium free of Cu^{2+}, Zn^{2+}, and Ag^+; coefficients c_1, c_2, and c_3 (L/μmol) represent the strength of toxicity of Cu^{2+}, Zn^{2+}, and Ag^+ individually as well as in non-interactive mixtures, respectively; d (dimensionless) is the slope parameter describing toxicity of these metals individually as well as in non-interactive mixtures; and $\{Cu^{2+}\}_0$, $\{Zn^{2+}\}_0$, and $\{Ag^+\}_0$ (μmol/L) are the free ion activity of Cu^{2+}, Zn^{2+}, and Ag^+ at the PM surface, respectively.

In the RA model, mixture components are supposed to have different modes of action of toxicity (Kinraide, 1999). Therefore, based on the RA concept, the response of lettuce exposed to non-interactive mixtures can be expressed as a multiplicative function of the response of the plants following exposure to each constituent separately (Eqn. S6.4) (Kinraide, 1999):

$$\text{Growth} = \frac{b}{\prod \exp[(c_i \times \{M^{n+}\}_0)^{d_i}]} = \frac{b}{\exp[\sum(c_i \times \{M^{n+}\}_0)^{d_i}]} \tag{S6.4}$$

For example, when Cu^{2+}, Ag^+, and Zn^{2+} do not affect toxicity of one another, the growth of lettuce exposed to mixtures of these three metal ions can be written as a multiplication of the responses of lettuce following exposure to these metal ions individually (Eqn. S6.5):

$$\text{Growth} = \frac{b}{\exp[(c_1 \times \{Cu^{2+}\}_0)^{d_1} + (c_2 \times \{Zn^{2+}\}_0)^{d_2} + (c_3 \times \{Ag^+\}_0)^{d_3}]} \tag{S6.5}$$

where coefficient b (mm) is the growth of lettuce roots in the medium free of Cu^{2+}, Ag^{+}, and Zn^{2+}; coefficients c_1, c_2, and c_3 (L/µmol) represent the strength of toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually as well as in non-interactive mixtures, respectively; d_1, d_2, and d_3 (dimensionless) are slope parameters describing toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually as well as in their non-interactive mixtures; and $\{Cu^{2+}\}_0$, $\{Zn^{2+}\}_0$, and $\{Ag^{+}\}_0$ (µmol/L) are the free ion activity of Cu^{2+}, Ag^{+}, and Zn^{2+} at the PM surface, respectively.

Equations S6.2-S6.5 are based on the conventional concepts of the CA and RA models assuming no interactions between mixture components, i.e., the presence of one metal does not affect the toxicity of another. In other words, toxicity of metals following exposure to these non-interactive mixtures is similar to the toxicity of these metals individually.

Toxicity of interactive mixtures

When metals in the mixtures are interactive, the interactions can be taken into account in determining the mixture toxicity by expanding the conventional CA and RA models. In particular, strength coefficients c_1, c_2, and c_3 in Equations S6.3 and S6.5 are linearly extended to include expansion coefficients. For example, based on an assumed linear interaction between Cu^{2+} and Zn^{2+}, the expanded strength coefficient describing the Cu^{2+} toxicity in interactive mixtures with Zn^{2+} (c_1^*) can be expanded as in Equation S6.6 or S6.7 (Kinraide et al., 1999):

$$c_1^* = c_1 \times (1 + c_{12} \times \{Zn^{2+}\}_0) \tag{S6.6}$$

or

$$c_1^* = \frac{c_1}{1 + c_{12} \times \{Zn^{2+}\}_0} \tag{S6.7}$$

where c_{12} (L/µmol) is the expansion coefficient, representing interactive effects of Zn^{2+} on Cu^{2+} toxicity. In Equation S6.6, Zn^{2+} enhances toxicity of Cu^{2+} if c_{12} is positive while in Equation S6.7, Zn^{2+} increases Cu^{2+} toxicity if c_{12} is negative. By contrast, alleviative effects of Zn^{2+} on Cu^{2+} toxicity occur when c_{12} in Equation S6.6 is negative or when c_{12} in Equation S6.7 is positive. The interactive effects are found to be statistically significant when the 95% confidence interval (CI) of c_{12} does not encompass zero. Similar equations can be written to integrate ion-ion interactions in modelling toxicity of Zn^{2+} in mixtures with Cu^{2+}. If interactions affect toxicity of only one component of the mixture, the strength coefficient of only this substance is extended. The expansion approach that results in all statistically significant coefficients will be chosen in predicting toxicity of metal mixtures, taking into account interactions between different mixture components.

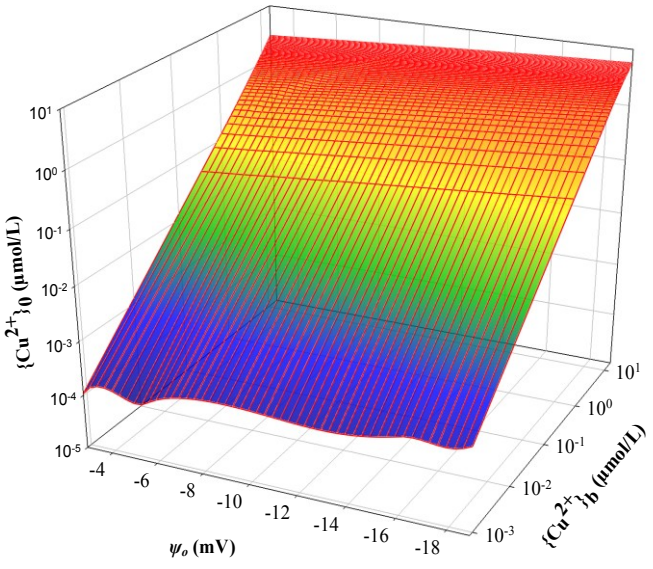

Figure S6.1. Surface interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} affect the Cu^{2+} activity at the plasma membrane (PM) surface: The free Cu^{2+} activity at the PM surface ($\{Cu^{2+}\}_0$; µmol/L) is plotted as a function of the free Cu^{2+} activity in the bathing medium ($\{Cu^{2+}\}_b$; µmol/L) in toxicity tests and the electrical potential at the PM surface (ψ_0; mV), taking into account effects of major cations (i.e., Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}). The surface potential ψ_0 (mV) was calculated according to the model developed by Kinraide and Wang (2010).

Table S6.2. Estimates of coefficients describing the root growth (Growth; mm) of lettuce exposed to Cu^{2+} in the presence of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} according to the Weibull Equation based on the free Cu^{2+} activity in the bulk-phase medium ($\{Cu^{2+}\}_b$; µmol/L) (Equation 6.2) and the free Cu^{2+} activity at the plasma membrane surface ($\{Cu^{2+}\}_0$; µmol/L) (Equation 6.3): *b* (mm) is the root growth in the medium free of Cu^{2+}; *c* (L/µmol) represents the strength of Cu^{2+} toxicity; and *d* (dimensionless) is the slope parameter; and statistical parameters, 95% confidence intervals (CI), determined by the regression analysis.

Parameter	$\{Cu^{2+}\}_b$ (µmol/L)	$\{Cu^{2+}\}_0$ (µmol/L)
b (mm; 95% CI)	40.43 (34.86-46.00)	37.28 (29.56-45.00)
c (L/µmol; 95% CI)	2.94 (1.93-3.95)	0.769 (0.49-1.05)
d (dimensionless; 95% CI)	0.65 (0.42-0.89)	0.751 (0.55-0.95)
n	180	180
r^2	0.65	0.79

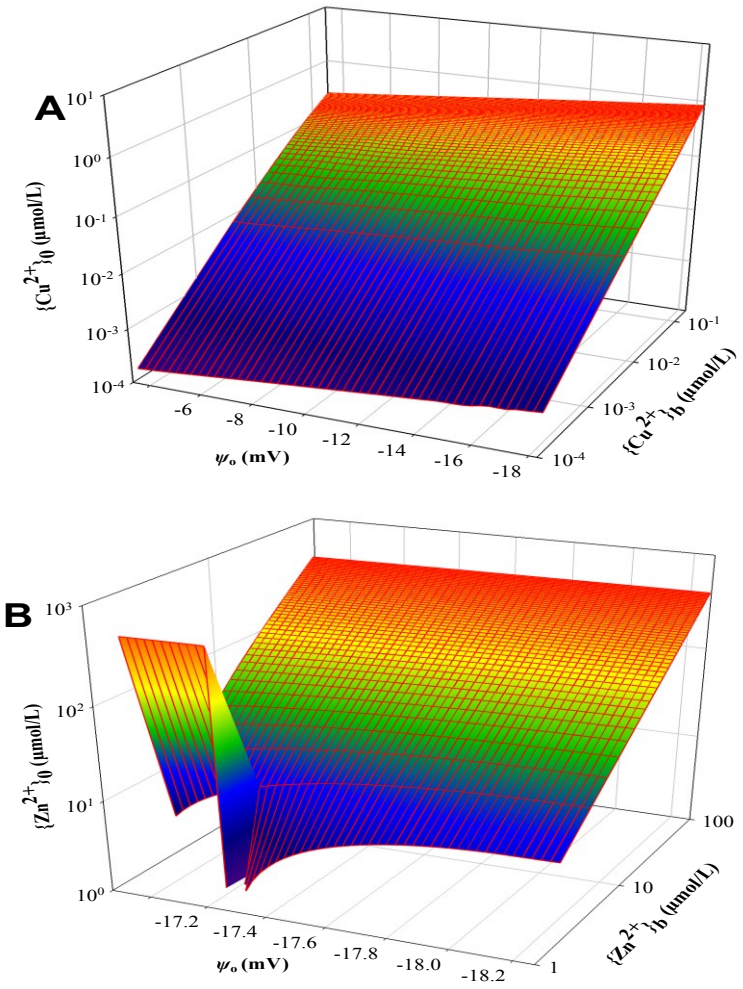

Figure S6.2. Surface interactions between Cu^{2+} and Zn^{2+} affect their activities at the plasma membrane (PM) surface through changes in the surface potential (ψ_0): (A) The activity of Cu^{2+} at the PM surface ($\{Cu^{2+}\}_0$; µmol/L) is plotted as a function of the free activity of Cu^{2+} in the solution ($\{Cu^{2+}\}_b$; µmol/L) and the surface potential (ψ_0; mV) which is influenced by the free Zn^{2+} activity in the solution; and (B) The activity of Zn^{2+} at the PM surface ($\{Zn^{2+}\}_0$; µmol/L) is plotted as a function of the free activity of Zn^{2+} in the solution ($\{Zn^{2+}\}_b$; µmol/L) and the surface potential (ψ_0; mV) which is influenced by the free Cu^{2+} activity in the solution.

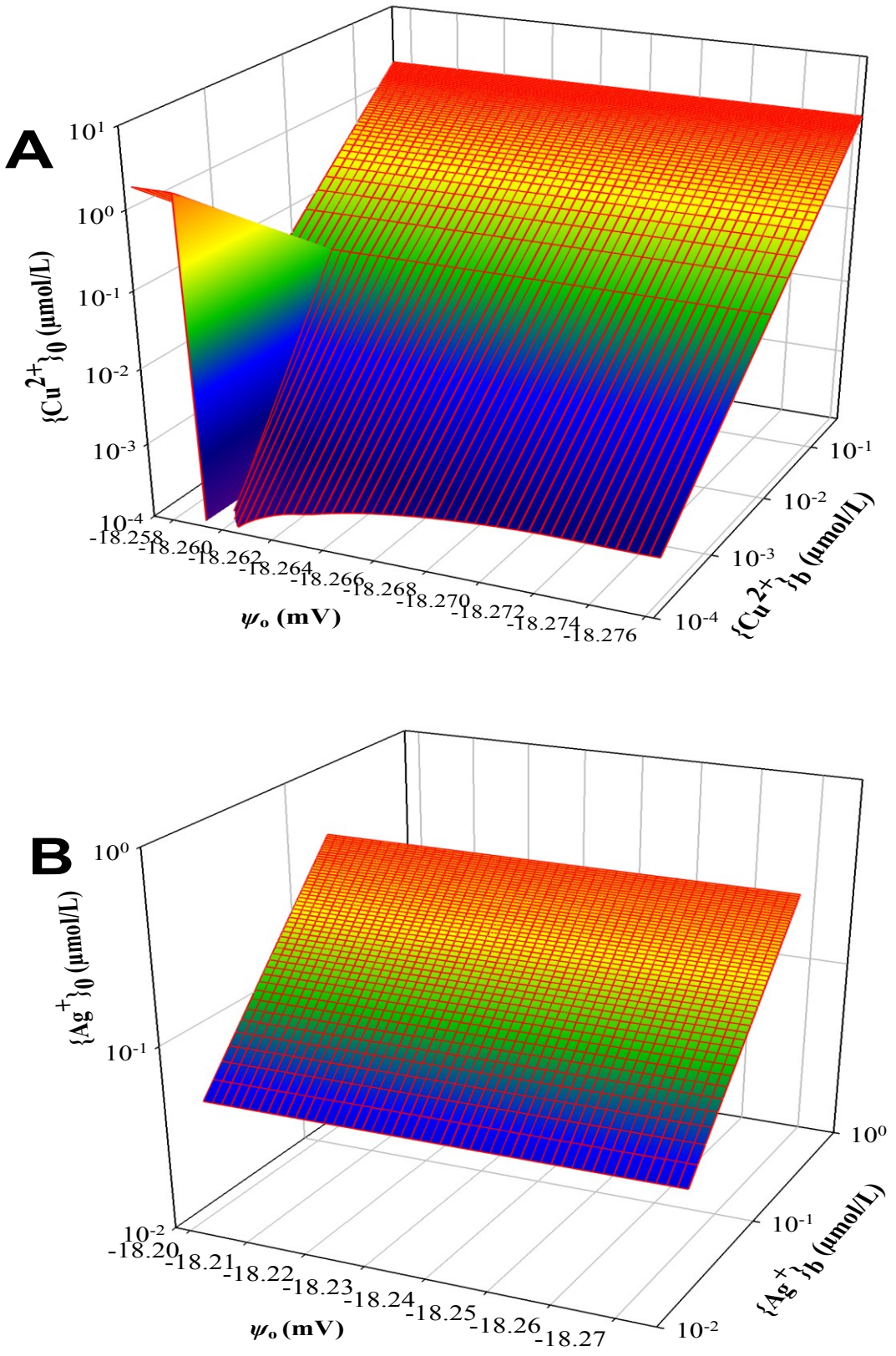

Figure S6.3. Surface interactions between Cu^{2+} and Ag^{+} affect their activities at the plasma membrane (PM) surface through changes in the surface potential (ψ_0): (A) The activity of Cu^{2+} at the PM surface ($\{Cu^{2+}\}_0$; µmol/L) is plotted as a function of the free activity of Cu^{2+} in the solution ($\{Cu^{2+}\}_b$; µmol/L) and the surface potential (ψ_0; mV) which is influenced by the free Ag^{+} activity in the solution; and (B) The activity of Ag^{+} at the PM surface ($\{Ag^{+}\}_0$; µmol/L) is plotted as a function of the free activity of Ag^{+} in the solution ($\{Ag^{+}\}_b$; µmol/L) and the surface potential (ψ_0; mV) which is influenced by the free Cu^{2+} activity in the solution.

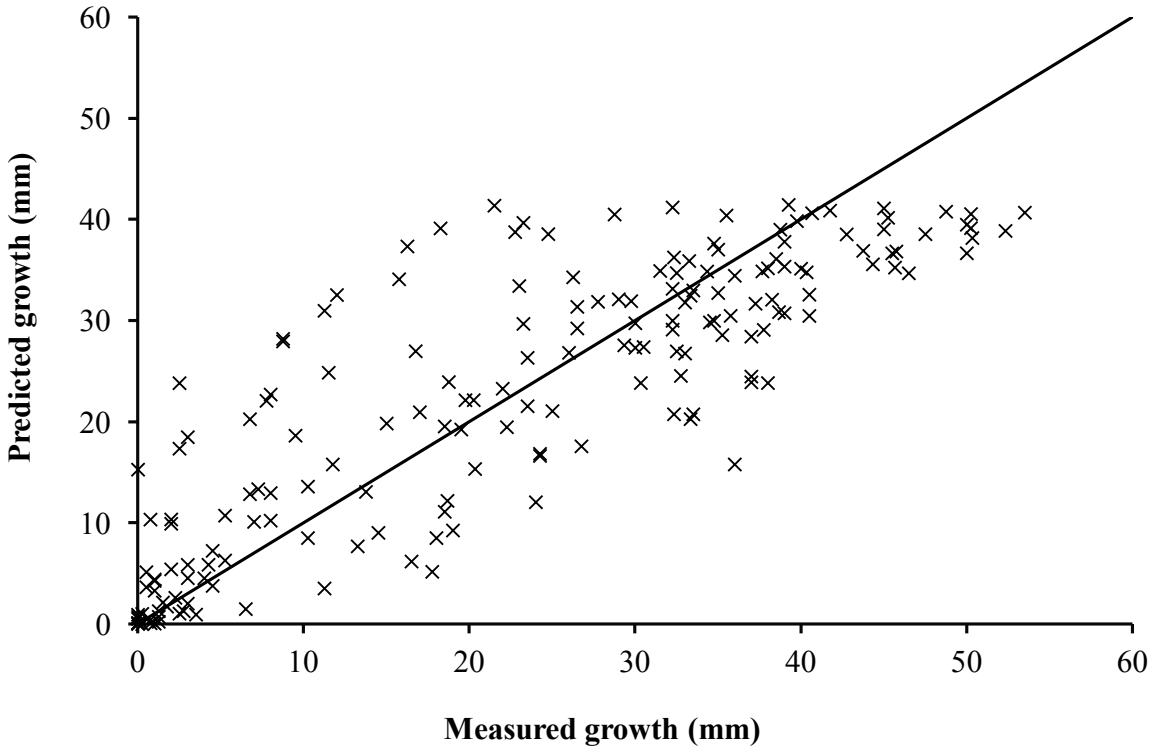

Figure S6.4. The relationship between the measured growth and the predicted growth of lettuce roots exposed to Cu^{2+} in the presence of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}. The solid line presents a 1:1 relationship.

Table S6.3. Estimates of the coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), in Equation 6.5 describing the toxicity of non-interactive mixtures of Cu^{2+}, Zn^{2+}, and Ag^{+} according to the concentration addition model and determined by the non-linear regression analysis (n = 238; r^2 = 0.83)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.99	1.19	42.11	47.65	52.33
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	0.67	0.04	15.71	0.59	0.76
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$1.66 \cdot 10^{-3}$	$0.11 \cdot 10^{-3}$	15.81	$1.45 \cdot 10^{-3}$	$1.87 \cdot 10^{-3}$
c_3 (L/µmol)	Strength of Ag^{+} toxicity	1.43	0.10	14.69	1.24	1.63
d (dimensionless)	Slope	1.38	0.12	11.65	1.14	1.61

Table S6.4. Estimates of the coefficients and statistical parameters, i.e., asymptotic standard error (ASE) and 95% confidence interval (95% CI), in Equation 6.6 describing the toxicity of non-interactive mixtures of Cu^{2+}, Zn^{2+}, and Ag^{+} according to the response addition model and determined by the non-linear regression analysis (n = 238; r^2 = 0.86)

Parameter	Definition	Estimate	ASE	Parameter/ASE	95% CI	
					Lower	Upper
b (mm)	Control growth	49.95	0.90	54.33	47.27	50.83
c_1 (L/µmol)	Strength of Cu^{2+} toxicity	0.73	0.05	14.88	0.63	0.83
d_1 (dimensionless)	Slope of Cu^{2+} toxicity curve	1.03	0.10	10.63	0.84	1.21
c_2 (L/µmol)	Strength of Zn^{2+} toxicity	$1.75 \cdot 10^{-3}$	$0.11 \cdot 10^{-3}$	16.65	$1.54 \cdot 10^{-3}$	$1.95 \cdot 10^{-3}$
d_2 (dimensionless)	Slope of Zn^{2+} toxicity curve	1.65	0.19	8.85	1.28	2.02
c_3 (L/µmol)	Strength of Ag^{+} toxicity	1.81	0.07	26.14	1.68	1.95
d_3 (dimensionless)	Slope of Ag^{+} toxicity curve	2.92	0.38	7.59	2.16	3.67

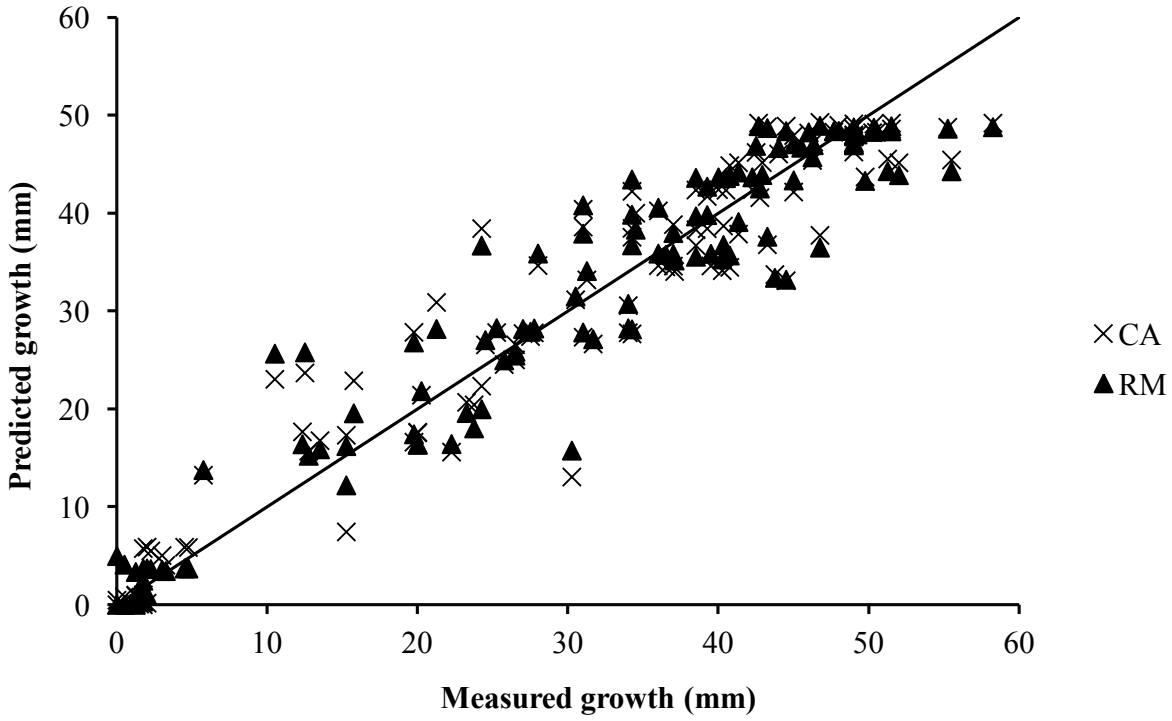

Figure S6.5. The relationship between the measured growth and the predicted growth of lettuce roots exposed to interactive mixtures of Cu^{2+} and Zn^{2+}. The estimated growth was determined based on the extended concentration addition (CA) and response addition (RA) models. The solid line represents a 1:1 relationship.

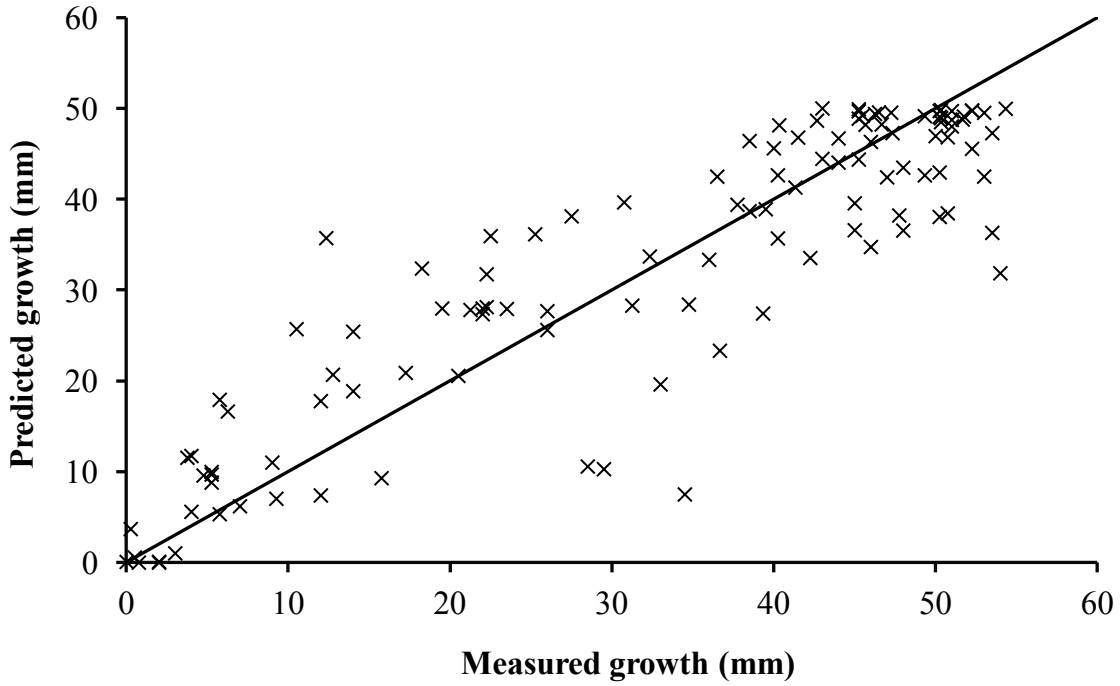

Figure S6.6. The relationship between the measured growth and the predicted growth of lettuce roots exposed to interactive mixtures of Cu^{2+} and Ag^{+}. The solid line represents a 1:1 relationship.

Chapter 7

Synthesis

7.1. Introduction

In this PhD thesis, the affinity of metals for biological ligands was used as the unifying factor for modelling bioaccumulation and bioavailability of metal mixtures. Bioaccumulation of metals (i.e., the net accumulation of the metals in a particular tissue or in the whole organism) results from exposure to different pathways, e.g., environmental media (e.g., air, water, soil, sediment) and diet, and is the result of a mass balance between uptake and elimination (SAB, 2006). Bioavailability of metals represents the amount of the metals that absorb onto, or into, and across the biological membrane of organisms (Fairbrother et al., 2007). The generalisation of metal bioaccumulation and bioavailability by using the affinity of metals for biological ligands as applied in this thesis is based on:

1) The involvement of the biological ligands, e.g., transporters and other metal-binding proteins, in uptake and subcellular partitioning of metals and the metal-specific affinity of the ligands (Simkiss and Taylor, 1995; Fairbrother et al., 2007; Roesijadi and Robinson, 1994; Rainbow, 2007; Sokolova and Lannig, 2008; Flemming, 1995; Kapoor and Viraraghavan, 1997; Tsezos et al., 1997; Diels et al., 1995; Kratochvil and Volesky, 1998; Hall and Willams, 2003; Yang et al., 2005)
2) The metal-specific affinity for the biological ligands as a determinant of metal uptake and toxicity (Seregin and Kozhevnikova, 2006; Rudakova et al., 1988; Merce et al., 2001)

The generalisation of metal bioaccumulation and bioavailability on the basis of the affinity of metals for biological ligands might result in significant progress in modelling metal toxicity in several aspects:

1) The integration of the metal-specific affinity for biological ligands may provide a better understanding of ion-ion interactions and may allow integrating the interactions in modelling joint toxicity of multiple metals.
2) This approach facilitates the incorporation of the interactions between ions and organisms at the biological surface into estimating metal bioavailability. This integration may then increase the reliability of metal risk assessments as in addition to chemical speciation, interactions between metals and biological ligands determine metal bioavailability (Fairbrother et al., 2007; Pagenkopf et al., 1974; McGeer et al., 2003; Chapman, 1996; Newman and Jagoe, 1994; Langston and Bryan, 1984; Pagenkopf, 1983; Playle, 1998; Worms et al., 2006; Ahlf et al., 2009). The integration of ion-ion and ion-organism interactions will result in more reliable estimations of bioaccumulation and toxicity of metal mixtures.
3) The development of models based on the affinity of metals for biological ligands, which is an intrinsic property of the metals, enables extrapolation to other metals in different conditions.

The contribution of the integration of the affinity of metals for biological ligands to modelling toxicity of metal mixtures was evaluated by comparing assessments based on the free metal ion activity in the solution (*Chapter 4*), the Biotic Ligand Model (BLM) (*Chapter 5*), and the electrostatic toxicity model (ETM) (*Chapter 6*).

A semi-mechanistic model was developed to estimate bioaccumulation for a number of metals simultaneously present in the environment in various species and across different exposure conditions (*Chapter 2*; Sub-question 1 in the *Introduction* Chapter). The accumulation of metals from both dissolved and dietary sources is included in the model. The development of the model is based on the relationship between metal uptake and the covalent index initiated by Veltman et al. (2008). The model simulates the accumulation of metals

mechanistically as a function of a metal-specific property, i.e., the covalent index, and a species-specific characteristic, i.e., the size-based filtration rate. Particularly, kinetics of metal uptake and elimination were modelled based on the covalent index of the metal and the species-specific size-based filtration rate. The development of the model based on the intrinsic properties facilitates a wide extrapolation to a variety of metals and exposure conditions. The use of the metal-specific covalent index that reflects the specificity of metal affinity for proteins to simulate metal bioaccumulation in mussels is the most important feature of the model developed in the present study, distinguishing it from previously-developed kinetic models. The model developed was then validated by field measurements of mussels from the Rhine and Meuse rivers.

The affinity of ions for biotic ligands at the water-organism interface was used to study effects of major cations, i.e., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, on Cu^{2+} toxicity to lettuce *Lactuca sativa* (*Chapter 3*; Sub-question 2 in the *Introduction* Chapter). Specifically, a BLM was developed to predict Cu^{2+} toxicity in the presence of these cations. According to the principle of the BLM, the toxicity of Cu^{2+} was determined by the accumulation of Cu^{2+} at the biotic ligands expressed by the fraction of the total number of biotic ligands occupied by Cu^{2+}. This fraction depends on the stability constants of binding of Cu^{2+} and the major cations to the biotic ligands. Effects of H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity were assessed separately. The stability constants for the binding of each major cation to the biotic ligand were determined using a linear relationship between the median effective concentration of Cu^{2+} and the concentration of the competing cation while keeping the concentrations of other cations constant.

Interactions between different ions were modelled based on the free ion activity in the solution and integrated in estimating toxicity of binary metal mixtures (Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+}) (*Chapter 4*; Sub-question 3 in the *Introduction* Chapter). Interactions between metal ions and biological ligands were excluded in this assessment. Metal toxicity was expressed by the strength coefficient of toxicity, calculated on the basis of the relationship between the response of lettuce and the free ion activity of metals in the solution. According to the conventional concepts of concentration addition (CA) and response addition or response multiplication (RA or RM, respectively), mixture substances do not interact with each other. In *Chapter 4*, the interactions were predicted and incorporated in estimating toxicity of the mixtures by extending the strength coefficient of toxicity. This was obtained by using mathematical relationships to express assumptions of non-interactions and linear interactions in binary metal mixtures.

The affinity of ions for biotic ligands at the water-organism interface was included in estimating toxicity of mixtures of Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+} to lettuce, *Lactuca sativa*, and thus allows integrating interactions between ions and interactions of ions with organisms in predicting toxicity of these mixtures (*Chapter 5*; Sub-question 4 in the *Introduction* Chapter). In particular, the BLM was combined with the toxic equivalency factor (TEF) approach to predict toxicity of the metal mixtures, integrating interactions between different mixture components in terms of their competition for binding sites at the biotic ligands. According to the BLM concept, toxic effects are determined by the fraction of the biotic ligands occupied by metal ions. In *Chapter 5*, the applicability of this principle to assessment of metal mixtures was examined. The fraction of the total number of biotic ligands bound to metal ions was applied to determine the TEF for each metal as well as the toxic equivalency quotient of metal mixtures. As such, the interactions between different metal ions as well as between metal ions and the biotic ligands are integrated in predicting toxicity of the binary metal mixtures.

The affinity of ions for biological ligands at the membrane surface was integrated in modelling ion-ion interactions and effects of the interactions were incorporated in predicting

joint toxicity of multiple metal ions in the Electrostatic Toxicity Model (ETM) (*Chapter 6*; Sub-question 5 in the *Introduction* Chapter). This was performed by assessing the activity of ions at the plasma membrane (PM) surface and modelling metal toxicity based on the free metal ion activity at the PM surface. Moreover, this approach allows investigating interactions at different levels. Firstly, ions interact with each other in the proximity of the outside of the membrane (surface interactions). These surface interactions affect the activity of ions at the PM surface and were assessed by the electrostatic approach. Secondly, interactions occur within the cells between adsorbed ions, directly affecting metal toxicity (internal interactions). These internal interactions were quantified and integrated in modelling toxicity of metal mixtures by fitting the experimental data to mathematical equations similar to the approach applied in *Chapter 4*, but based on the free metal ion activity at the PM surface rather than the free metal ion activity in the exposure solution.

Based on the results described in each of the chapters, the contribution of the incorporation of metal affinity for biological ligands is synthesised in the following sections of this chapter. This is carried out for toxicity assessment of single metals in the presence of common cations (*Section 7.2*) and for toxicity assessment of metal mixtures (*Section 7.3*). Furthermore, possibilities for application of the developed models as well as recommendations for further research are given (*Section 7.4*).

7.2. Affinity of metals for biological ligands in modelling toxicity of single metals

7.2.1. Quantitatively estimating toxicity of single metals

In this thesis, single metal toxicity of Cu^{2+}, Zn^{2+}, and Ag^{+} was assessed by the BLM, the ETM, and the free ion activity model (FIAM) and expressed by different parameters (Table 7.1). The BLM parameters include the fraction of the total number of biotic ligands occupied by metal ions at the 50% response level ($f50_M$; dimensionless) and corresponding affinity constants (K_{MBL}; L/mol) (*Chapter 5*). In the FIAM, toxicity is expressed by the free ion activity of metals in the solution that results in a 50% inhibition in the growth of lettuce roots ($EA50\{M^{n+}\}_b$; µmol/L) (*Chapter 5*). In the ETM, toxicity of single metals is presented by the free ion activity of the metals at the PM surface at the 50% response level ($EA50\{M^{n+}\}_0$; µmol/L) predicted by the CA and RA models (*Chapter 6*).

According to the BLM (based on $f50_M$) and the electrostatic approach (based on $EA50\{Cu^{2+}\}_0$), toxicity decreased in the sequence of $Ag^{+} > Cu^{2+} > Zn^{2+}$ (Table 7.1). However, the assessment based on the free ion activity of metals in the solution indicated that Cu^{2+} was the most toxic, followed by Ag^{+} and Zn^{2+} (Table 7.1). All three models were based on the same assumption, namely that free ions are the main reactive species of metals determining metal toxicity, where the difference is attributed to the inclusion of ion-organism interactions in estimating metal toxicity in the BLM and the electrostatic approach. Furthermore, these two latter approaches provide a mechanistic understanding of metal binding and toxicity. Particularly, the BLM parameters indicate that the lowest fraction of the total biotic ligands occupied by metal ions was required to result in a 50% reduction in the root growth of lettuce was in case of Ag^{+}. Moreover, Cu^{2+} had the strongest affinity for binding sites at the biotic ligands, contributing to the lowest activity of Cu^{2+} in the solution at the 50% response level. A similar explanation can be derived for the electrostatic approach. According to this method, Cu^{2+} ($\log K_{Cu} = 2.76$) had a stronger affinity for binding sites at the PM surface compared to Ag^{+} ($\log K_{Ag} = 0.8$). These results underpin the significance of integrating the interactions between metal species and the biological ligands in assessing

intrinsic toxicity of single metals. This inclusion is of importance for a mechanistic understanding of metal binding and toxicity.

Table 7.1. Toxicity of Cu^{2+}, Ag^{+}, and Zn^{2+} individually was assessed by the Biotic Ligand Model (BLM), the Free Ion Activity Model (FIAM), and the Electrostatic Toxicity Model (ETM). The most important BLM parameters describing toxicity of single metals include the fraction of the total number of biotic ligands occupied by metal ions at the 50% response level ($f50_M$; dimensionless) and the stability constant of the metal ion-biotic ligand binding (K_{MBL}; L/mol). In the FIAM, toxicity of metals is expressed by the free ion activity of the metals in solution at the 50% response level ($EA50\{M^{n+}\}_b$; µmol/L). In the ETM, metal toxicity is represented by the free ion activity of the metals at the plasma membrane that results in a 50% inhibition in the root elongation ($EA50\{M^{n+}\}_0$; µmol/L). This value was predicted according to the concepts of concentration addition (CA) and response addition (RA) models. 95% confidence intervals are provided.

Metals	Biotic Ligand Model (Chapter 5)		Free Ion Activity Model (Chapter 5)	Electrostatic Toxicity Model (Chapter 6)	
	$f50_M$ (dimensionless)	$\log K_{MBL}$ (L/mol)	$EA50\{M^{n+}\}_b$ (µmol/L)	$EA50\{M^{n+}\}_0$ (µmol/L) (CA)	$EA50\{M^{n+}\}_0$ (µmol/L) (RA)
Cu^{2+}	0.36 (0.29-0.43)	7.40	$2.60 \cdot 10^{-2}$ ($1.87 \cdot 10^{-2}$-$3.61 \cdot 10^{-2}$)	1.14	0.96
Zn^{2+}	0.42 (0.38-0.44)	4.00	$1.06 \cdot 10^{2}$ ($9.11 \cdot 10^{1}$-$1.24 \cdot 10^{2}$)	461.90	457.61
Ag^{+}	0.22 (0.20-0.24)	6.39	$1.34 \cdot 10^{-1}$ ($1.19\ 10^{-1}$-$1.50 \cdot 10^{-1}$)	0.54	0.49

7.2.2. Interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}

Both BLM and ETM allow incorporation of interactions between toxic ions, e.g., Cu^{2+}, with competing cations, e.g., H^{+}, Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+}, at the biological surface in modelling metal toxicity in principle. As a result of this integration, the accumulation of metal ions at the biological surface (biotic ligands in the BLM and the PM surface in the ETM) might be a better indicator of metal toxicity in the presence of common cations than the exposure level in the environment. In this PhD thesis, interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} were modelled by these two approaches (Table 7.2).

The integration of the affinity of ions for biological ligands in modelling Cu^{2+} toxicity in the presence of common cations, i.e., Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, results in substantial progress in studying interactions between Cu^{2+} and these major cations. For example, the free ion activity of Cu^{2+} at the PM surface $\{Cu^{2+}\}_0$ was found to be a better indicator of Cu^{2+} toxicity in the presence of these common cations than the free ion activity of Cu^{2+} in the solution $\{Cu^{2+}\}_b$ (*Chapter 6*). This was caused by the integration of surface interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} in estimating Cu^{2+} toxicity.

Table 7.2. Assessment of interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} by the Biotic Ligand Model (BLM) and the Electrostatic Toxicity Model (ETM). According to the BLM concept, these major cations may compete with Cu^{2+} for binding sites at the biotic ligands, affecting Cu^{2+} toxicity. In the ETM, effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity may result from surface and internal interactions between these major cations and Cu^{2+}. Surface interactions are expressed as effects of these major cations on the free ion activity of Cu^{2+} at the plasma membrane $\{Cu^{2+}\}_0$ through changes in the surface potential ψ_0 (mV) and calculated by the Nernst Equation (Eqn. 7.1). ψ_0 is determined by the ionic composition of the bathing medium (Eqn. 7.2). Internal interactions are represented by the osmotic effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} adsorbed (Os; µmol/L) on Cu^{2+} toxicity (Growth; mm) (Eqn. 7.3).

Biotic Ligand Model (Chapter 3)	**Electrostatic Toxicity Model (Chapter 6)**	
	Interactions	**Quantification**
Interactive effects cannot be modelled	Surface	$\{M^{n+}\}_0 = \{M^{n+}\}_b \times \exp\left[-\frac{F \times n \times \psi_0}{R \times T}\right]$ (7.1*)
		$\psi_0 = f(\{Na^{+}\}_b, \{K^{+}\}_b, \{Ca^{2+}\}_b, \{Mg^{2+}\}_b)$ (7.2*)
	Internal	$\text{Growth} = \frac{b}{\exp[(c_1 \times (1 + c_{10} \times Os + c_{20} \times Os^2) \times \{Cu^{2+}\}_0)^d]}$ (7.3*)

(7.1*) $\{M^{n+}\}_0$ and $\{M^{n+}\}_b$ (mol/L) are the activities of free ion M^{n+} at the PM surface and in the bulk phase medium, respectively; ψ_0 (mV) is the electrical potential at the PM surface; n (dimensionless) is the charge on the ion; F (J/mV) is the Faraday constant (F = 96.485); R (J/mol/K) is the universal gas constant (R = 8.314); and T (K) is the temperature.

(7.2*) $\{Na^{+}\}_b$, $\{K^{+}\}_b$, $\{Ca^{2+}\}_b$, and $\{Mg^{2+}\}_b$ (mol/L) are the free ion activities of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} in solution, respectively; ψ_0 (mV) is calculated as a function of the ionic composition of the bathing medium according to the model developed by Kinraide and Wang (2010).

(7.3*) The response of lettuce following metal exposure was assessed in terms of the root growth (Growth; mm); b (mm) is the growth of lettuce roots without Cu^{2+} adsorbed on the membrane surface or osmotic effects caused by Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}; c_1 (L/µmol) is the strength coefficient of Cu^{2+} toxicity without osmotic effects caused by Na^{+}, K^{+}, Ca^{2+}, or Mg^{2+}; c_{10} and c_{20} (L/µmol) are expansion coefficients representing osmotic effects of Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on Cu^{2+} toxicity; Os (µmol/L) is the osmolarity that is determined by the common cations in solution; $\{Cu^{2+}\}_0$ (µmol/L) is the free ion activity of Cu^{2+} at the PM surface; and d (dimensionless) is the slope of the adjacent curve describing Cu^{2+} toxicity under the influence of the osmotic effects.

Moreover, the inclusion of the affinity of ions for biological ligands allows investigating interactions and accumulation of metal ions at the biological surface. The BLM predicts the accumulation of metals at biotic ligands at the water-organism interface, while the ETM provides estimations of the adsorption of free metal ions at the PM surface. In this PhD thesis, the ETM was shown to have a higher capacity than the BLM in quantifying the interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} (Table 7.2). Particularly, the effects of Na^{+}, K^{+},

Ca^{2+}, and Mg^{2+} could not be modelled by the BLM due to the lack of a significant relationship between Cu^{2+} toxicity and the concentrations of the common cations in solution. According to the BLM investigated in this thesis, only H^+ effectively competes with Cu^{2+} for binding sites at the biotic ligand, significantly affecting Cu^{2+} toxicity. By applying the ETM, surface interactions can be explicitly quantified while the free metal ion activity at the PM surface could explain about 70% of the internal interactions. However, similar to the BLM, the assessment of the internal interactions by the ETM strongly depends on experimental data, e.g., the conclusion that whether the interactive effects are found to be statistically significant or not depends on the fitting of the experimental data and certain mathematical relationships. The limitations of the BLM in modelling interactions between Cu^{2+} and Na^+, K^+, Ca^{2+}, and Mg^{2+} might be attributed to the exclusion of specific physiological processes that involve the major cations. For instance, a number of processes may affect tolerance of organisms to toxic metals while according to the BLM principle, only competition for binding sites is included in determining metal accumulation at the biotic ligands and toxicity is completely determined by this accumulation (*Chapter 3*). In the ETM, the dependence of its contribution in integrating internal interactions in modelling metal toxicity on the fit between the experimental data and mathematical equations results from the exclusion of kinetic mechanisms.

7.3. Contribution of the integration of affinity of metals for biological ligands to modelling toxicity of metal mixtures

7.3.1. Modelling bioaccumulation of metal mixtures

In this thesis, a semi-mechanistic model was developed based on the covalent index and the size-based filtration rate (*Chapter 2*). In both the zebra and quagga mussels, modelled concentrations of all test metals were within one order of magnitude of the measurements. In addition, for a number of metals including Mn, Fe, Co, Ni, Se, Cd, Sn, and Pb, 71–99% of the variance in their accumulation in zebra mussels at different locations could be explained by the model (r^2 = 0.71–0.99). Furthermore, the integration of the affinity of metals for biological ligands in the model allows for extrapolation to a number of metals without the necessity for case-specific validation. In previous studies, kinetic bioaccumulation models were usually only able to estimate concentrations of a limited number of metals for which experimental data about physiological processes such as the uptake constant and the ingestion rate are available (Luoma and Rainbow, 2005). The semi-mechanistic bioaccumulation model developed in this PhD thesis results in reliable estimations of concentrations of a variety of metals, e.g., 13 elements in two different species and various exposure conditions in the present study as described above. Additionally, the uptake rate constant, that is modelled based on the covalent index and the size-based filtration rate in this thesis, is comparable to experimental data of Roditi et al. (2000). However, the exclusion of some physiological processes in the model developed in this PhD thesis accounts for over- or underestimations for some metals, e.g., Cu^{2+} (*Chapter 2*).

7.3.2. Interactions in mixtures of Cu^{2+}–Ag^+ and Cu^{2+}–Zn^{2+} and toxicity of the mixtures

By integrating interactions between different ions at the biological surface in estimating metal bioavailability, the BLM and the ETM possess potential to investigate interactions between metals in mixtures. But the application of these two approaches in assessment of toxicity of metal mixtures is limited. In this PhD thesis, these methods were applied in modelling interactions between Cu^{2+} and Zn^{2+} and between Cu^{2+} and Ag^+ besides the assessment based on the free metal ion activity in the solution (Table 7.3).

Table 7.3. Assessment of interactions in mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} by the Biotic Ligand Model, the Free Ion Activity Model for mixtures, and the Electrostatic Toxicity Model based on the concepts of concentration addition (CA) and response addition (RA)

Mixture		Biotic Ligand Model (Chapter 5)	Free Ion Activity Model for mixtures (Chapter 4)		Electrostatic Toxicity Model (Chapter 6)	
Cu^{2+} –Zn^{2+}	Interactions	Competitive binding	CA	Zn^{2+} significantly reduced Cu^{2+} toxicity	Surface	Zn^{2+} reduced $\{Cu^{2+}\}_0$; Cu^{2+} did not substantially affect $\{Zn^{2+}\}_0$
			RA	Zn^{2+} significantly reduced Cu^{2+} toxicity	Internal	CA: Zn^{2+} significantly reduced Cu^{2+} toxicity; Cu^{2+} increased Zn^{2+} toxicity RA: Zn^{2+} significantly reduced Cu^{2+} toxicity; Cu^{2+} did not affect Zn^{2+} toxicity
	Mixture toxicity	$r^2 = 0.65$	$r^2 = 0.92$		$r^2 = 0.92$	
Cu^{2+} –Ag^{+}	Interactions	Non-competitive binding	CA	Cu^{2+} significantly reduced Ag^{+} toxicity; Ag^{+} increased Cu^{2+} toxicity	Surface	No substantial interactive effects
			RA	No significant interactive effects	Internal	CA: Cu^{2+} reduced Ag^{+} toxicity; Ag^{+} increased Cu^{2+} toxicity RA: No significant interactive effects
	Mixture toxicity	$r^2 = 0.69$	$r^2 = 0.80$		$r^2 = 0.80$	

Based on the affinity of metal ions for biological ligands as expressed by the stability constant of binding of metal ions to biotic ligands (BLM) and by the binding constant of metal ions to ligands on the membrane surface (ETM), the BLM and the ETM allow to quantitatively integrate interactive effects in estimating the concentration of metal ion-biotic ligand complexes at the water-organism interface and in estimating the accumulation of free metal ions at the PM surface. While in the ETM this estimation can be always achieved, in the BLM this can be carried out only provided that certain assumptions are included, i.e., the main assumption being that metal ions in the mixture bind to either the same or to different binding sites. The BLM facilitates predicting the accumulation of different components in metal mixtures at the biotic ligands based on toxicological data for single metals, i.e., the stability constants of binding of metals to biotic ligands. However, an understanding of the relationship between this accumulation and metal toxicity following exposure to mixtures is lacking. In this thesis, the principle of toxic equivalency was applied, i.e., the equivalent fraction of the biotic ligand occupied by Cu^{2+}, Zn^{2+}, and Ag^{+} results in similar effects. In the FIAM for mixture and the ETM, a direct link between the free ion activity of metals in the solution and at the PM surface, respectively, and metal toxicity is not available following exposure to metal mixtures. In this PhD thesis, the experimental data were fit to the mathematical equations based on assumptions about ion-ion interactions. The justification of this approach is not confirmed yet because of a limited understanding of metal binding and toxicity, especially following mixture exposure. The approach of expressing ion-ion interactions by mathematical equations in the FIAM for mixture and the ETM shows better predictive power than the combination of the BLM and the TEF approach in estimating toxicity of metal mixtures (Table 7.3).

Compared to the approach based on the free metal ion activity in the solution, the ETM allows explicitly delineating surface interactions. Although the ETM does not explicitly delineate internal interactions, the correlation between the variations in the electrical potential at the PM surface and the internal interactions provides a potential explanation and interpretation of ion-ion interactions within the organism. In the present study, modelling approaches based on the free ion activity in the solution and based on the free metal ion activity at the PM surface performed equally well in estimating mixture toxicity as shown by the same value of r^2 (Table 7.3). This similarity is related to statistically significant relationships between the free ion activity of metals in the solution and the activity at the PM surface resulting from negligible variations in the electrical potential at the PM surface. These small variations in the surface potential as found in this thesis are ascribed to a high ionic strength of the nutrient solution used. Furthermore, in this PhD thesis, the ETM based on the role of the electrical potential at the PM surface provides additional explanations in interpreting ion-ion interactions, besides competition for binding sites as assumed in the BLM.

7.4. Comparison of the Biotic Ligand Model and the Electrostatic Toxicity Model in modelling metal toxicity

In both the BLM and the ETM, interactions between metal ions and organisms are included in estimating metal bioavailability, allowing predicting metal accumulation at the biological surface. However, there are substantial differences between the two approaches, each leading to their own advantages and disadvantages in metal assessment.

The advantage of the BLM is the direct link between metal accumulation at the biotic ligands and metal toxicity. However, effects of one competing cation, e.g., H^{+}, Na^{+}, or Ca^{2+}, on the accumulation of one toxic metal at the biotic ligand could be modelled only if the

variations in the toxicity of the toxic metal with varying concentrations of the competing cation are significantly related to the variations in the concentration of the competing cation. In addition, effects of the common cations on metal toxicity can only be assessed separately. Another disadvantage of the BLM is its assumption of competition between ions for binding sites at the biotic ligands, excluding other mechanisms of ion-ion interactions.

In modelling toxicity of metal mixtures, the BLM allows estimating the accumulation of one metal in mixtures taking into account effects of other components, which determine metal toxicity. However, this prediction must be based on certain assumptions about competitive or non-competitive binding, which are usually not verified because of a limited understanding of modes of action of metals, especially following exposure to metal mixtures. The binding of metals to multiple sites adds another disadvantage of the application of similar or dissimilar binding sites in the BLM. In other words, different metal ions may have both similar and dissimilar binding sites and the patterns of interaction will be different for metals sharing the same biotic ligands as compared to metals interacting with different ligands. These phenomena cannot be expressed by the BLM on forehand. Moreover, the validity of the application of the stability constants of metal ions as determined by means of single exposure assessments to the assessment of metal mixtures is not verified.

The ETM shows both advantages and disadvantages in studying interactions between Cu^{2+} and common cations and interactions in metal mixtures. This modelling approach allows including effects of multiple common cations into modelling metal toxicity simultaneously and might provide insight into additional mechanisms in simulating ion-ion interactions, i.e., through changes in the electrical potential at the PM surface, besides the competitive binding. Moreover, the accumulation of toxic metals at the PM surface taking into account interactions with major cations or with other toxiciants in the mixture can be always estimated. However, the ETM does not provide a mechanistic link between the accumulation of metal ions at the PM surface and metal toxicity. Therefore, the capacity of the ETM for taking into account internal interactions between different ions in estimating metal toxicity depends on the fit of the experimental data and mathematical equations.

7.5. Conclusions

From the results presented in the previous chapters and the synthesis of these chapters as shown in the previous sections, the following main conclusions can be distilled.

1) The covalent index might be used as a unifying factor in generalisation of metal bioaccumulation (*Chapter 2*). Metal accumulation levels predicted by the semi-mechanistic model developed based on this property in this thesis were within about one order of magnitude of the measurements. In addition, 70% to 99% of the variability in the concentrations in mussels of a number of metals could be explained by the model.

2) Common cations, e.g., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, reduce Cu^{2+} toxicity (*Chapter 3*). For H^+, this alleviation might result from competition between H^+ and Cu^{2+} for binding sites at the biotic ligands as assumed by the BLM. In other words, competition for binding sites might be a mechanism by which ions interact with each other, influencing metal accumulation at the biotic ligands and subsequently affecting metal toxicity. However, ion-ion interactions cannot be completely interpreted by the competitive binding only. Interactions between Cu^{2+} and Na^+, K^+, Ca^{2+}, and Mg^{2+} cannot be modelled by their competition for transport sites at the biotic ligands as assumed in the BLM.

3) The free ion activity of metals at the root surface is a better indicator than the metal activity in solution when assessing bioavailability and toxicity of single metals (*Chapters 5–6*). The BLM parameters are better predictors of intrinsic toxicity of single metals than the median effective activity expressed by the exposure level in the solution (*Chapter 5*). The free metal ion activity at the PM surface is a better indicator than the free metal ion activity in the solution in estimating toxicity of Cu^{2+} in the presence of the common cations (r^2 = 0.79 compared to 0.65, respectively; *Chapter 6*). This advantage is caused by the possibility to include the interactions between ions at the biological ligands in the predictions.

4) The free metal ion activity at the membrane surface and the free metal ion activity in solution perform equally well in estimating metal mixture toxicity as shown by similar values of the coefficient of determination (r^2 = 0.80–0.92; *Chapters 4 and 6*). This might be deduced from the statistically significant relationship between the free metal ion activity at the PM surface and the free metal ion activity in solution. This correlation, in turn, results from small variations in the electrical surface potential with varying free ion activity of metals in the bathing medium, which are related to a high ionic strength of the Steiner default solution.

5) Approximately 65% of the variability in toxic effects of mixtures of Cu^{2+}–Zn^{2+} and 64–84% of the variability in toxicity of mixtures of Cu^{2+}–Ag^{+} could be explained by a combination of the BLM and the TEF approach (*Chapter 5*). This result indicates that the assumption that metal accumulation at the biotic ligands determines metal toxicity might be applicable to metal mixtures.

6) Interactions between different ions may occur at different levels, i.e., surface and internal interactions (*Chapter 6*). While surface interactions can be quantified, the possibility to integrate internal interactions in modelling metal toxicity is limited, depending on the fit between the experimental data and mathematical equations. This disadvantage is encountered because a mechanistic link between the accumulation of metal ions at the PM surface and metal toxicity is still lacking.

7) Electrostatic interactions as expressed by variations in the electrical potential at the PM surface may provide an alternative mechanism for competition for binding sites in interpreting interactions between different ions (*Chapter 6*). Dose-dependent internal interactions between Cu^{2+} and Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, as predicted based on the free metal ion activity at the PM surface, might contribute to explain the inconsistent trend in Cu^{2+} toxicity with varying concentration of these major cations. Moreover, internal interactions between metals in mixtures might be related to changes in the electrical potential.

In general, this PhD thesis shows substantial contribution of incorporating the affinity of metals for biological ligands in metal assessment. The accumulation of metals at the biotic ligands at the water-organism interface and the activity at the membrane surface are better indicators than the exposure level in solution in estimating toxicity of single metals. Although the free metal ion activity at the PM surface and the activity in the solution perform equally well in estimating metal mixture toxicity, the electrostatic toxicity model allows delineating ion-ion interactions and integrating the ion-biological ligand interactions in the predictions of metal bioavailability and toxicity. In addition, the accumulation of metals at the biotic ligands, which is influenced by ion-ion interactions, might be a potential predictor of toxicity of metal mixtures. These results and conclusions indicate that the use of metal affinity for biotic ligands as a unifying factor in generalisation of metal bioaccumulation and bioavailability might result in considerable progress in assessments of metal toxicity and ion-ion interactions.

7.6. Recommendations

The results in this PhD thesis open possibilities for further research as well as potential application of the approaches applied. The different modelling approaches applied in this thesis might be integrated in current regulations and research on risk assessment, especially for metal mixtures.

7.6.1. Recommendation for an integrated approach

As presented in *Chapter 4*, metal ions interact with each other via two main ways. Ions may lead to changes in the electrical surface at the membrane surface, which is accompanied by alterations in the membrane permeability (indirect interactions). Alternatively, direct interactions occur between different metal ions that bind to the same biotic ligands, e.g., protein transporters, i.e., replacement of one metal ion by another. Both mechanisms influence toxicity of metal mixtures and should be therefore considered in combination in predicting ion-ion interactions. Indirect interactions could be quantified by the electrostatic toxicity model while direct interactions could be estimated by the BLM. Consequently, an approach integrating these two methods potentially allows taking into account both indirect and direct interactions between different ions in predicting joint toxicity of multiple metals. Furthermore, these two mechanisms are related to each other. On the one hand, the changes in the membrane permeability caused by alterations in the surface potential have effects on the stability constant of the metal ion-biotic ligand binding. On the other hand, direct interactions between different ions at the biological surface determine activities of metal ions at the PM surface, and consequently influence the ionic composition of the exposure compartment in direct contact with the biological surface. These observations emphasise the importance and create possibilities of combining the BLM and the ETM in investigating interactions between different metals and modelling their joint toxicity. In particular, in the current application of the BLM, effects of one metal ion on the toxicity of another can be modelled only if statistically significant relationships are found between the exposure level of one cation and the toxic effects of another expressed by the median effective concentration/activity. However, as reported in *Chapter 3* of this PhD thesis, although common cations, i.e., Na^+, K^+, Ca^{2+}, and Mg^{2+}, led to a substantial difference in the median effective activity of Cu^{2+}, these effects could not be taken into account in the BLM of Cu^{2+} toxicity. Moreover, negligible changes in the electrical potential at the PM surface do not necessarily indicate weak interactions between different ions. Particularly, metals with similar physicochemical properties may strongly interact with each other, i.e., replacement by each other at binding sites while the surface potential is relatively constant because of their similar properties. These disadvantages of the BLM and the ETM could be overcome by combining these two approaches, for example, integrating the electrical potential of the membrane surface in modelling the stability constant of the metal ion-biotic ligand binding. As such, effects of cations on the toxicity of another can be always modelled. The stability constant of binding to biotic ligands for one cation when no other cations are present in solution can be considered as the intrinsic affinity. When other cations are added to the solution, the "conditional" stability constant of the metal can be determined by both the intrinsic affinity and the varied electrical potential at the PM surface. Furthermore, this conditional stability constant can be integrated into calculating the fraction of the total number of biotic ligands occupied by the metal ion, which is the key indicator determining toxicity of the metal. This modelling approach potentially allows taking into account both direct and indirect interactions between different ions in estimating joint toxicity of multiple metal ions.

7.6.2. Recommendations for management

Integration of the mechanistic bioaccumulation model in exposure assessment. Results in this PhD thesis demonstrate that the covalent index is potentially a suitable unifying factor for estimating metal bioaccumulation in mussels (*Chapter 2*). Because of the similarities in uptake mechanisms of terrestrial and aquatic organisms especially in terms of the transport proteins involved (*Chapter 1*), a mechanistic bioaccumulation model based on the covalent index might be developed and able to accurately estimate metal accumulation in plants. Furthermore, this model may be integrated in the exposure analysis in risk assessment. For instance, the application of the mechanistic accumulation model may improve the current approach for human exposure assessment. Consumption of crops is an important pathway of metal uptake in humans and estimations of metal concentrations in crops are therefore required to assess human exposure (Swartjes, 2007). The bioaccumulation model can be integrated in, e.g., the CSOIL model, as an alternative for the current approach of using single and generic experimental values of the bioconcentration factor or empirical relationships between the bioconcentration factor and soil characteristics in estimating metal accumulation in vegetables (Brand et al., 2007).

Application of the BLM and the ETM for setting environmental quality standards for single metals. The current approaches of setting environmental quality standards for single substances (e.g., benchmark doses, NOAEL/NOEC) strongly depend on critical values, e.g., EC50 for individual metals obtained at specific conditions. This disadvantage can be overcome by using the BLM and the ETM to link critical values to environmental conditions, e.g., pH, alkalinity, and hardness of water. Additionally, the accuracy of the BLM in estimating toxicity of single metals to plants indicates possibilities of the model for use in setting soil quality criteria.

Application of the TEF approach in assessments of metal mixtures. The conventional TEF approach based on environmental critical values, e.g., median effective concentration EC50 expressed by the exposure level in solution, has been integrated in REACH in the assessment of the risks of dioxins, furans, and dioxin-like PCBs in the EU (EC, 2008a). So far, one of the reasons for not applying this approach to metal mixtures is the large variability in metal bioavailability. The determination of TEF values based on the accumulation of metal ions at the biotic ligands may overcome this limitation. This may provide the "intrinsic" relative toxic potency of different metal ions.

Integration of uncertainty factors in mixture assessment. Uncertainty factors are often used in risk assessment to cover inter- and intra- species variability. It has been suggested that the uncertainty factor currently applied in risk assessment does not sufficiently cover mixture effects (Kortenkamp et al., 2009). In addition, a specific mixture assessment factor is not employed in the current chemical-by-chemical risk assessment. The main reason is the lack of a validated approach for derivation of such a factor. Metal toxicity, as applied in this thesis, is determined by the accumulation of metals at the biological surface, which is a function of the stability constants of the metals and other metals in the environment. Consequently, effects of one metal on the accumulation of another metal at the biological surface are influenced by the difference in their stability for biological ligands. Therefore, an uncertainty factor based on the difference in the affinity constants between different metals in mixtures may improve the risk assessment of metal mixtures.

Integration of both whole mixture and component-based approaches in a tiered approach. Generally, in the present risk assessment, two approaches have been applied in studying mixture toxicity: whole mixture and component-based approaches. In the whole mixture approach, the toxicity of given mixtures is empirically assessed in particular conditions (EC,

2008b). Therefore, any synergistic and antagonistic interaction between mixture components is inherently included in the measured response of the organism. However, this approach is usually not suitable for developing environmental quality standards or extrapolation to different environmental conditions (Gennings et al., 2000). In the component-based approach, toxicity of mixtures is predicted based on the response of the organism to individual components using common models such as CA and RA. In this PhD thesis, both whole mixture and component-based approaches were used. Particularly, the combination of the BLM and the TEF approach is component-based while the whole mixture approach is included in the method of fitting toxicological data on metal mixtures to mathematic equations. Both whole mixture and component-based approaches have their own specific advantages and disadvantages and should be combined, instead of being used as alternatives to each other (Kortenkamp et al., 2009). This thesis provides possibilities to improve current approaches in assessment of metal mixtures. Although the approach of fitting empirical data to mathematical equations is based on assessment on metal mixtures, the concepts of CA and RA are included in this method, potentially allowing extrapolation to different conditions. Moreover, the methods applied in this thesis might be combined in a tiered approach to include both whole mixture and component-based methods. In the first step, the whole mixture approach is applied to determine whether the interactive effects are significant or not. If the effects are insignificant, conventional approaches of using the CA and RA models can be applied in the second step to predict toxicity of metal mixtures. If the interactive effects are significant, some approaches integrating the interactions as applied in the present study may be applied.

7.6.3. Recommendations for further research

Combination of the covalent index and other metal-specific properties to improve the predictive power of the mechanistic bioaccumulation model. The affinity of membrane transport proteins is metal specific, depending on metal charge and atomic radius, and preferences for coordination and ligands (Bell et al., 2002; Handy and Eddy, 2004; Veltman et al., 2008). Metal charge and radius are included in the covalent index. However, other factors should be combined with the covalent index to develop a better unifying factor for generalisation of metal bioaccumulation. For example, it was found that molecular weight can explain approximately 70% of the variability in metal bioaccumulation (Hendriks et al., 1998). A combination of the molecular weight and the covalent index may improve the predictive power of the model further. In the bioaccumulation model developed, a relation of the assimilation efficiency to the covalent index was not included in because of the lack of a statistically significant relationship between these two parameters, hindering the development of a mechanistic model. Therefore, other metal-specific properties should be considered in order to increase the potential for predicting the metal assimilation efficiency. A correlation between the assimilation efficiency and metal properties facilitates the development and validation of a mechanistic model in which metal accumulation is predicted without necessity for experimental measurements of physiological parameters.

Application of the covalent index and/or other chemical properties in modelling metal subcellular distribution. The internal fate of metals may vary substantially, due to the intracellular transport and trafficking of metals. These processes also involve proteins and other metal-binding ligands. Consequently, chemical properties that represent the affinity for these ligands like the covalent index considered in this thesis might also contribute to determine the subcellular partitioning of metals and might consequently be useful to estimate the subcellular distribution of the metals.

Application of the combination of the BLM and the TEF approach as well as the ETM approach for mixtures of more than two metals. The principle of these approaches may be applicable to mixtures of more than two metal ions. Particularly, in the BLM it is possible to incorporate effects of several metal ions in estimating the accumulation of one given metal assuming that these ions have the same or different binding sites at the biotic ligands. In the ETM, it is possible to estimate accumulation of one metal ion at the PM surface in the presence of a number of other metal ions if their binding constants are available, which allow computation of the electrical potential at the PM surface. With the development in the measurements of PM surface potential and the surface charge density, the number of metal ions for which the binding constants with the PM surface are available is increasing.

Investigation of the application of different mathematical relationships to express ion-ion interactions. In this PhD thesis, ion-ion interactions in metal mixtures were assumed to follow linearity. However, non-linear relationships may occur. Moreover, the conclusion about the interactions depends on the mathematical equations used. Therefore, the possibility that interactions between different metal ions follow non-linear patterns should be examined as well.

Investigation of effects of the surface potential in the transport of ions via the membrane. In the present study, negligible variations in the surface potential were observed. Therefore, the effects of the variations in the surface potential were not included in modelling internal interactions as well as toxicity of metal mixtures based on the activity of metal ions at the PM surface. In other words, effects on the free metal ion activity at the PM surface are the main consequences of the changes in the surface potential. However, besides effects on the surface activity, the electrical potential at the PM surface affects the transport of ions via the membrane (Wang et al., 2011). Therefore, methods aiming at integrating the influence of the electrical potential at the PM surface on the internal transport of ions should be developed, amongst others including studies in media of low ionic strength.

The results presented in this thesis show evident advantages of integrating the interactions between ions and biological ligands into the estimations of metal bioaccumulation, bioavailability, and toxicity. Moreover, different approaches applied in this PhD thesis show substantial potential in predicting toxicity of metal mixtures, integrating ion-ion interactions. These approaches may be integrated into present regulations in risk assessment, increasing the reliability and reducing uncertainties in the analysis.

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SUMMARY

There are a number of challenges in modelling metal bioavailability and toxicity resulting from the highly specific behaviour of metals in the environment and in organisms. Metal bioavailability and toxicity are further complicated because of interactions between different metals and between metals with organisms. Consequently, a unifying factor, which allows generalisation of metal bioaccumulation, bioavailability, and toxicity, is not well validated. Moreover, proteins and other metal-binding ligands play an important role in metal uptake and subcellular trafficking. Therefore, affinity of metals for the biological ligands may be able to describe the variability in metal bioaccumulation, bioavailability, and toxicity (*Chapter 1*). This method may improve the reliability of estimations, facilitate assessment of metal-metal interactions and metal mixture toxicity, and increase the potential for extrapolation to a number of metals. The affinity of metals for the biological ligands was expressed by the covalent index (semi-mechanistic bioaccumulation model), by the stability constant of binding of metals with biotic ligands at the water-organism interface (Biotic Ligand Model, BLM), and by the stability constant of binding of metals and the membrane (Electrostatic Toxicity Model, ETM).

The covalent index, which was suggested to represent the affinity of metals for proteins, was used to develop a semi-mechanistic model simulating metal bioaccumulation in zebra (*Dreissena polymorpha*) and quagga (*Dreissena bugensis*) mussels (*Chapter 2*). Particularly, kinetics of metal uptake and elimination via food and water were modelled based on the covalent index and the size-based filtration rate. This modelling approach increases the potential for extrapolation to a variety of metals, species, and exposure conditions without the need for case-by-case calibration. The estimated internal concentrations of 13 metals studied in zebra and quagga mussels were approximately within one order of magnitude of the measurements. For several metals, 70–99% of the variability in the internal metal concentrations in differently-sized mussels and at various sampling locations could be explained by the model. These results demonstrate that the covalent index is a potential unifying factor in the generalisation of metal bioaccumulation.

A BLM was developed to investigate effects of common cations, i.e., H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, on Cu^{2+} toxicity to lettuce *Lactuca sativa* (*Chapter 3*). According to the BLM principle, toxic cations, e.g., Cu^{2+}, may compete with the common cations for binding sites at the biotic ligands on the water-organism interface. Consequently, cations like H^+ are expected to have alleviative effects on the Cu^{2+} toxicity. H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}, in deed, were found to reduce toxicity of Cu^{2+} to lettuce. However, only effects of H^+ could be described by the BLM, i.e., toxicity of Cu^{2+} at different solution pH conditions could be predicted based on the BLM parameters. More than 95% of the variability in the toxic effects of Cu^{2+} with varying solution pH could be explained by the BLM. Moreover, the inconsistent trend in the effects of Na^+, K^+, Ca^{2+}, and Mg^{2+} may be related to specific physiological processes caused by these elements that are not included in the BLM.

The contribution of the incorporation of the affinity of metals for biological ligands to modelling toxicity of metal mixtures was evaluated by separate assessments based on the free metal ion activity in the solution (*Chapter 4*), the accumulation of metal ions at the biotic ligands (*Chapter 5*), and the free metal ion activity at the membrane surface (*Chapter 6*). Interactions in mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^+ were incorporated in estimating their toxicity. This was obtained by extending the conventional models of concentration addition (CA) and response addition (RA). About 80–92% of the variability in the toxicity of the mixtures could be explained by the mathematical equations developed. Based on the assumption of the absence of interactions in the metal mixtures, Zn^{2+} was found to be the least

toxic element compared to Cu^{2+} and Ag^{+} while there was no significant difference in toxicity of Cu^{2+} and Ag^{+}. On the basis of an assumption of potential interactions in the metal mixtures, Zn^{2+} significantly reduced Cu^{2+} toxicity while Cu^{2+} did not significantly affect Zn^{2+} toxicity. In addition, Cu^{2+} had significant alleviative effects on Ag^{+} toxicity while Ag^{+} enhanced Cu^{2+} toxicity according to the extended CA model. The most important disadvantage of the method applied is the lack of underlying mechanisms of the mathematical relationships developed because of a limited and controversial understanding of metal binding.

The BLM was combined with the toxic equivalency factor (TEF) approach in order to estimate toxicity of mixtures of Cu^{2+}–Zn^{2+} and Cu^{2+}–Ag^{+} to lettuce (*Chapter 5*). In particular, the fraction of the total number of biotic ligands occupied by metal ions was used to determine the relative toxic potency of Cu^{2+}, Zn^{2+}, and Ag^{+} expressed by TEF and the toxic equivalency quotient (TEQ) of metal mixtures. In other words, both TEF and TEQ were expressed based on the fraction of the total number of biotic ligands occupied by metals in mixtures. This approach allows integrating metal-metal interactions in terms of competition for binding sites at the biotic ligands in predicting toxicity of metal mixtures. 60–84% of the variability in toxic effects of the mixtures could be explained by TEQ. Moreover, toxicity of metal mixtures depended not only on their TEQ, but also specific composition of the mixtures. This finding is attributable to metal properties, e.g., essentiality, that lead to dose-specific physiological effects.

The accumulation of ions at the biological interface expressed by the free metal ion activity at the membrane surface was used to model interactions between Cu^{2+} and common cations, i.e., Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, and between Cu^{2+} with Zn^{2+} and with Ag^{+} (*Chapter 6*). This approach explicitly revealed ion-ion interactions at the proximate outside of the membrane surface (surface interactions) and allowed investigating interactions between cations adsorbed (internal interactions). The major cations reduced the free ion activity of Cu^{2+} at the membrane surface while effects of the internal interactions on Cu^{2+} toxicity (i.e., reducing or enhancing) were dose-dependent. At the exposure levels studied, Zn^{2+} substantially reduced the accumulation of Cu^{2+} at the membrane surface while Cu^{2+} did not affect the surface activity of Zn^{2+}. Additionally, the exposure levels of Cu^{2+} and Ag^{+} tested were not sufficient to result in substantial effects on the surface activity of each other. Furthermore, significant internal interactions following exposure to the metal mixtures were predicted by expanding the conventional concepts of CA and RA. Particularly, according to extended models of these concepts, Zn^{2+} adsorbed significantly reduced Cu^{2+} toxicity. The extended RA model also revealed significant effects of the interactions between Cu^{2+} and Ag^{+} on their toxicity, i.e., Ag^{+} increased Cu^{2+} toxicity while Cu^{2+} alleviated Ag^{+} toxicity.

The contribution of the affinity of metals for biological ligands to estimating bioaccumulation, bioavailability, and toxicity of metals was summarised and synthesised (*Chapter 7*). The affinity of metals for proteins as expressed by the covalent index was shown to potentially contribute to the metal bioaccumulation. The accumulation of metal ions at the biotic ligands or the membrane surface is a better indicator of toxicity of single metals than the level in the exposure solution. The assumption that toxic effects are determined by the concentration of ion-biotic ligand complexes may be applicable to toxicity of metal mixtures. In spite of a lack of an advantage of the free metal ion activity at the membrane surface compared to the exposure level in the solution in estimating toxicity of metal mixtures, the affinity of metals for the membrane allows distinguishing surface and internal interactions. Moreover, effects of internal interactions on toxicity of metal mixtures might be explained in relation to the surface potential, which is influenced by the affinity of metals for the membrane.

In summary, using the affinity of metals for biological ligands as a unifying factor in generalisation of metal bioaccumulation and toxicity has substantial significance for metal assessment. Firstly, this approach allows including interactions between metals and organisms in the predictions of metal bioavailability. The accumulation of metals at the biological surface is a better predictor of intrinsic toxicity than the exposure level in the environment. Secondly, the modelling method based on the affinity of metals for biological ligands enables incorporating metal-metal interactions in estimations of metal bioaccumulation, bioavailability, and toxicity, especially following exposure to metal mixtures. Thirdly, this approach facilitates extrapolation potential. Consequently, the generalisation of metal assessment based on the affinity of metals for biological ligands may create considerable progress in risk assessment of metals.

SAMENVATTING

Het gedrag van metalen in het milieu en in organismen verschilt sterk van het gedrag van organische stoffen. Deze constatering heeft geleid tot een aantal uitdagingen in het modelleren van de biobeschikbaarheid en de toxiciteit van metalen. De modellering van de biologische beschikbaarheid en de toxiciteit van metalen wordt verder gecompliceerd door specifieke interacties tussen metalen en tussen metalen en organismen. Als gevolg hiervan is er nog geen verbindende factor bekend die het mogelijk maakt om bioaccumulatie, biologische beschikbaarheid en de toxiciteit van metalen te generaliseren. Eiwitten spelen een belangrijke rol in de opname en het subcellulaire transport van metalen. Daarom zou de affiniteit van metalen voor eiwitten de variatie in metaal bioaccumulatie, biobeschikbaarheid en toxiciteit kunnen verklaren (hoofdstuk 1). Een nieuw ontwikkelde methode die gebaseerd is op de affiniteit van metalen voor eiwitten kan de betrouwbaarheid van schattingen van metaalopname, biobeschikbaarheid en toxiciteit verhogen. Tevens kunnen met behulp van deze methode de interacties tussen metalen worden gekwantificeerd en kunnen beperkt beschikbare gegevens over opname, biobeschikbaarheid en effecten van metalen naar andere metalen worden geëxtrapoleerd. De affiniteit van metalen voor eiwitten is uitgedrukt door de covalentie index (mechanistisch bioaccumulatie model), door de stabiliteitsconstante van binding van metalen met biotische liganden aan het water-organisme interface (Biotische Ligand Model; BLM) en door de stabiliteitsconstante van binding van metalen aan membranen (Elektrostatisch Toxiciteits Model; ETM).

De covalentie index, die de affiniteit van metalen voor eiwitten weergeeft, is gebruikt om een semi-mechanistisch model te ontwikkelen dat de bioaccumulatie van metalen in driehoeksmosselen (*Dreissena polymorpha*) en in quaggamosselen (*Dreissena bugensis*) voorspelt (hoofdstuk 2). De kinetiek van metaal-absorptie en -eliminatie via voedsel en water zijn gemodelleerd op basis van de covalentie index en de filtratiesnelheid. Deze modelmatige benadering verhoogt de potentie voor extrapolatie naar andere metalen, soorten en blootstellingsomstandigheden, zonder de noodzaak om van geval tot geval de kinetiek te valideren. De geschatte interne concentraties van de 15 bestudeerde metalen in de driehoeks- en quaggamosselen waren ongeveer binnen een orde van grootte van de metingen. Voor verschillende metalen wordt 70-99 % van de variatie in de interne metaalconcentraties in mosselen van verschillende grootte en van verschillende locaties verklaard door het model. Deze resultaten demonstreren dat de covalentie index een mogelijke verbindende factor is in de generalisatie van metaal bioaccumulatie.

Een BLM is ontwikkeld om de effecten van belangrijke kationen, zoals H^+, Na^+, K^+, Ca^{2+} en Mg^{2+}, op Cu^{2+} toxiciteit voor sla (*Lactuca sativa*) te onderzoeken (hoofdstuk 3). Volgens het principe van het BLM kunnen toxische kationen zoals Cu^{2+} concurreren met deze kationen voor binding aan de bindingsplaatsen op de biotische liganden op het water-organisme grensvlak. Zoals verwacht is gevonden dat kationen zoals H^+ de Cu^{2+} toxiciteit verminderen. Ook Na^+, K^+, Ca^{2+} en Mg^{2+} blijken de toxiciteit van Cu^{2+} voor sla te verminderen. Echter, alleen de effecten van H^+ kan worden beschreven door het BLM, hetgeen wil zeggen dat het nieuw ontwikkelde model de toxiciteit van Cu^{2+} bij verschillende pH-waardes accuraat kan voorspellen. Het BLM verklaart meer dan 95% van de variabiliteit in de toxische effecten van Cu^{2+} onder de verschillende pH-condities. De inconsistente trend in de effecten van Na^+, K^+, Ca^{2+} en Mg^{2+} is gerelateerd aan specifieke fysiologische processen die niet in het BLM worden meegenomen.

De integratie van de affiniteit van metalen voor biologische liganden in de modellering van de toxiciteit van metaalmengsels is geëvalueerd door afzonderlijke bepalingen van de activiteit van vrije metaalionen in de oplossing (hoofdstuk 4), van de accumulatie van

metaalionen op biotische liganden (hoofdstuk 5) en door het meenemen van de activiteit van het vrije metaalion op het membraanoppervlak van de slawortels (hoofdstuk 6). Interacties in mengsels van Cu^{2+}-Zn^{2+} en Cu^{2+}-Ag^{+} zijn meegenomen bij het schatten van de toxiciteit van mengsels. Dit is gedaan door het uitbreiden van de gebruikelijke basismodellen voor mengseltoxiciteit van concentratie additie en (CA) en response additie (RA). Ongeveer 80-92% van de variabiliteit in toxiciteit van de mengsels wordt verklaard door de ontwikkelde wiskundige vergelijkingen. Op basis van de aanname van afwezigheid van interacties tussen Cu^{2+}, Zn^{2+} en Ag^{+}, blijkt Zn^{2+} het minst toxische element te zijn, terwijl er geen significant verschil in toxiciteit is gevonden tussen Cu^{2+} en Ag^{+}. Door mogelijke interacties tussen de metaalionen in de mengsels mee te nemen, werd gevonden dat Zn^{2+} de Cu^{2+}-toxiciteit significant verminderde terwijl Cu^{2+} geen significante invloed had op de toxiciteit van Zn^{2+}. Verder verminderde Cu^{2+} de Ag^{+} toxiciteit, terwijl Ag^{+} op zijn beurt de Cu^{2+} toxiciteit juist versterkte. De evaluatie van de resultaten toont aan dat het belangrijkste nadeel van de ontwikkelde methode het gebrek aan kennis over de onderliggende bindingsmechanismen is.

Het ontwikkelde BLM werd in hoofdstuk 5 gecombineerd met de toxische equivalentie factor (TEF)-aanpak om de toxiciteit van mengsels van Cu^{2+}-Zn^{2+} en Cu^{2+}-Ag^{+} voor sla te schatten. Met name de fractie van het totale aantal biotische liganden dat bezet was met metaalionen werd gebruikt om de relatieve toxiciteit van Cu^{2+}, Zn^{2+} en Ag^{+} te bepalen, uitgedrukt als TEF en als het toxische equivalentie quotiënt (TEQ) van metaalmengsels. Met andere woorden: TEF en TEQ werden uitgedrukt op basis van de fractie van het totale aantal biotische liganden dat bezet was door de in de mengsels aanwezige metalen. Deze aanpak maakt het mogelijk om metaal-metaal interacties in termen van competitie voor bindingsplaatsen op de biotische liganden te integreren in het voorspellen van de toxiciteit van metaalmengsels. 60-84% van de variabiliteit in de toxische effecten van de mengsels kon worden verklaard door het TEQ. Bovendien is aangetoond dat de toxiciteit van metaalmengsels niet alleen afhangt van hun TEQ, maar ook van de specifieke samenstelling van de mengsels. Deze bevinding is toe te schrijven aan specifieke metaaleigenschappen die leiden tot verschillende fysiologische effecten op verschillende niveaus van blootstelling, zoals bijvoorbeeld metaalessentialiteit.

De accumulatie van ionen aan het membraanoppervlak, uitgedrukt als de activiteit van de vrije metaal ionen op het membraanoppervlak, werd gebruikt om interacties tussen Cu^{2+} en de belangrijkste kationen in het medium, zoals Na^{+}, K^{+}, Ca^{2+} en Mg^{2+}, en tussen Cu^{2+} en Zn^{2+} en Ag^{+} te modelleren (hoofdstuk 6). Deze benadering toonde expliciet de aanwezigheid aan van ion-ion interacties aan de buitenkant van het membraanoppervlak (oppervlakte-interacties) en maakte het mogelijk om interacties tussen de aan het membraan geabsorbeerde kationen te onderzoeken. De belangrijkste kationen verminderden de activiteit van Cu^{2+} aan het membraan oppervlak, terwijl effecten van de interne interacties op Cu^{2+} toxiciteit (dat wil zeggen, het verminderen of vergroten van de interacties) dosisafhankelijk waren. Bij de in deze studie toegepaste blootstellingsniveaus verminderde Zn^{2+} de accumulatie van Cu^{2+} aan het membraan oppervlak sterk, terwijl Cu^{2+} geen invloed had op de vrije metaalion activiteit van Zn^{2+} op het membraanoppervlak. Daarnaast werd gevonden dat de gebruikte blootstellingsniveau's van Cu^{2+} en Ag^{+} onvoldoende waren om hun wederzijdse oppervlakteactiviteiten substantieel te beïnvloeden. Verder werden significante interacties in mengsels voorspeld door de concepten van concentratie additie en respons additie uit te breiden. In het bijzonder toonden de aangepaste modellen van deze concepten aan dat aan het membraan geadsorbeerd Zn^{2+} de Cu^{2+} toxiciteit aanzienlijk verminderde. Het aangepaste respons-vermenigvuldigingsmodel bleek ook significante effecten van de interacties tussen Cu^{2+} en Ag^{+} op hun toxiciteit te verklaren, dat wil zeggen: Ag^{+} verhoogde de Cu^{2+} toxiciteit, terwijl Cu^{2+} de Ag^{+}-toxiciteit verminderde.

De bijdrage van de affiniteit van metalen voor biologische liganden op de schatting van bioaccumulatie, biologische beschikbaarheid en toxiciteit van metalen is samengevat in hoofdstuk 7. De affiniteit van metalen voor eiwitten, zoals uitgedrukt door de covalentie-index, is bepalend voor de bioaccumulatie van metalen. De accumulatie van metaalionen op biotische liganden of op het membraanoppervlak is een betere indicator van de toxiciteit van een metaal dan de totaalconcentratie van het metaal in het testmedium. De aanname dat toxische effecten bepaald worden door de concentratie van complexen tussen het metaal ion en het biotische ligand kan worden toegepast voor het bepalen van de toxiciteit van mengsels van metalen. Ondanks het feit dat het gebruik van de vrije metaalion activiteit op het membraanoppervlak geen significante verbetering levert van schattingen van de toxiciteit van mengsels van metalen ten opzichte van het uitgaan van het blootstellinsniveau van de metalen in het testmedium, wordt geconcludeerd dat het meenemen van de affiniteit van metalen voor het membraan het toelaat om interacties aan het membraanoppervlak en interne interacties te onderscheiden. Bovendien kunnen op deze manier de effecten van interne interacties op de toxiciteit van mengsels van metalen worden verklaard in relatie tot de oppervlaktepotentiaal die wordt beïnvloed door de affiniteit van metalen voor het membraan.

Samenvattend is het gebruik van de affiniteit van metalen voor biologische liganden als verbindende factor in de veralgemening van metaal bioaccumulatie en toxiciteit, van aanzienlijke betekenis voor de beoordeling van de risico's van metalen in het milieu. Ten eerste, laat deze benadering het toe om interacties tussen metalen en organismen mee te nemen in de voorspelling van de biobeschikbaarheid van metalen. De accumulatie van metalen op biologische membranen is hierbij een betere indicator van de intrinsieke toxiciteit van de metalen dan de blootstellings-concentratie van de metalen. Ten tweede laat de hier ontwikkelde modelleringsmethodiek op basis van de affiniteit van metalen voor biologische liganden het toe om metaal-metaal interacties mee te nemen in schattingen van de bioaccumulatie, biologische beschikbaarheid en toxiciteit van metalen. Ten derde vergemakkelijkt deze benadering de mogelijkheden voor extrapolatie van testgegevens tussen verschillende blootstellingsmedia en tussen verschillende metalen. De generalisatie van de beoordeling van de risico's van metalen op basis van de affiniteit van metalen voor biologische liganden wordt beschouwd als een belangrijke innovatie van de risicobeoordeling van metalen.

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CURRICULUM VITAE

T.T. Yen Le (Lê Thị Thu Yến) was born in Hung Yen province, Vietnam on the 10th of December 1985. After completing a regular fundamental programme, she studied at a specialised secondary school majoring on science, particularly mathematics, physics, and chemistry. At secondary school, she was awarded the second prize in a contest in chemistry for talented students in the province of Hung Yen. Subsequently, she had a strong desire to acquire further knowledge in chemistry passing the competitive entrance exam to enter the chemistry-specialised high school for talented students. This programme is managed by Hanoi University of Science (HUS) and organised by the Vietnam National University Hanoi (VNU) with the main objective of training highly skilled labour force. Afterwards, Yen Le decided to follow environmental sciences, a multidisciplinary field at HUS, VNU with the aim to focus on applications of chemistry knowledge. She carried out her first project by assessing the safety of a bioproduct for improving soil quality at the Vietnamese Academy of Science and Technology. Her next projects during her Bachelor studies were related to absorption of metals by microorganisms. These projects helped her to obtain the third and second prizes in scientific research competition for students in two consecutive years. After graduating from HUS with the highest grade in the Bachelor programme in Environmental Sciences, she was provided with an opportunity to follow a Doctoral programme in the same area, skipping the Master education. Yet, she was also awarded a Huygens Scholarship to follow the Master programme in Environmental Sciences at Radboud University Nijmegen (RU), the Netherlands. As chemical risk assessment has not been developed in Vietnam yet, she chose Human and Environmental Risk Assessment as her major, focusing on modelling of bioaccumulation and toxicity. She has worked in the National Institute for Public Health and the Environment (RIVM), the Netherlands for about one year to carry out her second Master internship and parts of her PhD research. Afterwards she was appointed as a junior researcher at Department of Environmental Science, RU, to complete her thesis.

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