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Incorporating toxicokinetics and toxicodynamics in metal bioavailability models using *Enchytraeus crypticus*

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我不知道将去何方，但我已在路上

“Science is a wonderful thing if one does not have to earn one's living at it.”

Albert Einstein

Contents

Chapter 1

General introduction

1.1 Metals in the environment

Sources of metals

Metals are present in the aquatic and terrestrial environment as a result of both natural and anthropogenic processes. The background concentrations of metals in the natural environment, with the weathering of parent rock and diffuse atmospheric deposition as the sources, are relatively low in comparison to the concentrations that can be found at contaminated sites (Alloway, 1995). Apart from background levels, elevated concentrations of metals in the environment are mainly caused by human activities (Bak et al., 1997). Man's perturbation of nature's slowly occurring life cycle of metals includes (i) agricultural activities, where fertilizers, animal manures, and pesticides containing metals are widely used; (ii) industrial activities, including mining, smelting, metal finishing and energy production; (iii) the return of these metals in a concentrated form to the natural environment through waste disposal (D'Amore et al., 2005). Metals can be released into the environment in gaseous, particulate, aqueous, or solid form and emanate from both diffuse and point sources (Garrett, 2000). The metals released from these sources can reach aquatic and terrestrial ecosystems through various routes: directly (dumping, leakage and spilling), atmospheric emission followed by wet and dry deposition, and via sewage sludge.

Effects of metals

Metals playing a vital role in various biochemical and physiological processes in living organisms are generally recognized as essential elements for life. Essential metals include e.g. copper, manganese, nickel and zinc. The optimal biological performance can be obtained in a sometimes fairly narrow range of concentrations, which is called the "window of essentiality". A too low concentration of an essential metal (below the window of essentiality) may lead to deficiency, whereas a too high concentration may cause toxic effects (Hopkin, 1989). Nonessential metals are defined as those having no known physiological function or beneficial effects for organisms, and include e.g. cadmium, lead, mercury and silver (Peijnenburg and Vijver, 2007). However, non-essentiality is always difficult to prove and may not hold for all organisms.

Aquatic and terrestrial organisms can accumulate metals from water, soils, and sediments through either direct or indirect contact with them (e.g., ingestion, contact with (pore) water, inhalation of airborne particles and vaporized metals, etc.). These accumulated metals may exert adverse effects on organisms through their chemical behavior and their interactions with biomolecules in biological systems, leading to toxic effect (Mudgal et al., 2010). It has been widely recognized that metal concentrations exceeding physiological boundaries can result in toxicity to plants, animals and microorganisms. Phytotoxicity is caused when exposed to metal by changing the permeability of cell membranes, competing for sites with essential elements, enhancing oxidative stress, and affecting the reactions of sulphydryl (-SH) groups with cations (Kabata-Pendias, 2010). Metals can be strongly bound by –SH groups, leading to a change of the structure and enzymatic activities of proteins and causing negative effects on growth, reproduction and survival of aquatic and terrestrial animals (Hodson, 1988; Santorufo et al., 2012). A high level of metals may also affect the size or biomass of the microbial community, the activity of enzymes, and microbial processes like C and N mineralization and nitrification (Giller et al., 1998). Furthermore, food-chain

transfer of metals is an important route of human exposure and may cause severe effects on human health, like kidney damage, endocrine disruption, immunological disorders, and even death (Peralta-Videa et al., 2009). Toxicological studies on the effects of metals on organisms are therefore necessary in the context of risk assessment.

Metal pollution is a global problem that may have damaging effects on ecosystems and human health. This strengthens the need to develop accurate quality criteria and standards to evaluate to what extent metals pose a risk to ecosystems. Risk assessment requires toxicity evaluations of the effects of contaminants on plants, invertebrates and microbes. The results of risk assessment provide the principal basis for legislative decisions to reduce elevated risks (Rooney et al., 2007). Too stringent criteria may lead to increased societal costs for emission reduction and environmental sanitation measures, whereas criteria that are too loose may result in harm to ecosystems. Risk assessment not only relies on knowledge on the inherent toxicity of metals to organisms. Also exposure is of key importance, as it is the combination of exposure and hazard (toxicity) that determines the real risk. For metals, only a (small) fraction of the total amount present in the environment is available for uptake and therefore for causing effects on organisms. This means that the accuracy of the risk assessment for metals is constrained by our ability to determine the fraction that is actually available for organisms (Peijnenburg et al., 2007).

1.2 Bioavailability

Bioavailability was introduced as a concept that accounts for the fraction of the total amount of a chemical present that causes toxic effects. Knowledge of bioavailability therefore may improve the accuracy of the risk assessment. Bioavailability has been considered a dynamic process in which environmental availability of a metal causes exposure, resulting in actual uptake of metals (bioavailability) and subsequently effects due to metals interacting with a biological target (toxicological bioavailability) (Van Straalen et al. 2005; Peijnenburg et al., 2007).

Total concentration versus bioavailability

Until now risk assessment procedures of metals are still predominantly based on total metal concentrations. However, there is accumulating evidence showing that the relationship between total metal concentration and its toxic effect on biota is not straightforward (Van Gestel et al., 1995). In ecosystems, the aquatic and terrestrial ecotoxicity not only varies between species but environmental characteristics also greatly influence the effect concentration of metals (Plette et al., 1999; Lock et al., 2000; Lock and Janssen, 2001a). Therefore, total metal concentration alone is considered not to be a reliable indicator for the potential adverse effects (Janssen et al., 2000). Only the bioavailable fraction can actually be taken up by organisms and subsequently induce adverse effects (Peijnenburg et al., 2002). Rather than attempting to define bioavailability in a generic manner, a more profitable strategy may be to develop a series of indicators, such as free metal ion activity and body metal concentration.

Exposure in terms of free ion activity

The free metal ions are supposed as the most available/active species in water/soil solutions since trace metals are mainly transported into biological cells in ionic form due to the fact that ionic channels are involved (Ahlf et al., 2009). The observed variability in toxicity can be partly explained by attributing the toxic effect of metal to differences in the free ion activity in solution (Sauvé et al., 1998; Slaveykova and Wilkinson, 2002). As their speciation has a strong influence on the bioavailability of metals, any environmental factors that change the relative distribution of a metal over its different species will affect their bioavailability and toxicity. Trace metal speciation in the natural water systems is mainly dependent upon pH and which complexing ligands present (Pagenkopf, 1983; Van Gestel and Koolhaas, 2004). Dissolved organic matter (DOM) and inorganic ligands could reduce metal toxicity by complexation with metals and reduce the amount of free metal ions (Playle et al., 1993; Heijerick et al., 2003). Software programs like WHAM (Tipping, 1994) and MINEQL (www.mineql.com) have been developed to calculate free ion activities of metals from dissolved metal concentrations by incorporating a suit of chemistry parameters (e.g. pH, DOC and other cations).

Exposure in terms of body concentration

It has been proposed that an effective way of assessing the bioavailability of metals is by actually measuring the amount of metal accumulated in organisms (Peijnenburg et al., 2002, Van Straalen et al. 2005). As both the biotic (e.g. physiology) and abiotic (e.g. pH, cations, organic matter) modifying factors can be incorporated, metal body concentration may serve as a direct biological indicator of bioavailability and provide a more accurate estimation of the bioavailable fraction (Lanno et al., 2004). Many research efforts have been made to link metal body concentration to toxicity. It is assumed that when a chemical accumulates in an organism to a level above a theoretical toxic threshold, called critical body residue (CBR), toxic effects occur (McCarty and Mackay, 1993). However, the relationship between metal accumulation and toxicity may be influenced by physiological mechanisms, which could limit the interpretation of the concentration within organisms (Rainbow, 2007). After absorption, metals may be metabolized and excreted, accumulated in different tissues, sequestered internally, or transported in the organism to the sites of toxic action (Vijver et al., 2004). The portion of the body metal concentration that reaches and interacts with a critical receptor is toxicologically bioavailable, and is actually causing toxic effects. Hence, the ultimate goal of exposure assessment is to estimate the biologically effective dose also called target dose (Ahlf et al., 2009).

Characterization of the bioavailable fraction of metals is a prerequisite for an adequate risk assessment. Therefore, a more widespread appreciation of metal bioavailability is necessary.

1.3 Toxicokinetics and toxicodynamics

The accumulation of a metal by organisms can be affected by a range of factors, including characteristics of the metal, exposure concentration, and physiology of the species (Spurgeon and Hopkin, 1999; Ardestani et al., 2014b). The total amount of a metal present in

an organism expresses the balance between the amount assimilated and the amount which has been excreted (Janssen et al., 1991). When uptake rate is higher than excretion/detoxification, the metal could gradually accumulate in the organism, which eventually may lead to toxic effects (Broerse et al., 2012). Hence, exposure time is a very important factor in order to explain toxicity.

The differences found in the accumulation patterns of different metals in different organisms may have profound effects on the relationship between toxicity and exposure time (Spurgeon and Hopkin, 1999). The uptake pattern is linear if organisms are unable to eliminate the metal or do so at an extremely low rate. In that case, the likelihood that the body concentration exceeds the critical body concentration will increase with increasing exposure time, while the 50% effect concentration will decrease with time. For metals which can (actively) be excreted by organisms and reach equilibrium rapidly, effect concentrations will be only time-dependent during the time needed to reach equilibration (Crommentuijn et al., 1994). The 50% effect concentration will reach a stable value as soon as accumulation is at steady state. Studying accumulation kinetics and the factors that influence the uptake and elimination therefore is useful for predicting the physiological fate of a metal, and may help predicting its possible ecotoxicological effects.

In general, the time-dependent toxicity at the level of the organism can be simulated by two processes: toxicokinetics and toxicodynamics (Ashauer and Escher, 2010). Toxicokinetics link the environmental exposure concentration to the internal concentration as a function of time, and includes uptake, internal distribution, biotransformation and elimination of the chemical in organisms. Toxicodynamics relate the internal concentration to the toxic effect at the level of the individual organism (e.g. mortality) (Jager et al., 2011) (**Figure 1.1**). These processes play a vital role in determining metal bioavailability and toxicity. In the present thesis, attempts were made to incorporate toxicokinetics and toxicodynamics in bioavailability and toxicity modeling.

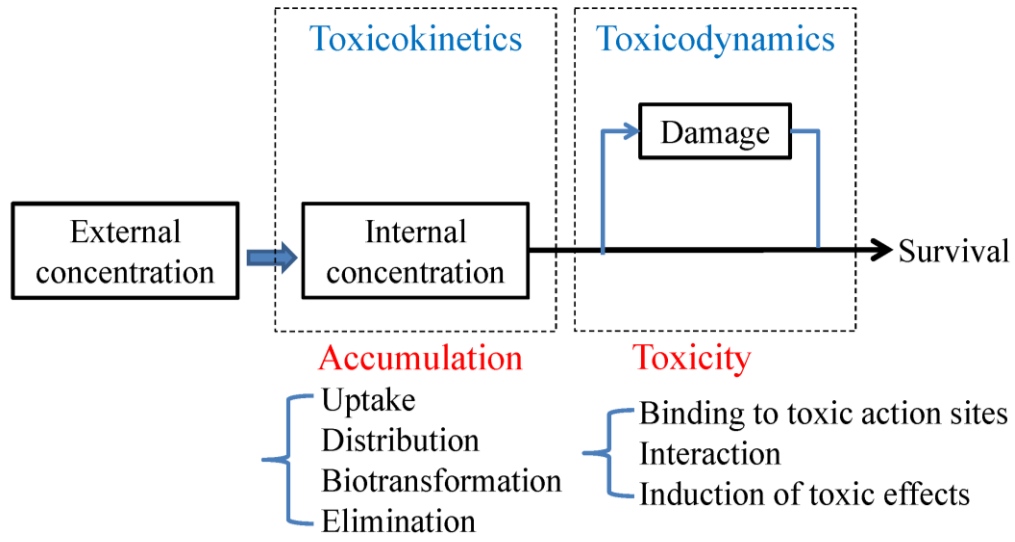

Figure 1.1 Processes leading to toxic effects of chemicals on organisms in time course

1.4 Biotic ligand model for predicting single metal toxicity

History

An important development with regard to the identification of the bioavailable fraction of metals was the finding that the free ion activity of metals in surface water correlated directly with their toxicity. These observations have led to the development of the conceptual free-ion activity model (FIAM) that describes how variations in the toxicity of metals can be related to their aqueous speciation and interactions with the organism (Morel, 1983). The FIAM is mainly based on the finding that the complexation of inorganic and organic ligands and DOM decreases metal toxicity by reducing free ion activity (Campbell, 1995). It is then assumed that a specific biological adverse effect (e.g. reduction of growth or reproduction, mortality) will occur at a fixed free ion activity of metals, independent of other water quality parameters. However, it appeared that metal toxicity is not determined by the free ion activity alone (Allen and Hansen, 1996). Meyer et al. (1999) found that the concentrations of Ni and Cu bound to fish gills were consistent predictors of acute toxicity when water hardness varied, better than free ion activities of Ni and Cu. The presence of cations was hypothesized to exert a protective effect on metal toxicity by competing with metal ions for binding sites, leading to the development of the gill site interaction model (GSIM) (Pagenkopf, 1983).

The conceptual framework for the chemical equilibrium-based biotic ligand model (BLM) was developed from the FIAM and GSIM, taking into account both the influence of metal speciation (e.g. DOC, inorganic ligands) and competitive binding of protective cations (e.g. Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) on the bioavailability and toxicity of metals (Di Toro et al., 2001; Santore et al., 2002). The most important assumption of the BLM is that metal toxicity is related to the binding of free metal ions to sites on the organism at the organism-water interface. These sites may be either physiologically active sites leading to a direct biological response, or transport sites leading to metal transport into cells, and further transport into the body, followed by an indirect biological response (De Schamphelaere and Janssen, 2002). In the context of the BLM framework, these active sites are defined as the biotic ligand. The (in)direct biological responses can be represented as the formation of a metal-biotic ligand complex and toxic effects will occur when the concentration of metal-biotic ligand complexes reaches a critical level or if a certain fraction of the biotic ligands is occupied by the metal (Di Toro et al., 2001). The primary role of BLMs within regulatory frameworks of metals is to remove the influence of test-specific abiotic conditions within ecotoxicity databases, and to provide a quantitative tool for assessing metal availability and toxicity with the variation of water chemistry (Paquin et al., 2000).

Build-up of biotic ligand model

Based on the concept of BLM, metal ions (M^{Z+}) and other cations can bind to the theoretical biotic ligand (BL) sites. The interaction between cations and BL is treated as a surface complexation reaction. The stability binding constant K_{MBL} (L mol^{-1}) for a metal ion (M^{Z+}) binding to the biotic ligand can be expressed by an equilibrium equation:

$$K_{\mathrm{MBL}} = \frac{\{\mathrm{MBL}\}}{\{\mathrm{M^{Z+}}\}\times\{\mathrm{BL}\}} \rightarrow \{\mathrm{MBL}\} = K_{\mathrm{MBL}} \times \{\mathrm{M^{Z+}}\} \times \{\mathrm{BL}\} \quad (1.1)$$

where $\{M^{Z+}\}$ is the free metal ion activity (mol/L, M), {MBL} is the concentration of biotic ligand sites bound by metal (M), {BL} is the concentration of unoccupied biotic ligand sites (M).

The magnitude of toxic effect is assumed to be related to the fraction of binding sites on the biotic ligand occupied by the metal, and this fraction (f_{MBL}) depends on the binding affinity of M^{Z+} to the BL, the free ion activity, and binding affinity of the competing cations:

$$f_{MBL} = \frac{\{MBL\}}{TBL} = \frac{K_{MBL} \times \{M^{Z+}\}}{1 + K_{MBL} \times \{M^{Z+}\} + \sum K_{CBL} \times \{C^{Z+}\}} \quad (1.2)$$

where TBL is total biotic ligand site concentration (M), $\{C^{Z+}\}$ is the activity of cations (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and H^{+}) in solution (M), K_{CBL} is the stability constant for the binding of cations to the biotic ligand sites (L mol^{-1}). The summation is over all cations that potentially influence the binding of metal to the biotic ligand.

According to BLM theory, the fraction of metal binding sites occupied by the metal at which 50% effect occurs f_{50}, is assumed to be constant and independent of water characteristics. Equation 1.2 can be rewritten as:

$$EC50\{M^{Z+}\} = \frac{f_{MBL}^{50\%}}{(1 - f_{MBL}^{50\%}) \times K_{MBL}} \times \{1 + \sum K_{CBL} \times \{C^{Z+}\}\} \quad (1.3)$$

where $EC50\{M^{2+}\}$ (M) is the free metal ion activity resulting in 50% adverse effect.

The biological response is related to the assumed bioavailable fraction (f_{MBL}) and fitted using a logistic model, which reads:

$$S = \frac{S_{max}}{1 + (\frac{f_{MBL}}{f_{MBL}^{50\%}})^{b}} \quad (1.4)$$

where S is the survival number, S_{max} is the control survival number, *b* is the slope parameter. By fitting the toxicity data with the above equations, the parameters of the BLM required for predicting toxic effects of metals (i.e., f_{50}, K_{MBL} and K_{CBL}) can be obtained. Instead of survival, the BLM may also be applied to sublethal endpoints like growth and reproduction, but most currently available BLMs still focus on mortality.

State of the art of biotic ligand model

The BLM has been proposed as a tool to quantitatively evaluate the manner in which water chemistry affects the speciation and bioavailability of metals in aquatic ecosystems (Paquin et al., 2002). Many aquatic BLMs have been successfully developed to predict metal toxicity to fish, algae and water fleas at different water conditions, within a factor of two of the observed values (De Schamphelaere and Janssen, 2004b; Bossuyt et al., 2004).

A vital assumption in BLM is that metal toxicity occurs as a consequence of free metal ions reacting with binding sites on the biotic ligand, which is the gill in the case of fish (Di Toro et al., 2001). Toxicity mechanisms for aquatic and terrestrial organisms are assumed to be similar since the general binding sites (e.g., Na^{+} and H^{+} transporters) are inherently the same for most living organisms (Niyogi and Wood, 2004). Assuming that the biotic ligand is a more general binding site, the principle underlying aquatic BLMs is likely also to be valid for terrestrial organisms. The uptake of Cu in lettuce was found to be inhibited by protons and cations (Ca^{2+} and Mg^{2+}) (Cheng and Allen, 2001). Protective effects of protons, Ca^{2+} and Mg^{2+} on the rhizotoxicity of Cu and Zn in wheat have been reported (Kinraide et al., 2004).

The competitive effect of cations on metal toxicities to plants, invertebrates and microbes have been reviewed, indicating a BLM-type interaction (Thakali et al., 2006a; Thakali et al., 2006b; Ardestani et al., 2014a). These studies suggest the basic BLM assumption of cation competition may also hold for terrestrial organisms. In this thesis, we therefore explored the applicability of the BLM to *Enchytraeus crypticus*, being a representative soil organism.

An important issue raised regarding BLMs is the effects of time. Nearly all of the existing BLMs are developed for a stationary situation with fixed exposure period, neglecting the toxicokinetics and toxicodynamics of metals in the test organisms. The binding constants of cations in the BLM are derived from the linear relationship between the effect concentration (e.g., LC50, EC50) and the activities of the cations in the exposure medium. As the effect concentration is often negatively correlated with exposure time, this linear relationship may also change with time. Therefore the derived binding constants of competitive cations may vary with exposure time. Although previous studies have found that the protective effect of competitive cations decreases with time, most of them just simply compare the developed chronic BLM with an acute BLM (Heijerick et al., 2002, 2005). In our study, attempts were made to extend the time scale of application of the BLM. It is still unclear which parameters are time-dependent and which are not, therefore direct evidence may be obtained by experimentally determining the effect of time on each BLM parameter (binding constants and f_{50}). We therefore systemically developed different BLMs at different exposure times, with an ultimate goal to develop a generic BLM which is capable of accurately describing metal toxicity at varying (pore)water chemistries and at different exposure times. Two approaches are possible: (1) allow the key parameters such as binding constants to be time-dependent, or (2) keep these parameters as fixed but include additional processes that cause the time-dependency, for example changes in the biological target.

1.5 Modeling mixture toxicity

Most of the studies dealing with toxic effects of metals focus on single metals, while organisms in aquatic and terrestrial ecosystems are typically exposed to mixtures of metals. Significant adverse effects have been found when estrogenic chemicals were combined at concentrations below their individual No-Observed Effect concentration (NOEC) (Silva et al., 2002). The effects of metal mixtures are predominantly antagonistic and additive, sometimes slightly synergistic, irrespective of the organism and environmental compartment tested (Vijver et al., 2010). This suggests that chemicals in mixtures may lead to different effects than the single metals. Ignoring mixture effects may lead to either overestimation or underestimation of the risk. However, until now, in the great majority of ecotoxicological risk assessments, toxic effects were evaluated based on single metal criteria assuming as simple model of addtivity. Little progress has been made for setting environmental quality criteria to evaluate true mixture effects. Therefore, it is necessary to develop appropriate approaches\models to predict the combined effects of metal mixtures for establishing a realistic metal regulation.

Concentration addition model (CA) and Independent action model (IA)

There are two classical reference models (CA and IA) which enable us to calculate the expected mixture toxicity, based on the toxicities of the individual components and their concentrations in the mixture.

The CA concept was initially introduced by Loewe and Muischnek (1926), and is also known as the Toxic Unit (TU) approach (Sprague, 1970). It has been developed for mixture components that have common target sites and a similar mode of action. Every concentration of a metal in a mixture can be replaced by an equally effective concentration of another metal (Berenbaum, 1985). In this approach, the concentration of each metal in the mixture (c_i) is divided by the toxic concentration for the metal causing *X*% effect (e.g., the concentration causing 50% mortality of the organisms or reducing reproduction by 50%, EC50\LC50), to convert the concentration into a toxic unit (TU_{Xi}). So, TU_{Xi} scales the concentration of the metal in the mixture for its relative toxicity. The CA model assumes that the overall toxic potency of mixtures (MT) can be expressed as the sum of toxic units of the individual chemicals.

$$\mathrm{MT} = \sum_{i=1}^{n} \mathrm{TU}_{Xi} = \sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_{Xi}} \quad (1.5)$$

The CA model will hold true if MT = 1 at a total concentration of mixture provoking *X*% effect, and the effect of the combined metals is then additive (Boedeker et al., 1993).

The IA concept, sometimes also mentioned Response Addition, is used for predicting the toxic effects of mixtures of chemicals that are believed to have dissimilar modes of action (Bliss, 1939). The combined effect is calculated from any effect of single metal as follows:

$$\mathrm{E}(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - \mathrm{E}(c_i)] \quad (1.6)$$

where $\mathrm{E}(c_{\mathrm{mix}})$ denotes the predicted effect (scale from 0-1) of an n-chemical mixture, c_i is the concentration of chemical *i*, $\mathrm{E}(c_i)$ is the effect of chemical *i* present singly at concentration c_i.

Generally, CA allows the prediction of an effect concentration of a mixture using known effect concentrations of the single chemicals. In contrast, IA relies on known effects of the individual chemicals for predicting the overall effect of a mixture (Backhaus et al., 2000). However, in practice, it is often difficult to determine which model should be applied for predicting mixture toxicity as metals may have multiple and/or unknown modes of action.

The CA and IA models are based on the assumption that the presence of one metal does not affect the biological action (uptake, distribution, or metabolization) of the others in mixtures. However, both physicochemical and biochemical behavior of a metal are known to change in the presence of other metals, which may lead to complex mixture effects that cannot properly be estimated from a single-metal point of view (Pokarzhevskii and Van Straalen, 1996; Vijver et al., 2010). Therefore, it is important to take into account possible metal-metal interactions in estimating mixture toxicity.

Deviations from CA and IA

In either CA or IA, mixture interactions can be detected when the observed impact of the mixture is greater than predicted (more than additive), the same as predicted (strictly additive) or less than predicted (less than additive). The terms “synergistic” and “antagonistic” are commonly used to describe “more” or “less” than additive effects, respectively (Norwood, 2003). However, recent data shows that more complex response patterns need to be addressed

by considering dose level– or dose ratio–specific synergism and antagonism to enable a correct assessment of mixture effects. A stepwise statistically based data analysis procedure (MIXTOX module) was therefore adopted to detect and quantify possible deviations from the CA and IA reference models (Jonker et al., 2005).

Metal interactions occur at various levels which complicates the toxicity assessment for metal mixtures. Firstly, at the environmental level outside the organism, metals can interact with substances in the surrounding media which may affect their bioavailability. Secondly, interactions between metals at the toxicokinetic phase may influence the uptake of mixtures by organisms. Thirdly, interactions that occur at the toxicodynamic phase may influence the accumulation of metals at the target sites, and subsequently affect joint toxicity of metal mixtures (Weltje, 1998; Vijver et al., 2011). In our study, the MIXTOX model was applied in conjunction with free metal ion activity in solution and body concentrations. This helps to identify where the interactions possibly occur. Insights into interactions at different levels are beneficial to the extrapolation of results across different studies with the same metal mixture. More insight into mechanisms of the interactions between metals may be obtained by applying mechanism-oriented models such as the BLM and WHAM-F_{TOX}.

Multi-metal BLM and WHAM-FTOX approach

The biotic ligand model (BLM) for predicting the toxicity of individual metals exists now for more than a decade (Di Toro et al., 2001; Paquin et al., 2002). However, the BLM has rarely been extended to consider mixture scenarios (Borgmann et al., 2008; Kamo and Nagai, 2008; Jho et al., 2011; Le et al., 2013). Theoretically, if two metals compete for binding to the same site of toxic action on the organism, it should be possible to model the amount of metal bound to that site, and hence to predict metal toxicity using a mechanistic BLM approach in what would be a much more advanced type of CA model. Alternatively, if competition between metals does not occur, bioavailability of each mixture component estimated by the BLM (i.e., f_{MBL}) could be a more reliable indicator of mixture toxicity in conjunction with the IA model (Norwood, 2003). By combining single-metal BLMs with the toxic unit approach, the overall amounts of metal ions bound to the biotic ligands or the toxic equivalency factor (Liu et al., 2014), it is possible to predict mixture toxicity using the single-metal BLMs where stability binding constants for metal ions and other coexisting cations are already known. However, this poses a challenge since these stability binding constants have only been determined for a limited number of metals and organisms (Antunes et al. 2012; Niyogi and Wood, 2004).

Recently, a new predictor based on the amount of metal ions binding to humic acid, which is assumed to be a proxy of non-specific biotic ligand sites, has been proposed (Iwasaki et al., 2013). A noteworthy feature is that it chemically incorporates the competition of metals with other cations as well as the competition among metals at the biotic ligand. The so-called WHAM-F_{TOX} approach has been successfully applied and tested to predict metal accumulation in stream bryophytes (Tipping et al., 2008) and copper toxicity to a duckweed species (Antunes et al., 2012). In addition, Stockdale et al. (2010) linked macroinvertebrate species richness to the amount of metals and protons binding to humic acid in rivers sampled from UK, US, and Japan.

In WHAM-F_{TOX}, a toxicity function was introduced and linked to biological responses:

$$F_{tox} = \sum \alpha_i v_i \quad (1.7)$$

where toxicity coefficient α_i is fitted from toxicity data, and organism metal load v_i (humic acid proxy) is calculated with WHAM. Toxicity in terms of the amounts of bound cations, expressed by F_{TOX}, is currently assumed to be additive, but further development of the model could include more complex relationships. For now, it is still unclear whether this approach can serve as a surrogate for the extended BLM in modeling mixture toxicity to soil invertebrates.

1.6 *Enchytraeus crypticus* as model species

Enchytraeids (class Oligochaeta, family Enchytraeidae) are widespread in many soil types and play an important role in decomposition and soil bioturbation, with exposure to chemicals in soil by dermal, intestinal and respiratory routes (Didden, 1993). It has been recognized that anthropogenic stress factors can have negative effects on enchytraeids, their numbers can be reduced and species composition can be changed by soil compaction or changes in land use (Römbke, 2003). In addition, these organisms react very sensitively towards both organic (pesticide) and inorganic (metal) chemicals (Castro-Ferreira et al., 2012). Generally, the choice of an organism as a model species for ecotoxicological testing should meet the following requirements: 1) play an important role in the functioning of the soil ecosystem; 2) be present in a wide range of soil ecosystems; 3) occur abundantly; 4) can easily be collected in the field and cultured under laboratory conditions; 5) exposure to stress factors through soil solution and solid phase; 6) sensitive to a variety environmental stresses (Edwards et al., 1996). Enchytraeids readily fulfill these criteria and could be considered as suitable test organisms for the risk assessment of metals. Moreover, enchytraeids can be investigated at the level of laboratory, semi-field and field to determine direct effects (Römbke et al., 1994), and serve as an early warning indicator of changes in the composition and functioning of the soil ecosystem by monitoring the change in the community to establish indirect effects (Didden and Römbke, 2001).

The genus *Enchytraeus* has been used in standard ecotoxicological tests. The largest and best known species of this genus is *Enchytraeus albidus*. The reproduction test (ERT) guidelines, ISO 16387 (ISO, 2004) and OECD 220 (OECD, 2004), have recommended the use of *E. albidus* as an indicator of chemical stress. In previous researches, *E. albidus* has been successfully used to determine the toxicity of metals (Cd, Ni, Zn), with reproduction and survival as endpoints (Lock and Janssen, 2001b; Lock and Janssen, 2002a; Lock and Janssen, 2002b). Compared to *E. albidus*, *Enchytraeus crypticus* has a shorter generation time, larger number of juveniles, and broader tolerance range to distinct soil properties (pH, texture, and organic matter content (Van Gestel et al., 2011; Castro-Ferreira et al., 2012). Hence, *E. crypticus* can serve as a better model species and was chosen as test organism in the present study.

1.7 Sand-solution system

The application of the BLM concept or other bioavailability models to predict the toxicity of metals to soil organisms is mainly hampered by two problems: 1) the uptake routes of metals in soils are generally more complex than those in water, since exposure may

take place via both pore water and ingestion of soil particles; 2) the difficulty in univariately controlling the soil pore water composition, since re-equilibration of the system would occur following any changes in soil properties (e.g., metal spiking) (Steenbergen et al., 2005). Simplified soil solutions were therefore tried to be used in soil ecotoxicity tests to minimize the influence of complex soil processes and to facilitate manipulating metal exposure and speciation. The mortality of *Folsomia candida* exposed to simplified soil solutions with 0.2 mM of Ca after 7 days was over 30% (Ardestani et al., 2013). This fails to meet the validity criteria, as mortality should not exceed 20% in the control according to OECD and ISO guidelines. The high mortality might be caused by the extra biological stress to soil organisms in water-only media. To solve this problem, a sand-solution system was developed in the present study. Inert sand was obtained by removing the organic matter and Fe and Mn oxides and hydroxides by combustion and acid washing of quartz sand, which avoids the binding of metals to solid soil particles. During the toxicity tests no food was offered to the test organisms to exclude the effects of organic matter on metal speciation. *Lumbricus rubellus* and *E. albidus* showed good performance over a period of 6 and 14 day, respectively, in such an inert sand matrix containing a solution, supporting the use of sand-solution systems as an alternative test medium for soil organisms (Vijver et al., 2003; Lock et al., 2006).

1.8 Aims of the thesis

Summarizing the literature reviewed above, the following knowledge gaps were identified:

1) A reliable indicator/model is needed to reflect the bioavailability processes and to normalize metal toxicity data obtained from different exposure conditions.

2) The applicability of BLM concept for predicting uptake and toxicity of metal (mixtures) to soil organisms has not been well determined.

3) It is still unclear whether the bioavailability models developed at a fixed exposure time can be used for predicting toxicity in a dynamic environment and how to take time factor into account?

4) Mixture interactions at toxicokinetic and toxicodynamic processes and their relative contribution have not been distinguished and quantified.

5) Development of a generic bioavailability-based model, accounting for possible modifying factors, is desired to delineate uptake and toxicity of metal and metal mixtures.

We hypothesized that the development of models, which integrate factors affecting metal bioavailability and the dynamics of metal accumulation and toxicity, will significantly improve the precision of toxicity predictions in the context of ecotoxicological risk assessment of metals. The general aim of this thesis is to understand the dynamic bioaccumulation and toxicity of metals and metal mixtures in *E. crypticus* under varying exposure conditions by quantitative modelling. To achieve the aim, the following research questions were addressed:

[1] Are the uptake and toxicity of metals time-dependent? Is body concentration a reliable indicator for metal toxicity in a dynamic environment? (Chapter 2)

[2] Which cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and H^{+}) exert significant influence on the uptake and toxicity of Ni? Is the BLM concept applicable for predicting metal uptake and toxicity in terrestrial organisms? (Chapter 3)

[3] How to develop a generic BLM model that incorporates the toxicity-modifying factors of both water chemistry and exposure time? (Chapter 4)

[4] How do the mixture components (Ni and Co) interact with each other? Do the interaction patterns vary with different exposure times and with different expressions of exposure (ion activity in solution, internal concentration in the animals)? (Chapter 5)

[5] Can competitive chemical reactions at the biotic ligand sites of the organism be represented by the competitive binding to the functional groups of natural organic matter (humic acid)? Can the WHAM-F_{TOX} model be used to delineate mixture toxicity in the course of time? (Chapter 6)

1.9 Outline of the thesis

In Chapter 1: An overview was given regarding metal bioavailability and toxicity, toxicokinetics and toxicodynamics, and mixture effects. Existing predictive models for delineating metal accumulation and toxicity were also reviewed. Based on the literature review, the knowledge gaps were identified and the main research questions were highlighted.

The research questions raised above are answered in the following chapters:

In Chapter 2: Uptake and toxicity of Ni in *E. crypticus* were investigated under different exposure concentrations and different exposure times using a solution-sand system. It was assumed that the body concentration and toxicity of Ni are both exposure concentration and time dependent before reaching equilibrium. A one-compartment model was used to describe the uptake and elimination kinetics of Ni. Further, enchytraeid survival was related to the body concentration to see its applicability for predicting Ni toxicity under changing environmental conditions. The results were interpreted from the viewpoint of toxicokinetics and toxicodynamics. Our findings highlight that time is an important factor which should not be ignored when assessing the risk of metals in a dynamic environment.

In Chapter 3: According to the concept of BLM, the coexistent protons and cations may compete with free metal ions for binding to the transporter sites at the biotic ligands, inhibiting metal uptake and subsequently reducing the toxicity of a metal. To verify this assumption, we examined the influence of solution chemistry (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and H^{+}) on the uptake and toxicity of Ni to *E. crypticus*. An extended Langmuir model and a BLM, incorporating the possible effects of competing cations, were developed for predicting the uptake and toxicity of Ni, respectively. The binding constants of cations to biotic ligands derived from uptake and toxicity data were then compared. A similar binding constant would indicate that the effect of the cation is mainly through competition for the binding sites on the surface of organism to affect the entry of Ni. If the binding constants differ, the interaction between the cation and Ni cannot simply be explained by competition. The results of this study shed light on the underlying mechanisms of the protective effect of cations.

In Chapter 4: The BLM was originally developed for predicting acute metal toxicity upon a fixed exposure time, and its performance in the realistic dynamic environment is unknown. In this study, we found that BLM parameters derived from short-term toxicity data

cannot be used to estimate the long-term toxicity of Ni to *E. crypticus*. Therefore, generic BLMs were developed by introducing either a time-dependent f_{50} (fraction of binding sites occupied by the metal causing 50% mortality) or time-dependent binding constants. Both models well quantified Ni toxicity with the variation of water chemistry and exposure time regardless of the different underlying assumptions. Although direct evidence cannot be provided to support model selection, this study does show the importance of incorporating toxicokinetics and toxicodynamics in assessing metal toxicity.

In Chapter 5: Mixture toxicity of Ni and Co to *E. crypticus* at different exposure time was investigated. Concentration addition was used as the reference model to determine whether there are any interactions between mixture components and whether the interaction patterns vary with time. More complex deviations from concentration addition (i.e., pure antagonism\synergism, dose-level and dose-ratio dependent antagonism\synergism) were further analyzed using the MIXTOX model. The interactions between metals associated with different expressions of exposure (free ion activity, body metal concentration) were compared to determine where the interactions may happen. This study provides insight into the mechanisms of the interactive effects of Ni-Co mixtures on the survival of *E. crypticus* at different interaction levels.

In Chapter 6: Assuming that competition acts as a mechanism for metal mixture interactions, the use of BLM-based models for interpreting mixture effects is possible. A challenge is that the conditional stability constants for single metals required by the mixture BLM are often unknown. This problem can be solved by a newly developed WHAM-F_{TOX} approach, which assumes that organisms accumulate metals by binding at non-specific ligand sites (humic acid). The binding affinities of metals for humic acid already exist in the database of WHAM. Here, the applicability of the WHAM-F_{TOX} model for delineating the dynamic uptake and toxicity of Ni and Co mixtures in *E. crypticus* was evaluated. The estimated toxicity coefficients of Ni and Co at different exposure times were compared to reveal the differences in accumulation patterns between the two metals. My results show that the WHAM-F_{TOX} model provides a new tool for evaluating the potential mixture toxic effects of metals to soil organism in a dynamic environment.

Figure 1.2 provides a schematic overview of the research conducted in Chapters 2-6.

In Chapter 7: A comprehensive discussion is provided regarding the results reported in Chapters 2-6. Further development of mechanistically-underpinned models for describing metal accumulation and toxicity to soil organisms in real soils will also be discussed, together with recommendations and an outlook for future research directions.

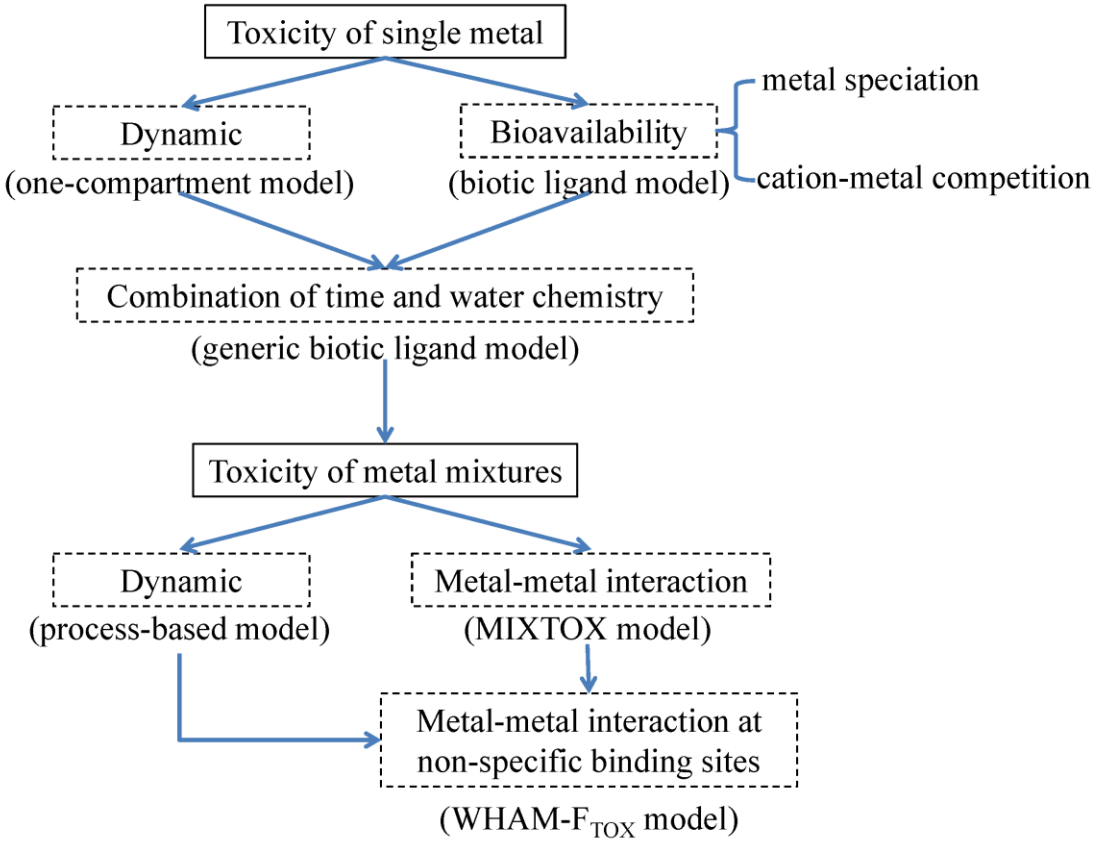

Figure 1.2 Schematic overview of the research performed in this PhD thesis.

Chapter 2

Toxicokinetics and toxicodynamics of nickel in *Enchytraeus crypticus*

Abstract

Metal toxicity is usually determined at a fixed time point, which may bias the assessment of risks associated with varied exposure time. Time-dependent accumulation and toxicity of nickel in the potworm *Enchytraeus crypticus* were investigated in solutions embedded in an inert quartz sand matrix. Internal Ni concentration and mortality were determined at seven different time intervals and interpreted from the perspective of toxicokinetics and toxicodynamics. A one-compartment model was used to describe the uptake and elimination kinetics of Ni. At each exposure concentration, Ni concentration in the organisms increased with increasing exposure time, reaching equilibrium after approximately 14 d. Median lethal concentration (LC50) decreased with time and reached an ultimate value of 0.182 mg/L. LC50 values expressed as internal Ni concentrations (LC50inter) were almost constant (16.7 mg/kg body dry weight) at each exposure time. The LC50inter was independent of exposure time, suggesting that internal concentration was a better indicator of Ni toxicity than external concentration. Uptake rate constant was 11.9 L/kg/d and elimination rate constants were 0.325 per day (based on internal concentration) and 0.070 per day (based on survival), indicating not all internal Ni contributes to toxicity. The present study highlights the importance of taking time into account in future toxicity testing and risk assessment practices.

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Environmental Toxicology and Chemistry, 2013, 32: 1835–1841

2.1 Introduction

Metal pollution in air, water, and soil is a global problem which may cause damaging effects on ecosystem and human health. Nickel is a metal of widespread distribution in the environment due to anthropogenic release, for example, the burning of fossil fuels, spreading of sewage sludge and manure, and mining activities (Phipps et al., 2002; Liber et al., 2011). Nickel is an essential element for several animal species, plants and microorganisms. It contributes to lipid metabolism, hematopoiesis and other biological functions at low concentrations, but causes toxic effects on organisms at elevated concentrations (Phipps et al., 2002). Elevated Ni ion concentrations have been reported to reduce the survival and reproduction of *Daphnia pulex*, *Enchytraeus albidus* and *Folsomia candida* (Kozlova et al., 2009; Lock and Janssen, 2002a) and inhibit the root growth of barley (Lock et al., 2007).

Factors determining metal toxicity can be organized into three basic categories: exposure, toxicokinetics and toxicodynamics (MaCarty and Mackay, 1993). Toxicokinetics describes the time course of metal accumulation, which includes metal uptake, distribution inside the body, biotransformation and elimination processes. It links external exposure to the internal concentration (Ashauer and Escher, 2010). Toxicodynamics describes the time course of toxic action at the target/active sites and subsequent toxic effects at the organism level (such as mortality, reproduction, and growth). It links the internal concentration to toxic effects. Toxicokinetics and toxicodynamics theory has revealed the importance of time for metal uptake and effects, while time has rarely been considered as a variable in toxicity testing or in risk assessment practices.

Internal concentrations depend not only on exposure concentration but also on exposure time. Spurgeon and Hopkin (1999), exposing earthworms to contaminated soil, found that the internal metal concentration increased with exposure time until it reached equilibrium. The relationship between toxicity and exposure time can be described by the Critical Body Residue (CBR) model (MaCarty and Mackay, 1993; Jager et al., 2011). The model assumes that there is an internal threshold concentration called CBR for organisms. Toxic effects will occur when the internal concentration exceeds this threshold. A metal present at a low concentration will be taken up slowly. If the uptake rate still exceeds the elimination rate it will slowly accumulate in organisms. In this case no or little toxic effects will be seen in an acute toxicity test when the exposure time is shorter than the time needed to reach the CBR, but significant toxic effects may occur upon chronic (long-term) exposure (Jager et al., 2011; Broerse and Van Gestel, 2010). In a study on zooplankton, Verma et al. (2012) found that the toxicity of metals is a function of exposure time and the LC50 declined with time. Without considering duration as a variable in toxicity tests, the resulting LC50 may underestimate the toxicity of metals (Jager et al., 2004). Nevertheless, currently most routine toxicity tests just focus on dose-effect relationships with fixed duration, ignoring the importance of time. More elaborate experimental designs are needed in order to enable the determination of time-dependent effects of metals on organisms.

It has been recognized that the internal concentration could serve as a better indicator than external exposure concentration for predicting metal toxicity to organisms. Unlike the internal concentration, external and bioavailable concentrations can be influenced by the environmental characteristics, the metal and the organism (Crommentuijn et al., 1994; Peijnenburg et al., 2002), which can affect the accumulation process. Borgmann et al. (1991)

found that the chronic toxicity of Cd to *Hyalella azteca* was much more constant when expressed as an internal concentration instead of measured Cd concentrations in the water. De Schamphelaere et al. (2005) reported that internal Cu is a better predictor for Cu toxicity to green algae than free Cu^{2+} activity in the exposure solution when pH is varied. Therefore, investigating the relationships between toxicokinetics (Ni accumulation in time) and toxicodynamics (survival in time) rather than the relationships between external exposure concentration and toxicity may provide a more accurate estimation of effects.

Enchytraeids play an important role in the functioning of terrestrial ecosystems and are sensitive to numerous chemical stressors (Didden and Römbke, 2001). They have close contact with soil pore water and the exposure takes place via the dermal and intestinal route (Römbke and Moser, 2002). *Enchytraeus albidus* is often used as test organism in soil toxicity tests because of its ecological relevance, practicability and data availability (Römbke, 2003). Moreover, *E. albidus* survives well in both soil and quartz sand medium (Lock et al., 2000; Lock and Janssen, 2001b; Lock et al., 2006). Recently, *Enchytraeus crypticus* is suggested to be a better model species than *E. albidus* in soil ecotoxicology, as it has better control performance, shorter generation time and higher reproduction rates, leading to a reliable and faster toxicity test (Castro-Ferreira et al., 2012). Quartz sand was chosen as test matrix in the present study. Using a solution only test medium for soil organisms can avoid the disturbance of complex soil processes, such as the complexation of metals with organic compounds and the competition of major cations. However, soil organisms perform best in solid medium, and water-only media may cause extra stress (Steenbergen et al., 2005). The use of sand-solution system as test medium is therefore a compromising choice to solve this problem.

So far, only limited information is available on the chronic toxicity of Ni to soil organisms, and the factor time was not taken into consideration in most cases. Therefore, the present study aims at investigating the relationship between internal Ni and exposure time in the potworm *E. crypticus*, and determining the effect of exposure time on Ni toxicity. Final aim is to build a relationship between toxicodynamics (survival in time) and toxicokinetics (Ni accumulation in time).

2.2 Material and methods

Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) has been cultured in VU University, Amsterdam for several years. For the preparation of culture media, a mixture of 1 kg of Lufa 2.2 soil and 3 L of tap water was shaken over night; the suspension was filtered over a 160 µm gauze and left for stabilization for 24 h. The supernatant was used to prepare agar media in which *E. crypticus* were cultured. Cultures were kept in a climate room at 16 °C, 75% relative humidity and in complete darkness. The animals were fed twice a week with a mixture of oat meal, dried yeast, yolk powder, and fish oil (Castro-Ferreira et al., 2012). Adult *E. crypticus* having eggs (white spots) in the clitellum region and with a length of approximately 1 cm were selected for testing.

Test medium

Quartz sand was used as test matrix. The characteristics of pretreated quartz sand are shown in Table 2.1. The sand was pre-combusted for 2 h at 600 °C to remove all organic matter (Lock et al., 2006), and then rinsed with 0.7 M HNO_3 (Sigma-Aldrich, 65%) to remove the remaining organic residues, carbonates and reactive Fe and Mn components (Vijver et al., 2003). After that, the acid was removed by flushing the sand several times with tap water and deionized water. In this way, inert sand was obtained and the pH of test solution was believed to be unaffected when contacting the inert sand.

Table 2.1 Characteristics of the pre-treated quartz sand used for solution only exposure toxicity tests with *Enchytraeus crypticus*.

Texture	Content
Clay (< 8 µm)	0.52%
Silt (8-63 µm)	0.55%
Sand (63-2000 µm)	98.93%
Very Fine Sand (63-125 µm)	6.03 %
Fine Sand (125-250 µm)	82.40 %
Middle Coarse Sand (250-500 µm)	10.03 %
Coarse Sand (500-1000 µm)	0.43 %
Very Coarse Sand (1000-2000 µm)	0.03 %

Test medium, composed of 0.2 mM Ca^{2+}, 0.05 mM Mg^{2+}, 2.0 mM Na^{+} and 0.078 mM K^{+}, was prepared by adding different amounts of $CaCl_2$, $MgSO_4 \cdot 7H_2O$, NaCl and KCl (Sigma-Aldrich; > 99%) to deionized water. For each treatment, the test medium was used as dilution to obtain different concentrations of Ni, added as $NiCl_2 \cdot 6H_2O$ (Sigma-Aldrich; > 98%). The solution pH was adjusted to 6.0 using 0.75 g/L MOPS (3-[N morpholino] propane sulfonic acid) (AppliChem; > 99%), 0.75 mg/L MES (2-[N-morpholino] ethane sulfonic acid) (Sigma-Aldrich; > 99%) and diluted NaOH when necessary.

Toxicity tests

Nickel toxicity was tested at 7 nominal concentrations (control, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 mg/L) and 7 exposure times (1, 2, 4, 7, 10, 14 and 21 d) were selected. Five replicates were used for each concentration and sampling time. For each treatment, 10 adult worms were exposed in a 100 mL glass jar (diameter 4.5 cm, height 8.0 cm), which was filled with 20.0 g pretreated quartz sand and 6.0 mL test solution. Before animal exposure the sand was kept in the test solution for 1 d (Steenburg et al., 2005). Tests were carried out at 20 °C with a cycle of 12 h light/12 h dark. The test jar with test medium was weighted twice a week and deionized water was added to compensate for water evaporation. Potworms were not fed during the experiment in order to avoid the influence of food on metal speciation in the test medium. Mortality was checked at each exposure time and the surviving animals were collected, washed with deionized water and frozen at -18 °C for further analysis.

Physical and chemical analysis

The particle size distribution of the pretreated quartz sand used in the present study was determined using a particle size analyzer (HELOS-QUIXEL; Sympaatec, Clausthal-

Zellerfeld, Germany) (Konert and Vandenberghe, 1997). At the end of experiment, the frozen animals were freeze dried for at least 24 h, weighted individually by microbalance, and digested in a 7:1 mixture of concentrated HNO_3 (Mallbaker Ultrex Ultra Pure, 65%) and $HClO_4$ (Mallbaker Ultrex Ultra Pure, 70%). Nickel body concentrations in animals were measured by graphite furnace atomic absorption spectrophotometry (AAS; Perkin Elmer 1100B). Porewater samples were collected by filtration of the test medium over a 0.45 µm membrane filter (Whatman®). Nickel concentrations in the initial solution and porewater were determined by flame AAS (Perkin ElmerAnalyst 100). Certified reference material DOLT-4 was used as quality control, and measured Ni concentrations were always within 15% of the certified value. The initial and porewater Ni concentrations of different treatments are shown in Table S2.1 (Supplemental data). The mean value of porewater Ni concentrations measured at different exposure times was used as actual exposure concentrations for data analysis in the present study.

Data analysis and modeling

Assuming that exposure concentration C_w (mg/L) is stable, a one-compartment model was used to describe the development of internal concentrations with time. This model reads:

$$C_0(t) = \frac{Kw \times Cw}{Ke_1} \times (1 - e^{-Ke_1 \times t}) \quad (2.1)$$

where $C_o(t)$ is Ni concentration in mg/kg dry body weight after exposure time t, K_w uptake rate constant in L/kg/d, K_{e1} elimination rate constant in per day. K_w and K_{e1} were obtained by fitting Equation 2.1 to all data from all exposure times and exposure concentrations together.

Median lethal concentrations (LC50) at each exposure time were calculated with the trimmed Spearman-Karber method (Hamilton et al., 1977). The relationship between survival and time was described by a logistic survival model (Crommentuijn et al., 1994):

$$S(t) = \frac{e^{-\mu t}}{1+(\frac{Cw}{LC50(t)})^b} \quad (2.2)$$

where $S(t)$ is the survival fraction at time t, C_w the exposure concentration of Ni in mg/L, $LC50(t)$ the LC50 value in mg/L calculated for each exposure time t, μ the natural mortality rate in per day, and b the slope parameter. This function was fitted to all survival data from all exposure times and exposure concentrations together.

Time-dependent toxicity is usually explained in terms of uptake and elimination kinetics. In this case, the relationship between toxicity and time may be expressed as:

$$LC50\ (t) = \frac{LC50\infty}{1-e^{-Ke_2 \times t}} \quad (2.3)$$

where $LC50(t)$ is the LC50 value in mg/L after t d of exposure, $LC50\infty$ the ultimate LC50 value in mg/L, and K_{e2} the elimination rate constant in per day.

The lethal body concentration (LBC) in mg/kg dry body weight is related to $LC50\infty$ as:

$$LC50\infty = LBC \times \frac{Ke1}{K_w} \quad (2.4)$$

The relationship between survival and internal concentration was described by a common logistic dose-response equation:

$$S = \frac{S_0}{1+(\frac{C_o}{LC50_{inter}})^b} \quad (2.5)$$

where S is the survival fraction, S_0 the control survival fraction, C_o Ni concentration in organisms in mg/kg dry body weight, LC50inter the LC50 based on internal concentrations in mg/kg dry body weight, and *b* the slope parameter. An overall LC50inter was obtained by fitting Equation 2.5 to all data together. Individual LC50inter values at each exposure time were also estimated using the same equation. Parameter estimation in equations (2.1-2.5) was done by nonlinear regression in SPSS 19.0 (IBM, Chicago, USA).

Chemical speciation of Ni in the test solutions was estimated using Visual MINTEQ. Input parameters included the pH and concentrations of Ni, Ca, Mg, Na, K, Cl^-, and SO_4^{2-}. In all treatments more than 99% of Ni existed as Ni^{2+}.

2.3 Results

Ni uptake kinetics

Internal Ni concentrations of *E. crypticus* exposed to different Ni concentrations in solution as a function of exposure time are plotted in Figure 2.1. Overall, the accumulation of Ni in the organisms depended on both exposure concentration and exposure time. At each time point, internal Ni concentration increased with the increasing exposure concentration. For all the treatments, internal Ni concentration increased upon increasing exposure time and leveled off reaching equilibrium after around 14 d. The highest concentration of Ni (32.9 mg/kg) in the organisms was found after 10 d exposure to 0.94 mg/L of Ni.

When Equation 2.1 was fitted to the data for different exposure concentrations (0.053, 0.072, 0.15, 0.28, 0.63, 0.94 mg/L) separately, the resulting K_w and K_{e1} values were 3.42, 19.8, 20.0, 30.6, 15.5, 10.1 L/kg/d and 0.069, 0.621, 0.339, 0.771, 0.395, 0.301 per day, respectively. Fitting Equation 2.1 to all data together gave overall uptake rate (K_w) and eliminate rate constants (K_{e1}) (± SE) for Ni in *E. crypticus* of 11.9 (± 1.43) L/kg/d and 0.325 (± 0.048)/d, respectively. The internal Ni concentrations estimated using the one-compartment model (Equation 2.1) with the overall K_w and K_{e1} are shown in Figure 2.1 (solid lines). The model fairly accurately described the Ni uptake, with $R^2 = 0.92$, $p < 0.01$.

Survival in time course

The relationships between the survival fraction of *E. crypticus* and exposure time at various Ni levels are presented in Figure 2.2. Mortality in the control groups was less than 15% after 21 d of exposure. Nickel toxicity to *E. crypticus* was associated with both exposure concentration and exposure time. Survival of the organisms decreased with increasing exposure concentration of Ni at each time point. At the same Ni exposure concentration, survival decreased with increasing exposure time. For the high exposure concentrations (0.28, 0.63 and 0.94 mg/L of Ni), toxicity of Ni to *E. crypticus* reached steady state in 21 d. For the low exposure concentrations (0.053, 0.072, 0.15 mg/L of Ni), steady state was not reached within the 21 d exposure period.

Natural mortality rate μ and slope *b* (± SE) derived from the logistic model (Equation 2.2) were 0.010 per day (± 0.002) and 1.57 (± 0.135) respectively. The solid lines in Figure 2.2 show the estimated survival fraction. The model fitted the data well with $R^2 = 0.89$, $p < 0.01$.

The LC50 values for the toxicity of Ni are plotted against exposure time in Figure 2.3. After 2 d of exposure, less than 50% mortality was observed and no LC50 could be calculated. The LC50 of Ni decreased from 0.75 mg/L at 4 d to 0.25 mg/L at 21 d. The fit of Equation 2.3 to the data is shown in Figure 2.3 as the solid line. The good fit is reflected by the $R^2 = 0.98$ and $p < 0.01$. Ultimate LC50, survival-based elimination rate (Ke_2) (± SE) and LBC obtained from the model (Equation 2.3 and 2.4) were 0.182 (± 0.031) mg/L, 0.070 (± 0.015)/d and 6.68 mg/kg dry body weight, respectively.

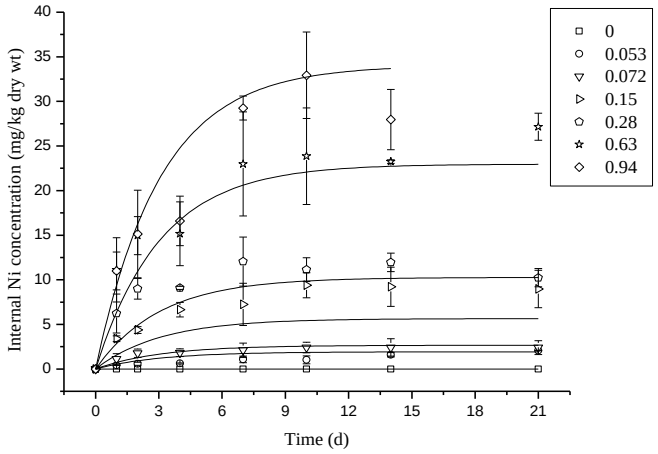

Figure 2.1 Relationship between internal Ni concentration (mg/kg dry body weight) and exposure time in *Enchytraeus crypticus* when exposed to different concentrations of Ni in solutions embedded in a quartz sand matrix. Data points show observed data with standard errors, and solid lines show the one-compartment model (Equation 2.1) fitted to the data. Each line corresponds to an individual exposure concentration.

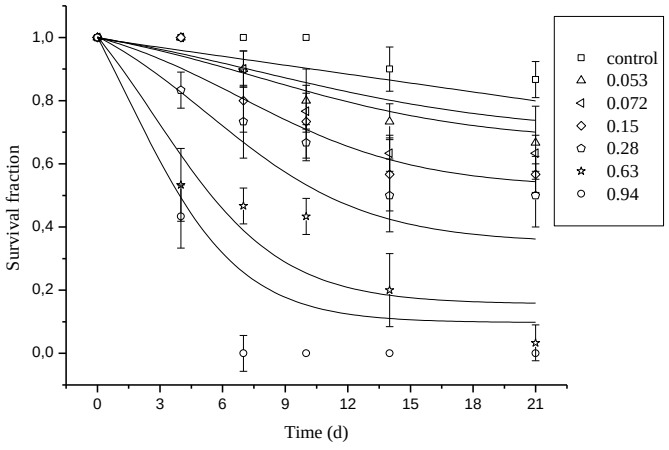

Figure 2.2 Survival fraction of *Enchytraeus crypticus* at different time intervals when exposed to different concentrations of Ni (mg/L) in solutions embedded in a quartz sand matrix. Data points represent observed data with standard errors, and solid lines show the logistic survival function (Equation 2.2) fitted to the data. Each line corresponds with an individual exposure concentration.

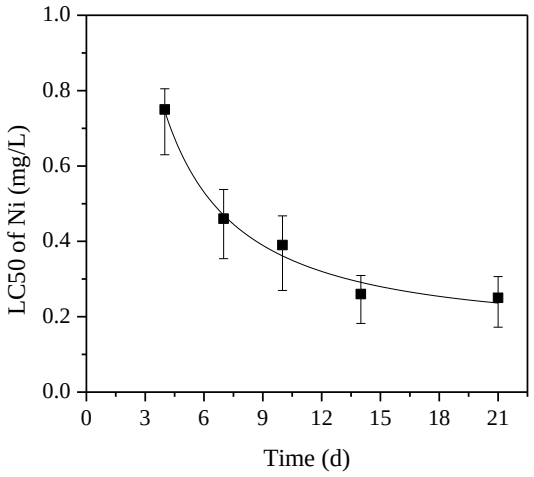

Figure 2.3 Development of LC50 values with time for the toxicity of Ni to *Enchytraeus crypticus* exposed in solutions embedded in a quartz sand matrix. Data points are LC50 values with 95% confidence interval calculated by the Trimmed Spearman-Karber method. The solid line shows exponential decline of LC50 following Equation 2.3 fitted to the data.

Linking survival to internal concentration

The relationship between survival of *E. crypticus* and internal Ni concentrations after 4, 7, 10, 14 and 21 d exposure is shown in Figure 2.4. Survival decreased with increasing internal Ni concentrations. When fitting Equation 2.5 to all data together, an overall LC50inter (± SE) value of 16.7 (± 1.45) mg/kg dry body weight was obtained. When fitting the survival data at each exposure time separately with the same equation, the obtained LC50inter values ranged from 16.9 to 17.1 mg/kg dry body weight.

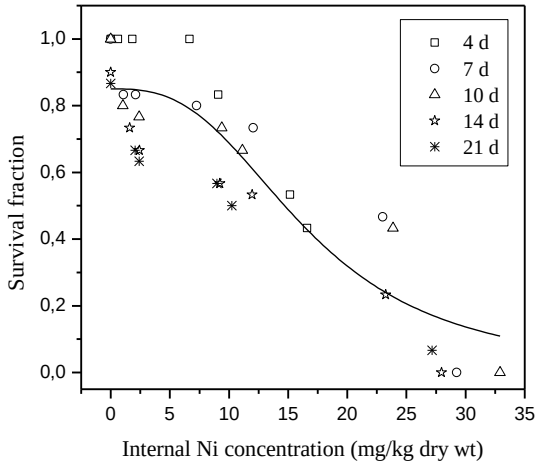

Figure 2.4 Survival of *Enchytraeus crypticus* exposed to Ni in solutions embedded in a quartz sand matrix as a function of Ni body concentrations measured at different exposure times. Solid line shows the fit of a logistic dose-response curve (Equation 2.5) to all data together.

2.4 Discussion

Internal Ni concentration increased with exposure time reaching equilibrium after approximately 14 d. In the beginning of the toxicity test, internal Ni concentrations were both exposure concentration and time dependent. After reaching equilibrium, internal concentrations were only related to exposure concentration. The relationship between internal Ni concentration and exposure time was nonlinear, indicating the ability of *E. crypticus* to eliminate Ni. Spurgeon and Hopkin (1999), investigating the accumulation pattern of metals in the earthworm *Eisenia fetida* exposed to contaminated field soil, found that it was able to eliminate Cu and Zn but not Cd and Pb. Janssen et al. (1991) compared the kinetics of Cd in 4 soil arthropod species and found that excretion rate constants were higher in *Notiophilus biguttatus* and *Orchesella cincta* than in *Neobisium muscorum* and *Platynothrus peltifer*. Generally, the accumulation pattern of metals is both metal- and species-dependent.

In the present study, LC50 of Ni for *E. crypticus* decreased with time from 0.75 mg/L at 4 d to 0.25 mg/L at 21 d. A number of studies have examined the toxicity of Ni for aquatic invertebrates, while toxicity data for terrestrial organisms is scarce. Moreover, most of the existing research just focused on the acute toxicity of Ni with short fixed exposure durations. Generally, the 48 to 96 h LC50 values of Ni for aquatic organisms ranged from 0.2 to 70 mg/L (Liber et al., 2011). Doig and Liber (2006), investigating the toxicity of Ni in water to the macro-invertebrate *Hyalella azteca*, found that 96 h LC50 was around 4.2 mg/L. Keithly et al. (2004) compared the acute and chronic toxicity of Ni to the cladoceran *Ceriodaphnia dubia* and found an acute 48 h LC50 of 81 mg/L and a chronic 7 d LC20 of less than 3.8 mg/L. Broerse and van Gestel (2010) found that the toxicity of Ni for the soil dwelling arthropod *Folsomia candida* was time dependent: LC50 decreased from approximately 1200 mg/kg at 6 d until 246 mg/kg after 5 wk, with an ultimate value of 157 mg/kg. Before organisms reach steady state, exposure time has a strong influence on toxicity (e.g. LC50), after that an ultimate effect concentration can be obtained. In most routine acute toxicity tests with aquatic organisms, exposure duration usually is no longer than 4 d which often is not sufficient to reach an ultimate LC50, and may cause the underestimation of toxicity.

The one-compartment model was used to describe Ni uptake in *E. crypticus* with time. Generally, the model fitted the data well (Figure 2.1). In the present study, the overall K_w and K_{e1} were used to predict internal concentrations. When fitting the data of internal concentration for different exposure concentrations separately, the individual K_w and K_{e1} values showed some variation. The K_w increased with the increasing exposure concentration and peaked (30.6 L/kg/d) at 0.28 mg/L Ni, and then decreased with increasing exposure concentration. The values of K_{e1} were almost constant except for the value at lowest exposure concentration (0.053 mg/L of Ni). Lock and Janssen (2001c) found that the uptake rate constant of Cd in *E. albidus* decreased with increasing exposure concentration. Ameh et al. (2012) reported that the uptake rate constant of Ni in the earthworm *Eudrilus eugenia* had a negative relationship with exposure concentration, but elimination rate constant showed a poor correlation with exposure concentration. These results are consistent with the findings of the present study. Uptake rate constant is determined not only by the characteristics of animal species and toxic substance but also by the characteristics of the environment. Elimination rate constant is mainly determined by characteristics of the organism (Crommentuijn et al., 1994). A possible explanation for the decrease of uptake rate constants with exposure

concentration is that there is a constant amount of metals ion transporters on the membrane of organisms (Li et al., 2009c). At high external concentrations the availability of these transporters may become limited and the physiological functioning of organisms may be disturbed, causing the decrease of uptake rates. So, toxicity may have affected Ni uptake kinetics also in the present study.

The estimated uptake rate constant of Ni in *E. crypticus* was 11.9 L/kg/d, with values at individual concentrations between 3.42 and 30.6 L/kg/d. Lock and Janssen (2001c) found that the uptake rate constant for Cd in *E. albidus* ranged from 0.104 to 0.214 kg/kg/d when exposed to Cd contaminated soil. Lister et al. (2011) investigated the toxicokinetics of Ni in the earthworm *Lumbricus rubellus* in field contaminated soil, and found that the uptake rate constant of Ni was around 0.103 kg/kg/d. The uptake rate constant found in the present study was much higher than the values reported previously, which is mainly due to the difference of environmental conditions. Compared to the studies in which the exposure medium was metal-contaminated soil, in the present study *E. crypticus* were exposed to a solution system. Most of the Ni existed as dissolved free Ni^{2+}, which is much more bioavailable and easier taken up by the animals. So, the higher uptake rate found in the present study may be explained from the higher Ni bioavailability in our test system.

Based on the uptake and elimination study, the elimination rate constant values of Ni in the earthworm *L. rubellus* ranged from 0.147 to 0.161/d (Lister et al., 2011). In the study of Broerse and van Gestel (2010), based on survival data the elimination rate constant of Ni in springtail *F. candida* was 0.024 per day. In the present study the elimination rate constant (K_{e2}) based on survival data for *E. crypticus* was 0.070 per day, which is lower than for *L. rubellus* but higher than for *F. candida*. These findings suggested that *L. rubellus* has a stronger capability of eliminating Ni than *E. crypticus* and *F. candida*. The differences of Ni elimination rate constant in different species may be related to the taxonomic position of the species (Janssen et al., 1991).

The toxicokinetic and toxicodynamic processes together determine metal toxicity to organisms. Development of internal concentration in time is supposed to reflect the toxicokinetic processes, survival in time the toxicodynamic processes (Ashauer and Escher, 2010). Two elimination rate constants (K_{e1} 0.325 per day and K_{e2} 0.070 per day) were obtained by using the one-compartment model. The K_{e1} was calculated on the basis of internal Ni concentrations, which is related to toxicokinetics. This K_{e1} value shows how fast Ni was accumulated and eliminated in the organisms. K_{e2} was calculated on the basis of toxic effects (i.e., mortality), and is related to toxicodynamics. K_{e2} therefore depends on the internal effective concentration which actually causes toxicity. The K_{e1} value was almost five-fold higher than K_{e2}, indicating that only approximately 20% of internal Ni in *E. crypticus* contributed to the observed effects. Vijver et al. (2004) reported that nonessential metals may be detoxified by storing them in non-toxic forms (e.g., binding to heat-stable proteins, specific metal-binding proteins, and storage in granules) so not the total internal concentration but only a fraction of the internal metal is responsible for toxicity in the organisms. Hence, the K_{e1} (relevant to total metal accumulation) should differ from the K_{e2} (relevant to toxic fraction).

In the present study, 21 d LC50 of Ni was 0.25 mg/L, but the estimated ultimate LC50 value was 0.182 mg/L, indicating that toxicity steady state was still not reached after 21 d.

However, after 21 d of exposure internal Ni concentrations did already reach equilibrium. This suggests that the toxicity process was delayed compared to the accumulation process. As described above, there are two steps (toxicokinetics and toxicodynamics) for metals before inducing toxic effects. After accumulation, the internal concentration leads to damage, and this damage leads to mortality. The concept of damage can explain why, at least in some cases, the kinetics of the body residues (time course of internal concentration) cannot explain the time course of mortality (Jager et al., 2011). The biological half-life of Ni was estimated to be 2.13 d and 9.90 d based on K_{e1} and K_{e2}, respectively, reflecting that the toxicodynamics was slower than the toxicokinetics.

The relationship between toxicokinetics and toxicodynamics was described by relating survival to internal concentrations. In the present study, when expressing LC50 on the basis of internal concentrations instead of external concentrations, LC50inter values at different exposure times were almost constant and more or less the same to the overall LC50inter. Different from LC50, the LC50inter of Ni in *E. crypticus* was almost fixed and independent of exposure time. When internal concentration is used to describe toxic effects the toxicokinetic processes have already been incorporated, so the influence of exposure time on toxicity can be reduced (McCarty and Mackay, 1993). Internal concentration predicted Ni toxicity well with a constant LC50inter. However, toxicity is also determined by toxicodynamic processes and internal concentration is not equal to the internal effective concentration which actually causes toxicity. A possible explanation for this paradox is that toxicokinetic processes determine the fraction that can interact with the target site (Escher and Hermens, 2004), indicating that internal effective concentration has an almost linear relationship with internal concentration. In the present study the LC50inter was 16.7 mg/kg, which is higher than the LBC of 6.68 mg/kg estimated by the one-compartment model. The value of LC50inter was expected to be similar to the lethal body concentration (Chaisuksant et al., 1997). The estimation of LC50inter was based on internal Ni concentrations, while the calculation of LBC was based on LC50∞ which links to the internal effective concentration. This may account for the observed differences of the two values. Generally, replacing exposure concentrations by internal concentrations is the first step toward a measure for actual toxicity and to partly eliminate or reduce the effect of time.

2.5 Conclusions

The findings in the present study revealed that Ni toxicity and bioaccumulation in *E. crypticus* are not only dependent on exposure concentration but also on exposure time before reaching a steady state. Internal Ni concentrations reached equilibrium after approximately 14 d of exposure. LC50inter was independent of exposure time, showing that internal concentration is a better predictor than exposure concentration for Ni toxicity. Exposure time is an important factor in determining metal uptake and toxicity and should not be ignored in future toxicity tests and risk assessments.

Supplemental information

Table S2.1. Measured Ni concentrations in different treatments of *Enchytraeus crypticus* in solutions embedded in an inert quartz sand matrix after different exposure times.

	Ni concentration (mg/L)					
Time (day) \ Nominal	0.1	0.2	0.4	0.8	1.6	3.2
0 (Initial solution)	0.075	0.14	0.30	0.60	1.5	3.0
1	0.058	0.079	0.16	0.32	0.70	1.3
2	0.068	0.084	0.16	0.32	0.74	1.4
4	0.057	0.085	0.15	0.31	0.63	1.1
7	0.066	0.078	0.15	0.22	0.63	1.1
10	0.049	0.060	0.14	0.28	0.59	0.79
14	0.035	0.065	0.17	0.22	0.60	0.42
21	0.035	0.056	0.15	0.28	0.49	0.49
Mean value	0.053	0.072	0.15	0.28	0.63	0.94

Chapter 3

Modelling uptake and toxicity of nickel in solution to *Enchytraeus crypticus* with biotic ligand model theory

Abstract

Protons and other cations may inhibit metal uptake and alleviate metal toxicity in aquatic organisms, but less is known about these interactions in soil organisms. The present study investigated the influence of solution chemistry on uptake and toxicity of Ni in *Enchytraeus crypticus* after 14 days exposure. Ca^{2+}, Mg^{2+} and Na^{+} were found to exert significant effects on both uptake and toxicity of Ni. An extended Langmuir model, which incorporated cation competition effects, well predicted Ni uptake. The LC50{Ni^{2+}} predicted by a developed Biotic Ligand Model matched well with observed values. These suggest that cation competition needs to be taken into account when modelling uptake and effects. The binding constants of Ni^{2+}, Mg^{2+} and Na^{+} on the uptake and toxic action sites were similar, but for Ca^{2+} they differed. This indicates that the effect of Ca^{2+} on Ni^{2+} toxicity cannot simply be explained by the competition for entry into organism.

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3.1 Introduction

It has been recognized that only a portion of the total amount of metal in the environment, that is the bioavailable fraction, can actually be taken up by organisms and subsequently induce adverse effects (Peijnenburg et al., 2002). Metal toxicity to aquatic and terrestrial organisms therefore cannot successfully be predicted from total external exposure concentrations (Plette et al., 1999; De Schamphelaere and Janssen, 2002; Van Gestel and Koolhaas, 2004; Steenbergen et al., 2005). Inaccurate estimation of the bioavailable fraction will either underestimate or overestimate the actual risks posed by metals. Therefore, improving ecological risk assessment for metals rests on better understanding bioavailability and developing appropriate bioavailability models.

An important step towards the identification of the bioavailable fraction of metals was the finding that there is a good relationship between effects and free metal ion activity. This led to the development of the Free Ion Activity Model (FIAM) which states that free metal ions in solution are responsible for metal uptake and toxicity in organisms (Morel, 1983; Campbell, 1995). Later, it was recognized that the coexisting protons and cations (e.g., H^+, Ca^{2+}, Mg^{2+}, K^+, and Na^+) can also affect metal accumulation and toxicity by competition with metal ions for uptake or toxic action sites (Plette et al., 1999; De Schamphelaere et al., 2005; Kalis et al., 2006). Li et al. (2009b) developed a competitive equilibrium model to quantify the effect of H^+ on Cd uptake by the earthworm *Eisenia fetida*, confirming that the pH-adjusted free Cd^{2+} activity was a better indicator for Cd body concentration than free Cd^{2+} activity alone. This suggested that the hypothetical competition for biotic uptake sites does exist, and the prediction of metal uptake based on free metal ion activity could be improved by the inclusion of the influence of the competitive cations.

The Biotic Ligand Model (BLM) was developed to take both metal chemical speciation and cation competition into account (Di Toro et al., 2001; De Schamphelaere and Janssen, 2002). The BLM assumes that organism response is directly related to the fraction of cellular sites at a critical site of toxic action (i.e., biotic ligand) which are occupied and deactivated by the reactive metal species (Di Toro et al., 2001) and that the competition of cations with metal ions for biotic ligands can alleviate toxicity. Considerable progress has been made in applying the principles of BLM to aquatic organisms (Di Toro et al., 2001; De Schamphelaere and Janssen, 2002; Paquin et al., 2000). But for soil organisms only few such BLMs are available, mainly for plants and earthworms (Steenbergen et al., 2005; Thakali et al., 2006a).

Originally, the BLM was developed to describe the toxic effects of metal bound to the gill of the fish. According to BLM theory, the gill is the hypothetical biotic ligand in fish. Meyer et al. (1999) found that the concentration of Ni and Cu bound to gills was approximately a constant predictor of acute toxicity to fathead minnows (*Pimephales promelas*). Further, the model has been applied to toxicity data for a series of other organisms. However, for plants and soil invertebrates it remains unclear where the biotic ligand is and whether the sites of toxic action are in direct contact with the external environment or not. Metal body concentration could be a reasonable approximation of the metal concentration binding at the toxic target sites (Escher and Hermens, 2004). A good relationship between metal toxicity and metal uptake will directly support the use of metal body concentration as an alternative for metal-biotic ligand complex for soil organisms. Van Gestel and Koolhaas

(2004) reported that internal concentration well described Cd toxicity to the springtail *Folsomia candida*. Lock et al. (2000), however, found that internal concentration is not a good predictor of acute toxic toxicity of Cd to the potworm *Enchytraeus albidus*. To explore the interaction of metals with cations on the biotic ligand, uptake mechanisms have to be studied, and linked to the toxic effects.

It has been widely recognized that elevated concentrations of Ni can exert toxic effects on aquatic and soil organisms (Rooney et al., 2007; Lock and Janssen, 2002a; Kozlova et al., 2009). Many empirical models have been developed to predict Ni bioavailability and toxicity to plants and invertebrates using different soil properties, such as pH, organic matter content, and cation exchange capacity (CEC) (e.g., Rooney et al., 2007; Smolders et al. 2009). The predictive ability of the developed models seemed to be good, however these empirical models did not provide much mechanistic insight into metal uptake and toxicity. Besides, the applicability of the developed models to other soil types is still unclear. For the purpose of mechanistic studies, a solution-sand system is therefore preferred as it facilitates univariately controlling solution composition and therefore exposure conditions.

The main objectives of the present study were to examine (1) whether the extended Langmuir model and the BLM, which incorporate possible cation competition, can be applied to predict Ni uptake and toxicity in *E. crypticus*, respectively; and (2) whether metal body concentration can serve as a surrogate for the metal-biotic ligand complex in predicting Ni toxicity to *E. crypticus*.

3.2. Materials and methods

Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) was chosen as test organism. *E. crypticus* has been cultured in VU University, Amsterdam for several years. They were cultured in agar prepared with an aqueous soil extract. Cultures were kept in a climate room at 16 °C, 75% relative humidity and in complete darkness. The animals were fed twice a week with a mixture of oat meal, dried yeast, yolk powder, and fish oil. Adult *E. crypticus* with eggs (white spots) in the clitellum region and with a length of approximately 1 cm were selected for testing. Enchytraeids play an important role in functioning of terrestrial ecosystems and are sensitive to chemical stressors (Didden and Römbke, 2001). Compared to *E. albidus*, *E. crypticus* is more suitable as it has better control performance, shorter generation time and higher reproduction rates, leading to reliable and faster toxicity testing (Castro-Ferreira et al., 2012). *E. crypticus* has a good performance in the exposure medium consisting of a solution embedded in inert quartz sand (He and Van Gestel, 2013).

Test medium

The pre-treatment of the quartz sand is described by He and Van Gestel (2013). The basic solution composed of 0.2 mM $CaCl_2$, 0.05 mM $MgSO_4$, 2.0 mM NaCl and 0.078 mM KCl was used as the control. Five sets of Ni toxicity tests were prepared: Ca-set, Mg-set, Na-set, K-set and pH-set (for details see Table 3.1). In order to distinguish the effect of different parameters, for each set only the concentration of the studied cation was varied, while the other parameters were kept constant at the same value as in the basic solution. Each set was

composed of at least 5 toxicity tests with different cation concentrations and a control. For each toxicity test, a series of Ni concentrations were used to obtain concentration-response curves covering 0-100% mortality of *E. crypticus*. Stock solutions of Ni, Ca, Mg, Na and K were prepared by adding different amounts of $NiCl_2 \cdot 6H_2O$, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, NaCl and KCl to deionized water. Test solutions with different levels of cations and different concentrations of Ni were prepared by adding different volumes of stock solutions to basic solution. All chemicals were obtained from Sigma-Aldrich and >99% pure. Except for the pH-set, all test solutions were adjusted to pH 6.0 (5.95-6.05) using 0.75 g L^{-1} MOPS (3-[N morpholino] propane sulfonic acid) (AppliChem; >99%), 0.75 mg L^{-1} MES (2-[N-morpholino] ethane sulfonic acid) (Sigma-Aldrich; >99%) and diluted NaOH when necessary. For pH-set, different pH values were obtained by adding MOPS buffer and diluted NaOH.

Table 3.1 Overview of the chemical characteristics of the test solutions used in different toxicity test sets with *Enchytraeus crypticus*.

Treatments	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	Ni (mg L^{-1})	pH
Control	0.2	0.05	2.0	0.078	0-6.4	6.0
Ca-set	0.5	0.05	2.0	0.078	0-25.6	6.0
	1.0	0.05	2.0	0.078	0-25.6	6.0
	2.0	0.05	2.0	0.078	0-25.6	6.0
	4.0	0.05	2.0	0.078	0-25.6	6.0
	8.0	0.05	2.0	0.078	0-25.6	6.0
Mg-set	0.2	0.25	2.0	0.078	0-25.6	6.0
	0.2	0.5	2.0	0.078	0-25.6	6.0
	0.2	1.0	2.0	0.078	0-25.6	6.0
	0.2	2.0	2.0	0.078	0-25.6	6.0
	0.2	4.0	2.0	0.078	0-25.6	6.0
Na-set	0.2	0.05	4.0	0.078	0-25.6	6.0
	0.2	0.05	8.0	0.078	0-25.6	6.0
	0.2	0.05	12.0	0.078	0-25.6	6.0
	0.2	0.05	16.0	0.078	0-25.6	6.0
	0.2	0.05	20.0	0.078	0-25.6	6.0
K-set	0.2	0.05	2.0	0.5	0-6.4	6.0
	0.2	0.05	2.0	1.0	0-6.4	6.0
	0.2	0.05	2.0	2.0	0-6.4	6.0
	0.2	0.05	2.0	4.0	0-12.8	6.0
	0.2	0.05	2.0	8.0	0-12.8	6.0
pH-set	0.2	0.05	2.0	0.078	0-6.4	6.0
	0.2	0.05	2.0	0.078	0-6.4	6.5
	0.2	0.05	2.0	0.078	0-6.4	7.0
	0.2	0.05	2.0	0.078	0-6.4	7.5
	0.2	0.05	2.0	0.078	0-6.4	8.0

Toxicity test

A 14-d toxicity test with *E. crypticus* was conducted using five replicates for each treatment. Ten adults were exposed in 100 mL glass jars filled with 20.0 g pretreated quartz sand and 5.4 mL test solution. The sand and the test solution were equilibrated for 1 day before introducing the animals. The experiments were incubated at 20 °C with a cycle of 12h light:12h dark. The jars were weighted every three days and water evaporation was compensated by adding deionized water. Mortality was checked after 14 days. All surviving animals were collected, washed with deionized water and frozen at -18°C for further analysis.

Physical and Chemical analyses

The particle size distribution of the quartz sand was determined using a particle size analyzer (HELOS-QUIXEL; Sympaatec, Clausthal-Zellerfeld, Germany). The concentrations of dissolved Ni, Ca, Mg, Na and K in test solutions were analyzed by flame atomic absorption spectrophotometry (AAS; Perkin ElmerAnalyst 100). At the end of experiment, the frozen animals were freeze dried for at least 24 h, weighted individually on a microbalance, and digested in a 7:1 mixture of concentrated HNO_3 (Mallbaker Ultrex Ultra Pure, 65%) and $HClO_4$ (Mallbaker Ultrex Ultra Pure, 70%). Nickel body concentrations in animals were measured by graphite furnace AAS (Perkin Elmer 1100B). The certified reference material DOLT-4 (dogfish liver; LGC Standards) was used for quality control, with the certified value (±SD) for Ni being 0.97 (±0.11) mg/kg. The reference material was measured once for each batch of 20 samples, and Ni concentrations were always within 15% of the certified value.

Data analysis

Activities of Ni and other cations in the test solutions were calculated using the Windermere Humic Aqueous Model (WHAM VI) (Tipping, 1998). The pH, DOC and measured dissolved concentrations of Ni, Ca, Mg, Na and K were used as input parameters. The animals stayed in tap water for about 0.5 h before being introduced into the test systems. In this way the gut content of organisms was mostly voided and no feces was produced to introduce DOC into test system. The concentration of DOC in the demi water used for the preparation of test solutions was 0.05 mg/L, and was taken into account when calculating the free ion activities of Ni and the other cations using WHAM VI. Median lethal Ni concentrations (LC50) based on measured Ni concentrations were calculated for each toxicity test using the trimmed Spearman-Karber (Hamilton et al., 1977).

Modelling descriptions

Extended Langmuir model predicting Ni uptake

A Langmuir isotherm was used to describe the relationship between Ni body concentrations and free Ni ion activity in exposure solution:

$$C_{Ni-body} = \frac{K_{NiS} \times \{Ni^{2+}\} \times C_{max}}{1 + K_{NiS} \times \{Ni^{2+}\}} \quad (3.1)$$

where $C_{Ni\text{-}body}$ is Ni body concentration (mol Ni kg^{-1} dry body weight), C_{max} is maximum Ni body concentration (mol Ni kg^{-1} dry body weight); $\{Ni^{2+}\}$ is free Ni^{2+} ion activity (M), K_{NiS} is the binding constant of Ni^{2+} for uptake sites on the biotic surface (L mol^{-1}).

The effect of competing cations on Ni uptake was described using an extended Langmuir model:

$$C_{Ni-body} = \frac{K_{NiS} \times \{Ni^{2+}\} \times C_{max}}{1 + K_{NiS} \times \{Ni^{2+}\} + \sum K_{CS} \times \{C^{z+}\}} \quad (3.2)$$

where $\{C^{z+}\}$ gives the activities of competing cations (e.g., Ca^{2+}, Mg^{2+}, and Na^{+}) (M), K_{CS} is the binding constant of cations for uptake sites on the biotic surface (L mol^{-1}). All body concentrations measured in animals from the toxicity tests were fitted to Equation 3.2. The constants for binding of Ni and cations to uptake sites (K_{NiS} and K_{CS}) were estimated using nonlinear regression analysis in SPSS 19.0. By incorporating the estimated K_{NiS} and K_{CS} into Equation 3.2, the $C_{Ni\text{-}body}$ could be predicted, using the free ion activities of metal ions and cations as inputs.

Biotic Ligand Model predicting Ni toxicity

Equilibrium equations for the binding of ions (e.g. Ni) to the biotic ligand sites can be written as stability constant expressions of the form:

$$K_{NiBL} = \frac{\{NiBL^{+}\}}{\{Ni^{2+}\} \times \{BL^{-}\}} \rightarrow \{NiBL^{+}\} = K_{NiBL} \times \{Ni^{2+}\} \times \{BL^{-}\} \quad (3.3)$$

where $\{NiBL^{+}\}$ is the concentration of biotic ligand sites bound by Ni (M), $\{BL^{-}\}$ is the concentration of unoccupied biotic ligand sites (M), K_{NiBL} is the stability constant of Ni^{2+} binding to the biotic ligand sites (L mol^{-1}).

The basic assumption of BLM is that the fraction of binding sites on the biotic ligand occupied by Ni dominates the magnitude of toxic effect, and this fraction (f_{NiBL}) equals:

$$f_{NiBL} = \frac{\{NiBL^{+}\}}{TBL} = \frac{K_{NiBL} \times \{Ni^{2+}\}}{1 + K_{NiBL} \times \{Ni^{2+}\} + \sum K_{CBL} \times \{C^{z+}\}} \quad (3.4)$$

where TBL is total biotic ligand site concentration (M), K_{CBL} is the stability constant for the binding of cations to the biotic ligand sites (L mol^{-1}).

According to the BLM concept, f_{NiBL} is constant at 50% lethal effect, and Equation 3.4 can be rewritten as:

$$LC50\{Ni^{2+}\} = \frac{f_{NiBL}^{50\%}}{(1 - f_{NiBL}^{50\%}) \times K_{NiBL}} \times (1 + \sum K_{CBL} \times \{C^{z+}\}) \quad (3.5)$$

where $LC50\{Ni^{2+}\}$ (M) is the $\{Ni^{2+}\}$ resulting in 50% mortality of *E. crypticus* after 14 days exposure; f_{50} is the fraction of binding sites occupied by Ni causing 50% mortality. A linear relationship could be observed between $LC50\{Ni^{2+}\}$ and the activity of each cation when the activities of other cations were kept constant. The slope and intercept corresponding to this linear relationship were used to derive the stability constants of competing cations by a matrix calculation following the method described by De Schamphelaere and Janssen (2002).

e.g., for Ca-set

$$\text{Slope}_{\text{Ca}} = \frac{f_{\text{NiBL}}^{50\%}}{(1-f_{\text{NiBL}}^{50\%})\times K_{\text{NiBL}}} \times K_{\text{CaBL}}$$

$$\text{Intercept}_{\text{Ca}} = \frac{f_{\text{NiBL}}^{50\%}}{(1-f_{\text{NiBL}}^{50\%})\times K_{\text{NiBL}}} \times (1 + K_{\text{MgBL}} \times \{\text{Mg}^{2+}\} + K_{\text{NaBL}} \times \{\text{Na}^{+}\} + K_{\text{KBL}} \times \{\text{K}^{+}\} + K_{\text{HBL}} \times \{\text{H}^{+}\}) \quad (3.6)$$

The estimation of K_{NiBL} and f_{50} is based on the best correlation between the logit percentage mortality (except for 0% or 100% mortality) and f_{NiBL} by varying value of K_{NiBL}. The solver programme in Microsoft Excel 2010 was used to minimize the root mean square error.

Logistic regression model

The dose-response relationships between overall survival and the assumed bioavailable fraction were fitted using a logistic model, which reads:

$$S = \frac{S_{\max}}{1 + (\frac{x}{x_{50}})^{b}} \quad (3.7)$$

where S is survival number, $S_{\max}$ is control survival number, b is the slope parameter. When using free ion activities, x is $\{Ni^{2+}\}$ (M), x_{50} is LC50$\{Ni^{2+}\}$ (M). When using BLM, x is f_{NiBL}, x_{50} is f_{50}; when using body concentration as dose, x is Ni body concentration (mol kg^{-1}), x_{50} is body concentration of Ni causing 50% mortality (denoted as LC50inter). Parameters were estimated by fitting Equation 3.7 to all data together using nonlinear regression in SPSS 19.0.

3.3 Results

Characterization of exposure medium

Particle size distribution of the quartz sand (Table S3.1) showed that it contained 98.9% sand, confirming that it can be considered as an inert matrix. The measured concentrations of Ni and cations in test solutions were always within 15% of the nominal concentrations.

Effect of cations on Ni uptake

The ratio of the Ni body concentration to $\{Ni^{2+}\}$ in the solution was defined as the organism-solution partition coefficients. The relationships between $C_{\text{Ni-body}}/\{Ni^{2+}\}$ (L kg^{-1}) and the activities of cations are shown in Figure 3.1. Increasing $\{Ca^{2+}\}$, $\{Mg^{2+}\}$, and $\{Na^{+}\}$ resulted in a significant decrease in the partition coefficients from 11.9 to 4.09 L kg^{-1} (R^2 = 0.52, $p < 0.005$), 8.04 to 2.37 L kg^{-1} (R^2 = 0.45, $p < 0.01$) and12.1 to 2.91 L kg^{-1} (R^2 = 0.25, $p < 0.05$), respectively. This indicated that Ca^{2+}, Mg^{2+} and Na^{+} inhibited the uptake of Ni^{2+} by *E. crypticus*. K^{+} and H^{+} had no effect on Ni uptake by *E. crypticus* since no significant relationship was observed between $C_{\text{Ni-body}}/\{Ni^{2+}\}$ and $\{K^{+}\}$ (R^2 = 0.24, p = 0.063), and $\{H^{+}\}$ (R^2= 0.015, p = 0.66).

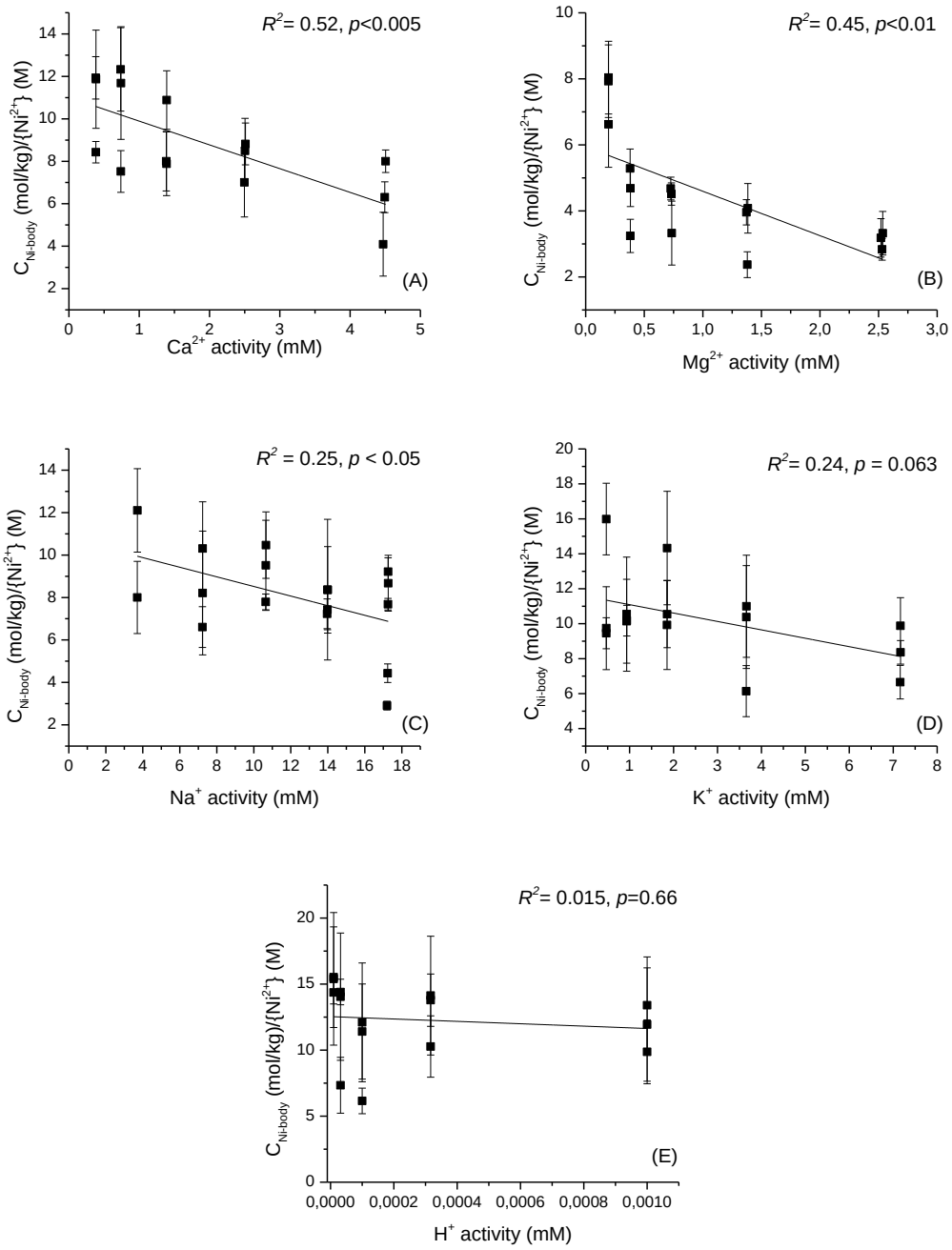

Figure 3.1 The relationship between the ratio of the body Ni concentration in *Enchytraeus crypticus* to the free ion activity of Ni^{2+} ($C_{Ni\text{-}body}/\{Ni^{2+}\}$ (L kg^{-1})) and the free ion activity of Ca^{2+} (A), Mg^{2+} (B), Na^{+} (C), K^{+} (D) and H^{+} (E) (mM) after 14 days exposure in different solutions (See Table 3.1) embedded in quartz sand matrix. Data points show the calculated values of $C_{Ni\text{-}body}/\{Ni^{2+}\}$. Solid lines show the linear regression.

Modelling Ni uptake by extended Langmuir

The measured Ni body concentrations of *E. crypticus* after 14 days exposure were fitted to the basic Langmuir model (Equation 3.1) for all data together. The relationship between predicted and observed Ni body concentrations is shown in Figure 3.2A. The obtained R^2

value was 0.69, indicating that free ion activity can explain only part of the variations in Ni body concentration among exposure media with different solution chemistry. Further, the extended Langmuir model (Equation 3.2) was applied to all accumulation data. The competition effect of Ca^{2+}, Mg^{2+} and Na^{+} was incorporated into this extended Langmuir model as these cations were identified to significantly affect Ni body concentration. The resulting binding constants log K_{NiS}, log K_{CaS}, log K_{MgS} and log K_{NaS} were 4.13, 1.44, 3.16, and 1.10, respectively. Figure 3.2B shows the Ni body concentrations predicted with extended Langmuir model plotted against the measured Ni body concentrations of *E. crypticus*. The model fit was greatly improved when the competition of Ca^{2+}, Mg^{2+} and Na^{+} with Ni^{2+} for biotic uptake sites was taken into account, with R^2 value of 0.84.

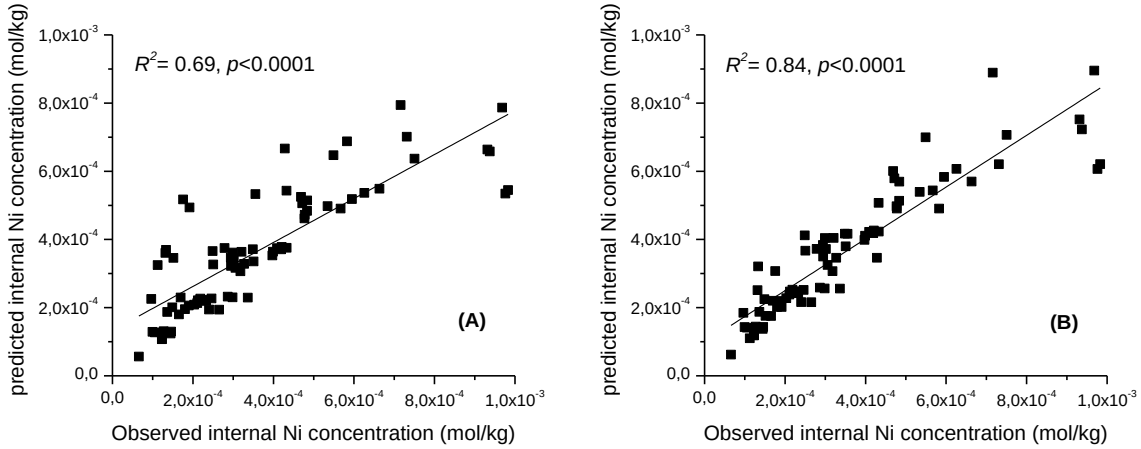

Figure 3.2 Linear regression relationship between the predicted and observed Ni body concentrations (mol kg^{-1}) of *Enchytraeus crypticus* after 14 days exposure in different solutions (See Table 3.1) embedded in quartz sand matrix based on (A) the basic Langmuir model (Equation 3.1) and (B) a BLM-based extended Langmuir model which corrected for the competing effects of Ca^{2+}, Mg^{2+} and Na^{+} (Equation 3.2).

Effects of cations on Ni toxicity

The relationships between LC50{Ni^{2+}} of *E. crypticus* and the activities of each cation are shown in Figure 3.3. The LC50{Ni^{2+}} values ranged from 16.9 to 186 μM, which is a 11-fold difference among different treatments. An increase in {Ca^{2+}}, {Mg^{2+}}, and {Na^{+}} effectively mitigated Ni toxicity to *E. crypticus*. Increasing {Ca^{2+}}, {Mg^{2+}}, and {Na^{+}} from 0.16 to 4.5 mM, from 0.040 to 2.5 mM and from 1.9 to 17 mM, resulted in a significant linear increase in LC50{Ni^{2+}} with 5-fold (R^2=0.94, p<0.005) (Figure 3.3A), 4-fold (R^2=0.95, p<0.005) (Figure 3.3B) and 2-fold differences (R^2= 0.74, p<0.05) (Figure 3.3C), respectively. When {K^{+}} increased from 0.073 to 7.16 mM, no obvious changes in LC50{Ni^{2+}} values were observed (R^2=0.51, p=0.11) (Figure 3.3D). The relationship between LC50{Ni^{2+}} and {H^{+}} was nonlinear and therefore the fit of the linear regression was not significant (R^2=0.38, p=0.27) (Figure 3.3E). Ni toxicity was not affected in the pH range of 6.0-7.0, but a further increase of pH up to 8.0 resulted in an increase in the toxicity of {Ni^{2+}}.

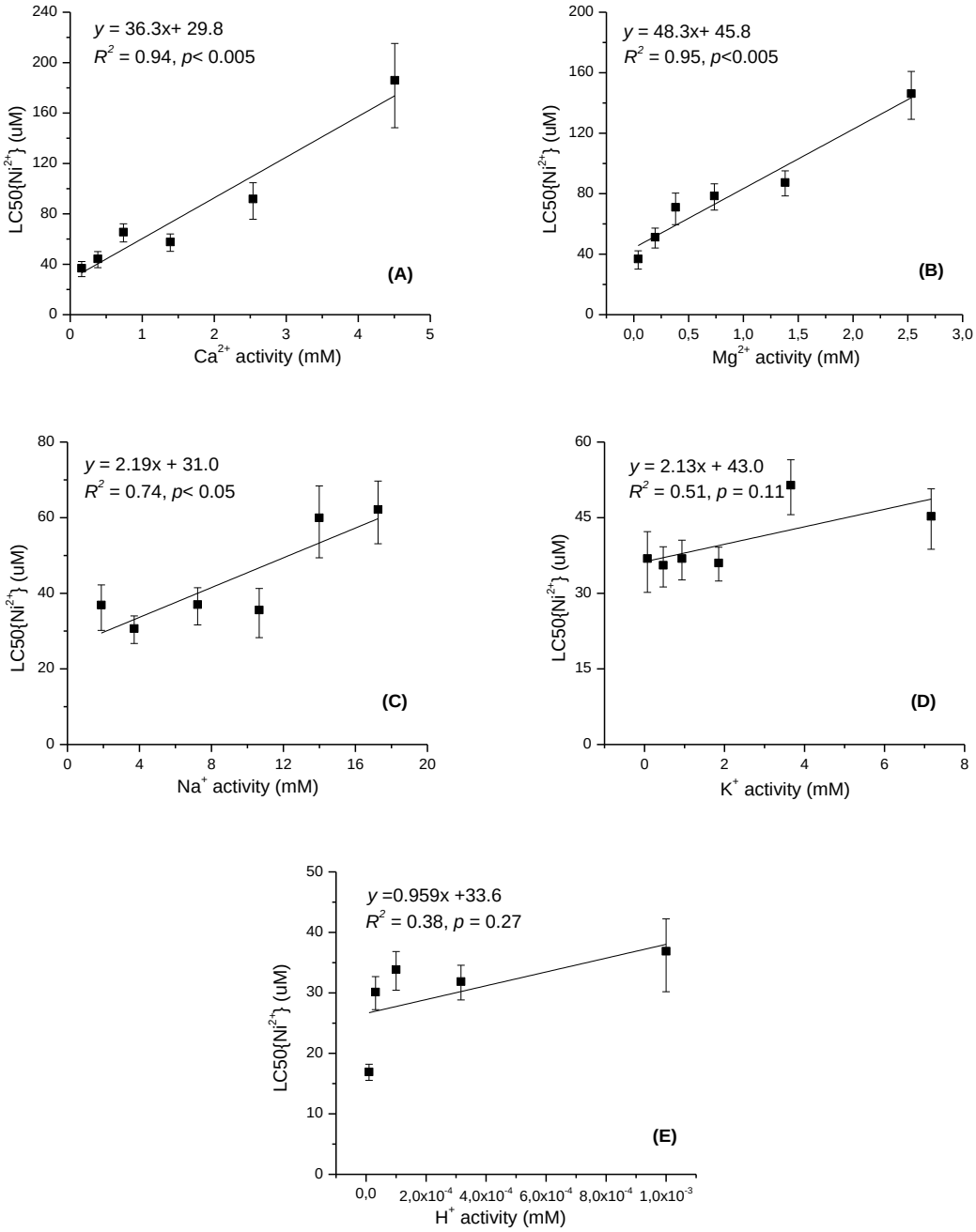

Figure 3.3 The 14-d LC50{Ni^{2+}} for the effect of Ni on the survival of *Enchytraeus crypticus* as a function of the activities of Ca^{2+}(A), Mg^{2+}(B), Na^{+}(C), K^{+} (D) and H^{+} (E) in test solutions embedded in quartz sand. See Table 3.1 for composition of the test solutions. Data points show the calculated LC50, error bars are 95% confidence intervals. Solid lines show the linear regression.

Modelling Ni toxicity by BLM

The effects of Ca^{2+}, Mg^{2+} and Na^{+} on Ni toxicity were incorporated into the BLM while pH and K^{+} were excluded as no significant relationship between LC50{Ni^{2+}} and {H^{+}} and {K^{+}} was observed. The derived stability constants log K_{CaBL}, log K_{MgBL} and log K_{NaBL} were

3.15, 3.09 and 1.97, respectively. The values of log K_{NiBL} and f_{50} were calculated using Equation 3.4 by changing the value of K_{NiBL} until the best fit between the logit value of the percentage mortality and f_{NiBL} was obtained. When log K_{NiBL} and f_{50} were 3.97 and 0.21, respectively, the best fit was reached with an R^2 value of 0.78 (Figure S3.1). All model parameters obtained above were incorporated in Equation 3.5 to yield the following relationship for Ni toxicity:

$$LC50\{Ni^{2+}\} = 3.15\times10^{-5}+0.046\{Ca^{2+}\}+0.046\{Mg^{2+}\}+0.0028\{Na^{+}\} \quad (3.8)$$

To examine the accuracy of the developed Ni-BLM model, LC50{Ni^{2+}} were predicted by Equation 3.8 for all sets of toxicity tests, using the activities of Ca^{2+}, Mg^{2+} and Na^{+} as input. The resulting relationship is shown in Figure 3.4. The predicted p(LC50{Ni^{2+}}) matched well with the observed values with an error less than a factor of two in most cases (except for the pH-8 set), indicating the applicability of the model for predicting Ni toxicity to *E. crypticus*.

The f_{NiBL} was calculated for all data using Equation 3.4 by incorporating the obtained BLM parameters. The survival of *E. crypticus* was plotted against {Ni^{2+}} and f_{NiBL}, respectively (Figure 3.5). Dose-response curves were fitted using the logistic regression model (Equation 3.7) and R^2 values were used to compare the goodness of model fits. Based on {Ni^{2+}} and f_{NiBL}, the obtained LC50{Ni^{2+}} and f_{50} (±SE) were 5.50×10^{-5} ($\pm0.35\times10^{-5}$) and 0.21 (±0.0045), respectively. The model fits were greatly improved by using f_{NiBL} instead of {Ni^{2+}} as dose, with R^2 value increasing from 0.75 for the Free Ion Activity Model to 0.90 for the Biotic Ligand Model.

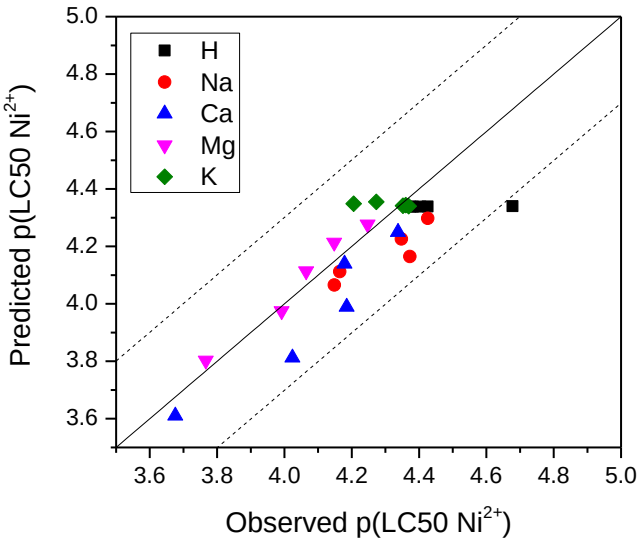

Figure 3.4 Relationship between the predicted and observed 14-d p(LC50{Ni^{2+}}) for the effect on the survival of *Enchytraeus crypticus* exposed to Ni^{2+} in different solutions embedded in a quartz sand matrix. The solid line indicates the 1:1 line and the dashed lines indicate a factor of two differences between the two values. See Table 3.1 for compostition of test solotions.

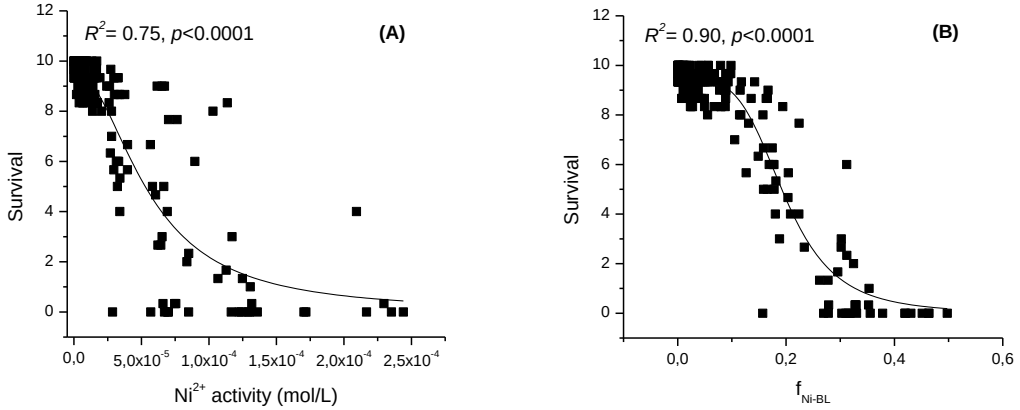

Figure 3.5 Dose response relationships for the effect of Ni on the survival of *Enchytraeus crypticus* after 14 days exposure in different solutions (See Table 3.1) embedded in a quartz sand matrix. Dose-response relationships based on (A) free ion activity of Ni^{2+} (M), (B) the fraction of biotic ligand sites occupied by Ni^{2+} (f_{NiBL}). Data points represent the observed values. Solid lines represent fit of logistic regression model (Equation 3.7) based on all data.

Linking Ni uptake to toxicity

When relating survival of *E. crypticus* to Ni body concentrations for all data sets together with the logistic dose-response model, the resulting R^2 was 0.68 (Figure 3.6A). Although large variations of data points existed especially in the Ca-set, the relationship between Ni uptake and toxicity was statistically significant at $p < 0.0001$. The obtained LC50inter (±SE) was 5.06×10^{-4} ($\pm0.35\times10^{-4}$) mol Ni kg^{-1} dry body weight. The model fit was significantly improved when the data of Ca-set was excluded, giving an R^2 value of 0.83 and an LC50inter of 4.63×10^{-4} ($\pm0.13\times10^{-4}$) mol Ni kg^{-1} dry body weight (Figure 3.6B). This showed that Ni accumulation well described Ni toxicity to *E. crypticus*, especially when the competition effect of Ca is ignored.

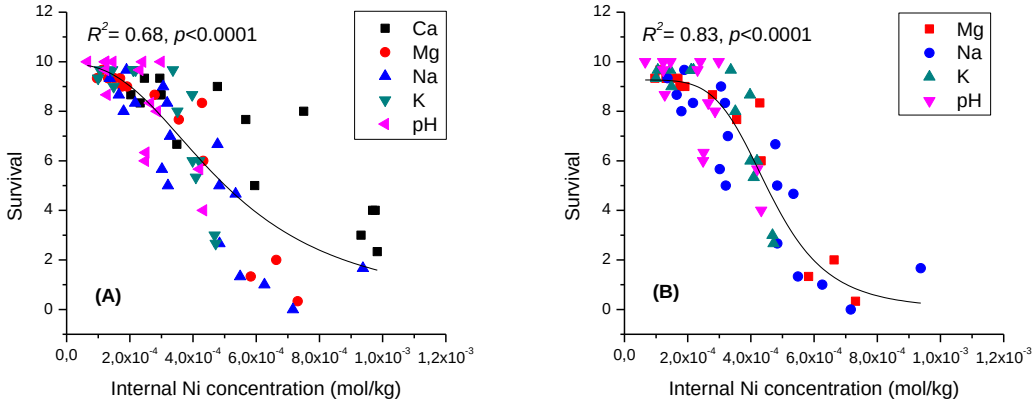

Figure 3.6 The relationship between survival of *Enchytraeus crypticus* and Ni body concentrations after 14 days exposure in different solutions (See Table 3.1) embedded in quartz sand matrix (A) for all sets together (Ca-set, Mg-set, Na-set, K-set, pH-set) and (B) excluding the Ca-set. Data points represent the observed values. Solid lines represent the fit of the logistic regression model (Equation 3.7).

3.4 Discussion

Prediction of Ni uptake

The FIAM proposes that free metal ion activity is the determining factor in metal uptake, and models based on free metal ion activity give better predictions for metal uptake than based on total soluble metal concentrations (Campbell, 1995). However, in the present study, $\{Ni^{2+}\}$ alone could not explain all the variation of metal accumulation in *E. crypticus* exposed to test media with different solution chemistry. The competition effect of cations appears to play an important role in metal uptake. A similar result has been found for Cu uptake by plants, which was affected by pH and the presence of competitive cations (e.g. Ca^{2+} and Mg^{2+}) (Cheng and Allen, 2001; Chen et al., 2013).

In the present study, a competition effect of Ca^{2+}, Mg^{2+} and Na^{+} on Ni uptake in *E. crypticus* was found, while K^{+} and H^{+} did not affect Ni uptake. Increasing concentrations of H^{+} and Ca^{2+} in test media significantly reduced Ni accumulation in the fish *Cirrhinus mrigala* (Karthikeyan et al., 2007). Mg^{2+} and H^{+} reduced Ni uptake in the green alga *Chlamydomonas ceinhardtii* through competition for the entry of Ni (Worms and Wilkinson, 2007). Toxic metal ions are generally thought to enter organisms by employing channels or carriers involved in uptake of essential cations (Bridges and Zalups, 2005). In several organisms, Ni has been shown to enter biological cells via Mg transporters (Chamnongpol and Groisman, 2002). The effects of other cations (Ca^{2+}, Na^{+}, H^{+}) on Ni uptake are less consistent, and the mechanism of their interaction with Ni accumulation should be further studied.

Based on our findings, an extended Langmuir model, which incorporates the defined competing cations (Ca^{2+}, Mg^{2+} and Na^{+}), was developed to predict Ni uptake by *E. crypticus*. The extended Langmuir model sufficiently explained the variation in Ni uptake with R^2 of 0.84, confirming that the competition of cations for uptake sites is an important factor in metal uptake.

Prediction of Ni toxicity

In the present study, Ca^{2+}, Mg^{2+}, and Na^{+} effectively alleviated Ni toxicity to *E. crypticus* while H^{+} and K^{+} had no significant effects. Positive linear relationships between LC50$\{Ni^{2+}\}$ and cation (Ca^{2+}, Mg^{2+}, and Na^{+}) activities were found. This supports the basic assumption of the BLM that competition occurs between metal ions and other cations for binding on the biotic ligands (transport sites or toxic action sites) on biological surfaces.

The alleviation of metal toxicity in general by Ca^{2+} and Mg^{2+} has been widely reported (De Schamphelaere and Janssen, 2002; Lock et al., 2007; Thakali et al., 2006a; Li et al., 2009a). An increase in the activity of Ca^{2+} and Mg^{2+} decreased the toxicity of Ni^{2+} to *Daphnia magna* (Deleebeeck et al., 2008) and *Daphnia pulex* (Kozlova et al., 2009). The $\log K_{CaBL}$ and $\log K_{MgBL}$ obtained in these two studies (Table 3.2) are comparable to the values derived in the present study. The observed effects of Mg^{2+} on Ni toxicity can be attributed to the similarity in the ionic diameter of the two ions, enabling Mg^{2+} to compete with Ni^{2+} for uptake through Mg^{2+} channels (Lock et al., 2007; Antunes and Kreager, 2009). Deleebeeck et al. (2008) reported that the addition of Ca and Mg concentrations from 0.25 mM to 3 mM increasingly protected *D. magna* against acute effects from Ni, further increasing Ca and Mg concentrations to 5 mM did not result in further decreases in Ni toxicity. This phenomenon

was not found in the present study at the highest concentrations of Ca (8 mM) and Mg (4 mM). This might be explained by the different sensitivity of species for water hardness.

In the present study, Na^+ showed slightly protective effects on Ni^{2+} toxicity, and the relationship between toxicity and Na^+ activity was not completely linear. In previous studies, no mitigating effect of Na^+ on Ni^{2+} toxicity has been reported for plants and aquatic organisms (Lock et al., 2007; Deleebeeck et al., 2008; Kozlova et al., 2009; Li et al., 2009a). A possible explanation is that the affinity of Na to physiological active binding sites of the organism could be different across species, or that it only has an effect at higher concentrations as shown in our study. The binding affinity of Na^+ for soil organisms might be stronger than that for plants and aquatic organisms (Steenbergen et al., 2005). Paquin et al. (2002) found that elevated concentration of metals can disturb ion uptake and efflux across the membrane, resulting in decreased plasma sodium level. Leonard and Wood (2013) investigated the effect of Ni on essential ion homeostasis of aquatic invertebrates, finding that the Na concentration in organisms was disrupted. An increased level of Na^+ in the exposure medium can enhance Na^+ uptake by organisms and relieve such physiological effects. Hence, Na^+ might exert protective effect through competing with Ni^{2+} for binding sites or reducing physiological disturbance of Ni^{2+} on *E. crypticus* especially at higher concentrations.

No linear relationship was found between LC50{Ni^{2+}} and {H^+} in the pH range (6.0-8.0) used in the present study. Our study showed that within the pH range of 6.0-7.0, Ni toxicity was not affected, while increasing pH from 7.5-8.0 resulted in an increase in Ni toxicity. The effects of pH on the Ni toxicity in different studies were inconsistent in the literature. Li et al. (2009a) found that when pH <7.0, an increase in H^+ activity decreased Ni toxicity to barley root elongation. However, Lock et al. (2007) found no significant effect of H^+ on Ni^{2+} toxicity to barley. The toxicity of Ni^{2+} to *D. pulex* increased with increasing H^+ activity (Kozlova et al., 2009). The findings of Deleebeeck et al. (2008, 2009) are consistent with our results. Increasing pH up to 7.5 and 6.5 had no effect on Ni^{2+} toxicity to *D. magna* and green algae (*Pseudokirchneriella subcapitata*), respectively. But the increase of pH caused increasing toxicity of Ni^{2+} when pH ranged from 7.5 to 8.2 and from 6.5 to 8.0, respectively. One possible explanation is the formation of other toxic Ni species at high pH levels. When pH $\leqslant$7.0, Ni^{2+} is the dominant species, while at pH >7.0 the fraction of Ni^{2+} decreased sharply, but the fraction of $NiHCO_3^+$ and $NiCO_3$ increased with increasing pH. Except Ni^{2+}, the toxicity of $NiHCO_3^+$ should also be considered (Li et al., 2009a).

An auto-validation showed that nearly all predicted LC50{Ni^{2+}} values were well within a factor of 2 of the observed values (see Figure 3.4), indicating that the BLM can be used to predict Ni toxicity to *E. crypticus* exposed to solution of varying properties. However, further research on the applicability of this BLM to predict metal toxicity in real soils is still needed. When developing a terrestrial BLM using tests in soils, the possible effect of each individual cation cannot be isolated and verified directly. The evident ameliorative effects of cations (e.g., H^+, Ca^{2+}, and Mg^{2+} on Ni^{2+} toxicity) is possibly due to the covariance of their activities with Ni^{2+} activities rather than their competitive binding at the BL sites (Thakali et al., 2006b). In our study, a sand-solution medium was used in the toxicity tests to provide direct evidence for the protective effects of cations by univariately controlling the composition of the test solutions. Our study therefore lends credence to the theory behind the terrestrial BLM. The obtained results in this sand-solution medium can be qualitatively or quantitatively

extrapolated to natural soils. For qualitative validation, it needs to be checked whether the identified uptake-modifying and toxicity-modifying factors in solution toxicity tests are also significantly affecting metal uptake and toxicity in soil toxicity tests. For quantitative validation, assuming soil pore water is the main route of exposure (Vijver et al., 2003; Van Gestel and Koolhaas, 2004), the free ion activities of metal and the competing cations in soil pore water can be calculated and used for predicting metal effects in real soils using the laboratory-derived BLMs. Examples of such kind of validation can be found for instance in Steenbergen et al. (2005) and Koster et al. (2006). Generally, these examples show that the results obtained in solution systems can be extrapolated to soils.

Bridging gaps between uptake and toxicity

The general principle of BLM is that the concentration of a metal at the biotic ligand determines its effect. The amount of metal bound to the biotic ligand (gill) can be experimentally determined for fish (Meyer et al., 1999). However, it is difficult to measure the amount of metal at the target sites of invertebrates and plants. Instead, models have been developed using the theoretical construct of the fish gill model. Toxicity data are often used as the empirical basis for determining binding affinity of metals to the biotic ligand (De Schamphelaere et al., 2002; Steenbergen et al., 2005). Antunes and Kreager (2009) found that the predicted concentration of Ni-biotic ligand complex accounted for nearly all of the Ni in the roots of barely. In the present study, the cations (Ca^{2+}, Mg^{2+} and Na^{+}) competing with Ni uptake were also found to be competitive for Ni toxicity, suggesting that the toxicity-modifying cations should be same as uptake-modifying cations. Within the concept of BLM, the biotic ligands are thought to be specific proteins that are involved in the uptake of essential elements (Niyogi and Wood, 2004). The competition cations are likely to exert their protective effect on organisms through inhibiting metal uptake by competition with the metal for biotic ligands. These findings indicate that metal body concentration may be considered a good approximation of target site concentration.

This assumption can be supported by a good relationship between toxicity and metal body concentration. In the present study, when relating all toxicity data to Ni body concentration no good relationship was observed. However, when the Ca-set data was removed, the predictive ability of metal body concentration for Ni toxicity was significantly improved. Borgmann et al. (2008) reported that whether the body metal concentration can describe toxicity depends on the location of the site of toxic action (i.e., biotic ligand). If the site of toxic action is on the surface of organisms, it is likely that the organism accumulates metal through the same action sites. If the site of toxic action is inside the organism, it may be completely different from the site of metal accumulation. In our study, there were no significant differences between $\log K_{MgBL}$ and $\log K_{MgS}$, and between $\log K_{NaBL}$ and $\log K_{NaS}$ (Table 3.2). However, $\log K_{CaBL}$ (= 3.15) was much higher than $\log K_{CaS}$ (= 1.44). These findings suggest that the protective effects of Mg^{2+} and Na^{+} on Ni^{2+} toxicity were mainly caused by the competition at the transport sites on the surface of organisms, which directly affected the entry of Ni^{2+} (Veltman et al., 2010). This is consistent with Worms and Wilkinson (2007), who found that Mg^{2+} competed against Ni for uptake through Mg^{2+} channels in green alga, however, the effect of Ca^{2+} on toxicity was stronger than that on uptake. So the interaction between Ni and Ca could not be simply explained by competition.

Hunn (1985) also found that except for competitive effects, Ca may have direct physiological functions by regulating membrane permeability when it binds to biotic ligands. Thus, we cannot simply equate the internal concentration with the concentration of the metal-biotic ligand complex to predict metal toxicity. When the actual concentrations of metals on the target-sites are considered instead of whole-body internal concentrations, the variations in toxicity can be further reduced, as demonstrated in our study.

3.5 Conclusion

Ca^{2+}, Mg^{2+} and Na^{+} significantly modified both the uptake and toxicity of Ni in *E. crypticus*. When these modifying factors were incorporated, Ni uptake could be described by an extended Langmuir model while a newly developed Biotic Ligand model well predicted toxicity of Ni. When linking uptake to toxicity, for the Mg-set and Na-set, Ni body concentrations in *E. crypticus* provide a good estimate of Ni toxicity but this is not the case for the Ca-set. These results suggest that the effect of Mg^{2+} and Na^{+} on Ni toxicity is mainly through competition with Ni^{2+} on the surface of the organism and inhibiting the entry of Ni^{2+}. However, the effect of Ca^{2+} cannot simply be explained by competition, suggesting different interactions at uptake and internal processing. Further research on the internal physiological mechanisms of ion interference with Ni^{2+} toxicity is needed.

Table 3.2 Parameters for biotic ligand models for Ni toxicity and bioaccumulation in invertebrates and plants from the present study and studies reported in the literature.

Organisms	Endpoint	Exposure medium	logK_{Ni}	logK_{H}	logK_{Ca}	logK_{Mg}	log K_{Na}	Sources
Enchytraeus crypticus	mortality	solution+sand	3.97	-	3.15	3.09	1.97	present study
	uptake	solution+sand	4.13	-	1.44	3.16	1.10	present study
Daphnia pulex	toxicity	solution	4.87	-	4.20	3.60	-	Kozlova et al. (2009)
Daphnia magna	immobilization	solution	4.00	-	3.10	2.47	-	Deleebeeck et al. (2008)
Hordeum vulgare	growth	solution	5.27	-	-	3.47	-	Lock et al. (2007)
Hordeum vulgare	elongation	solution	4.83	4.29	1.60	4.01	-	Li et al. (2009a)

Supplemental information

Table S3.1 Characteristics of the pre-treated quartz sand used for solution exposure only toxicity tests with *Enchytraeus crypticus*.

Texture	Content
Clay (< 8 µm)	0.63%
Silt (8-63 µm)	0.52%
Sand (63-2000 µm)	98.85%
Very Fine Sand (63-125 µm)	3.65 %
Fine Sand (125-250 µm)	42.76 %
Middle Coarse Sand (250-500 µm)	50.36 %
Coarse Sand (500-1000 µm)	2.07 %
Very Coarse Sand (1000-2000 µm)	0.00 %

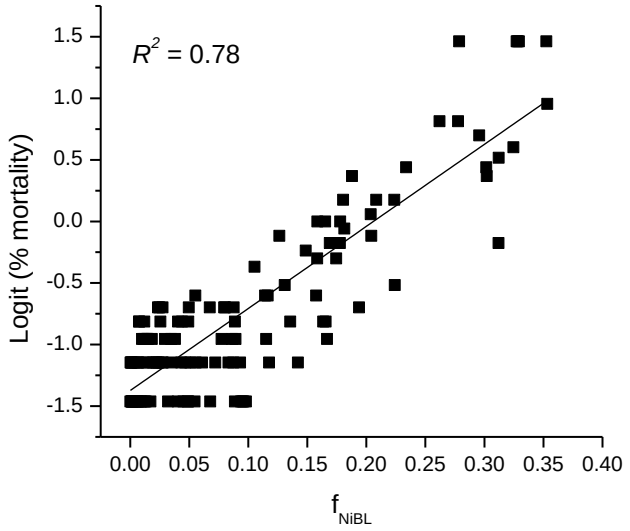

Figure S3.1 Relationship between the logit of the observed percentage mortality of *Enchytraeus crypticus* after 14-d exposure to different test solutions (see Table 3.1) and the calculated fraction of the biotic Ligand sites occupied by Ni (f_{NiBL}). The values of f_{NiBL} were calculated with Equation 3.4 using stability constants obtained in this study (Table 3.2).

Chapter 4

A generic biotic ligand model quantifying the development in time of Ni toxicity to *Enchytraeus crypticus*

Abstract

Biotic ligand models (BLMs) predicting metal toxicity for a fixed exposure time are available, but it is uncertain how to extrapolate predictions to a dynamic environment with time-variable exposure. Three BLM-based models were developed to describe change of Ni toxicity to *Enchytraeus crypticus* in time. These models assumed that: (a) biotic ligand binding constants of Ni and competing cations (K_{NiBL} and K_{CBL}) and the fraction of biotic ligands occupied by Ni to produce 50% mortality (f_{50}) are fixed with time, (b) K_{NiBL} and K_{CBL} remain constant while f_{50} varies with time, and (c) K_{NiBL}, K_{CBL}, and f_{50} are all time-dependent. Model (a) successfully described the 7-d toxicity of Ni but failed in explaining Ni toxicity at longer exposure times. Both models (b) and (c) well described Ni toxicity, within a factor of 2, at varying solution chemistries and different exposure times. This shows that the acute BLM cannot directly be applied for predicting chronic metal toxicity and that some BLM parameters may vary with time. Our findings provide plausible explanations for differences in mechanisms of acute and chronic toxicity, offering a framework for incorporating toxicokinetic and toxicodynamic processes in describing Ni toxicity in time.

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Chemosphere, 2015, 124: 170-176

4.1 Introduction

Metal contamination poses a great risk to environmental and human health. This strengthens the need to develop models to evaluate how large the risks of metals for organisms will be. The large variability of metal toxicity in different types of soil or water stresses the importance of explicitly considering bioavailability when assessing the environmental risks associated with a certain metal (Van Gestel and Koolhaas, 2004; Deleebeeck et al., 2009).

Many published studies have shown that metal bioavailability and toxicity are governed by (pore) water chemistry and properties of the organism (Sauvé et al., 1998; Meyer et al., 1999). The Biotic ligand model (BLM) is a mechanistic bioavailability model which incorporates both metal speciation (distribution over different chemical forms) and cation competition (protective effects of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, etc.) in (soil) solution (Di Toro et al., 2001; Paquin et al., 2002). Within the BLM, the free metal ion is assumed to be the main metal form available for uptake by organisms, and uptake and subsequent toxic effects are assumed to be affected by competition with other cations. The BLM assumes that toxicity is linked to f_{MBL}, which is the fraction of all biotic ligands on the organism occupied by metal ions. Bioaccumulation is the net result of the uptake, distribution and elimination of a metal in an organism during exposure. Toxicity is induced when internal concentration reaches the critical level (Crommentuijn et al., 1994). Accumulation and toxicity are dependent on exposure time, suggesting the importance of considering time as a factor in developing models for toxicity prediction. Many researchers have successfully developed BLMs to predict toxicity of metals to aquatic and soil organisms (Di Toro et al., 2001; Steenbergen et al., 2005; Thakali et al., 2006b). However, most existing BLMs were developed at a fixed exposure time, neglecting the time-dependent accumulation and toxicity processes.

The binding constants of metals and competitive ions derived in common BLMs are assumed to be intrinsic values that are metal- and species-specific and can only be derived in equilibrium conditions when steady-state concentrations of the metals at the biotic ligand are reached (Hatano and Shoji, 2010). According to this assumption, some models were developed based on the common BLM to predict the time course of toxicity of metals with fixed binding constants. These separate studies found that the fraction of binding sites occupied by the metal causing 50% effect (f_{50}) decreased with exposure time (Chen et al., 2009; Chen and Liao, 2010). Binding constants of Ca, Mg and Na obtained for *Daphnia magna* in a chronic (21 d) Zn-BLM were 0.2-0.4 log units lower than in an acute (48 h) Zn-BLM (Heijerick et al., 2002; Heijerick et al., 2005). Apparently, BLM parameters may be affected by exposure time. Thus, caution is needed when extrapolating the existing BLMs developed at a fixed exposure time to predict metal toxicity for organisms in a dynamic environment where metal speciation, accumulation and toxicity may be time-dependent.

When investigating how the common cations interact with Ni^{2+} in *E. crypticus*, Ca^{2+}, Mg^{2+}, and Na^{+} (but not H^{+} and K^{+}) were identified as competitive cations that have significant effects on 14 d Ni uptake and toxicity (He et al., 2014). This study aimed at (1) determining the effects of cations (Ca^{2+}, Mg^{2+}, and Na^{+}) on the toxicity of Ni to *E. crypticus* at different exposure times (7, 10 and 14 d); (2) developing a generic BLM to predict Ni toxicity to *E. crypticus* in time-course. To achieve the latter aim, predictabilities were compared of three models in which (a) no parameters are time-dependent, (b) binding

constants are fixed but f_{50} changes with time, and (c) all the parameters are time-dependent. Model (a) assumes that the developed BLM for Ni toxicity in the short term can also be applied to predict Ni toxicity in the long term. Model (b) assumes that binding constants of Ni^{2+} and competing ions are physical-chemical parameters that will remain constant throughout time, while f_{50} will change with time. Model (c) assumes that organisms may avail mechanisms that enable adjusting Ni uptake and toxicity in the course of time. These mechanisms could e.g., relate to dynamics of chemical reactions between Ni^{2+} and biotic ligands, and the possible alteration of the bioactive compartments such as cell surface and intracellular compartments.

4.2 Materials and methods

Test organism

Enchytraeus crypticus (Enchytraeidae; Oligochaeta; Annelida) were cultured in a climate room at 16 °C, 75% relative humidity and complete darkness. The animals were fed twice a week with a mixture of oat meal, dried baker's yeast, yolk powder, and fish oil. Adult *E. crypticus* having eggs in the clitellum region and measuring approximately 1 cm were selected for testing (OECD, 2004).

Test medium

A quartz sand-solution system was used to enable better control of metal exposure and speciation. This system provides a means to manipulate the composition of the pore water, with minimum influence of the soil solid phase. Quartz sand was pre-treated following He and Van Gestel (2013) and used as test matrix. All used chemicals were of reagent grade (Sigma-Aldrich; > 99%). The culture medium composed of 0.2 mM Ca^{2+}, 0.05 mM Mg^{2+}, 2.0 mM Na^{+} and 0.078 mM K^{+} was used as the control. Stock solutions of $NiCl_2$, $CaCl_2$, $MgCl_2$ and NaCl were prepared by adding different amounts of $NiCl_2{\cdot}6H_2O$, $CaCl_2$, $MgCl_2{\cdot}6H_2O$ and NaCl to deionized water. The test solutions were prepared by adding different volumes of stock solution to culture medium. All test solutions were adjusted to pH 6.0 (5.95-6.05) by using 0.75 g/L MOPS (3-[N morpholino] propane sulfonic acid) (AppliChem; >99%), 0.75 mg/L MES (2-[N-morpholino] ethane sulfonic acid) (Sigma-Aldrich; >99%) and diluted NaOH when necessary (Lock et al., 2006).

Toxicity tests

Three sets of test medium were performed: Ca-set, Mg-set and Na-set. Each set included five levels of these cations and five Ni concentrations (including control) (Table S4.1). The toxicity tests with adult *E. crypticus* were performed with three exposure times (7, 10 and 14 d) and three replicates for each treatment. The exposure times were selected based on the fact that equilibrium of Ni accumulation was reached in 10 to 14 d (He and Van Gestel, 2013). Ten adults were exposed in a 100 mL glass jar, containing 20.0 g pre-treated quartz sand and 5.4 mL test solution equilibrated for 1 d before starting exposures. Tests were carried out at 20 °C with a 12h light/12h dark cycle. Water evaporation was compensated for once every 3 days by adding deionized water. Enchytraeid mortality was assessed as the absence of response to a gentle mechanical stimulus to the front end (OECD, 2004).

Physical and chemical analysis

Particle size distributions of pre-treated quartz sand were determined using a particle size analyzer (HELOS-QUIXEL; Sympaatec); results are shown in Table S4.2. After contact of test solutions with quartz sand for different exposure times (7, 10 and 14 d), porewater samples were collected by filtration of the test medium over a 0.45 μm membrane filter (Whatman). Concentrations of Ni and cations in the initial test solutions and in porewater samples were analyzed by flame atomic absorption spectrophotometry (AAS) (Perkin Elmer AAnalyst 100). Since porewater concentrations did not show any significant change with time, for the data analysis mean values were used as actual exposure concentrations (Table S4.1).

Data analysis

Activities of Ni and other cations were calculated using the Windermere Humic Aqueous Model (WHAM VI) (Tipping, 1998). The measured pH values and porewater concentrations of Ni, Ca, Mg, Na and K were used as input parameters, and so were the nominal concentrations of the corresponding anion (i.e., Cl^-). Median lethal concentrations (LC50) and their 95% confidence intervals (95% CI) were calculated for each bioassay using the trimmed Spearman-Karber method (Hamilton et al., 1977).

Model description

Biotic Ligand Model (BLM)

According to the BLM hypothesis, the toxicity of Ni is determined by the fraction of all the biotic ligand sites (TBL) that is occupied by Ni (NiBL)

$$\frac{\mathrm{NiBL}}{\mathrm{TBL}} = f_{\mathrm{NiBL}} = \frac{\{\mathrm{NiBL^+}\}}{\{\mathrm{NiBL^+}\} + \sum\{\mathrm{CBL^{Z+}}\} + \{\mathrm{BL^-}\}} \tag{4.1}$$

where f_{NiBL} is the fraction of biotic ligand sites occupied by Ni^{2+}; $\{NiBL^+\}$ the concentration of Ni-biotic ligand complexes (M); $\{CBL^{Z+}\}$ the concentration of cation-biotic ligand complexes (M); $\{BL^-\}$ the concentration of unoccupied biotic ligand sites (M);

Stability binding constants are defined as, e.g. for Ni

$$K_{\mathrm{NiBL}} = \frac{\{\mathrm{NiBL^+}\}}{\{\mathrm{Ni^{2+}}\} \times \{\mathrm{BL^-}\}} \rightarrow \{\mathrm{NiBL^+}\} = K_{\mathrm{NiBL}} \times \{\mathrm{Ni^{2+}}\} \times \{\mathrm{BL^-}\} \tag{4.2}$$

where K_{NiBL} is the stability constant for binding of Ni to the Biotic ligand (L mol^{-1}); $\{Ni^{2+}\}$ the free ion activity of Ni (M).

By incorporating Equation 4.2, Equation 4.1 can be rewritten as,

$$f_{\mathrm{NiBL}} = \frac{K_{\mathrm{NiBL}} \times \{\mathrm{Ni^{2+}}\}}{1 + K_{\mathrm{NiBL}} \times \{\mathrm{Ni^{2+}}\} + \sum K_{\mathrm{CBL}} \times \{\mathrm{C^{Z+}}\}} \tag{4.3}$$

where $\{C^{Z+}\}$ is the free ion activity of cations (M); K_{CBL} the stability constant for the binding of cations to the Biotic ligand (L mol^{-1}).

According to the BLM concept, f_{NiBL} is constant at 50% effect (Di Toro et al., 2001), and Equation 4.3 can be rewritten as,

$$LC50\{Ni^{2+}\} = \frac{f_{NiBL}^{50\%}}{(1 - f_{NiBL}^{50\%}) \times K_{NiBL}} \times \{1 + \sum K_{CBL} \times \{C^{Z+}\}\} \quad (4.4)$$

where $LC50\{Ni^{2+}\}$ is the 50% lethal concentration expressed as $\{Ni^{2+}\}$ (M); f_{50} the fraction of biotic ligand sites occupied by Ni causing 50% mortality. Linear regressions between $LC50\{Ni^{2+}\}$ and activities of each cation were made in Excel 2007. The slopes and intercepts from these linear regressions were used for deriving stability binding constants for the different cations following De Schamphelaere and Janssen (2002).

Generic BLMs for quantifying time-course toxicity

Model (a)

The BLM parameters (K_{NiBL}, K_{CBL} and f_{50}) were assumed to be constant over time. A short term (7 d) Ni-BLM developed following Equation 4.3 and 4.4 was used as a generic model to predict $LC50\{Ni^{2+}\}$ for *E. crypticus* upon long-term exposure (10 and 14 d) with varying water compositions.

Model (b)

The values of K_{NiBL} and K_{CBL} were assumed to be constant, while f_{50} is time dependent. By incorporating the K_{NiBL} and K_{CBL} estimated after 7 d into Equation 4.3, f_{NiBL} was calculated for all treatments. A log-logistic dose-response model was used to correlate the survival of *E. crypticus* after 7, 10 and 14 d to f_{NiBL}.

$$S = \frac{S_{max}}{1 + (\frac{f_{NiBL}}{f_{NiBL}^{50\%}})^{\beta}} \quad (4.5)$$

where S is the survival number of *E. crypticus*, S_{max} the control survival number; β the slope parameter. The f_{50} values with corresponding standard error (SE) for each dose-response curve were estimated by nonlinear regression in SPSS 19.0. In the present study the f_{50} decreased with time (see Results section). Assuming that f_{50} will decrease with increasing exposure time until reaching an ultimate value, this can be expressed as,

$$f_{NiBL}^{50\%}(t) = f_{NiBL}^{50\%}\infty + a \times e^{-\frac{t}{b}} \quad (4.6)$$

where $f_{50}(t)$ is f_{50} at any exposure time t (d); $f_{50}\infty$ the ultimate value of f_{50}; a and b constants independent of time.

Incorporating the time dependent f_{50} (Equation 4.6) into Equation 4.4, the $LC50\{Ni^{2+}\}$ in time course can be expressed by Equation 4.7,

$$LC50\{Ni^{2+}\}(t) = \frac{f_{NiBL}^{50\%}(t)}{(1 - f_{NiBL}^{50\%}(t)) \times K_{NiBL}} \times \{1 + K_{CBL} \times \{C^{Z+}\}\} \quad (4.7)$$

where $LC50\{Ni^{2+}\}(t)$ is the LC50 value after t d of exposure.

Model (c)

Assuming that all BLM parameters are time-dependent, three individual BLMs were developed for exposure time (7, 10 and 14 d). The stability binding constants of Ni and

cations were found to be time dependent, while f_{50} remained almost constant (see Results section). It therefore was assumed that stability binding constants will decrease with time until reaching an ultimate value, which can be expressed as, e.g. for Ni

$$K_{\mathrm{NiBL}}(t) = K_{\mathrm{finalNiBL}} + c \times \mathrm{e}^{-\frac{t}{d}} \quad (4.8)$$

where $K_{\mathrm{NiBL}}(t)$ (L mol^{-1}) is the stability binding constants of Ni after exposure time t (d); $K_{\mathrm{finalNiBL}}$ the ultimate stability binding constans of Ni (L mol^{-1}); c and d constants independent of time.

Incorporating the time-dependent binding constants (Equation 4.8) into Equation 4.3, the fraction of biotic ligand sites occupied by Ni in time course (f_{NiBL}(t)) can be expressed as,

$$f_{\mathrm{NiBL}}(t) = \frac{K_{\mathrm{NiBL}}(t) \times \{\mathrm{Ni}^{2+}\}}{1 + K_{\mathrm{NiBL}}(t) \times \{\mathrm{Ni}^{2+}\} + \sum K_{\mathrm{CBL}}(t) \times \{\mathrm{C}^{Z+}\}} \quad (4.9)$$

where $K_{\mathrm{CBL}}(t)$ (L mol^{-1}) are the stability binding constants of cations (Ca, Mg and Na) after exposure time t (d).

The LC50{Ni^{2+}} over time can be expressed in terms of the time course stability binding constants of Ni and cations,

$$\mathrm{LC50}\{\mathrm{Ni}^{2+}\}(t) = \frac{f_{\mathrm{NiBL}}^{50\%}}{(1 - f_{\mathrm{NiBL}}^{50\%}) \times K_{\mathrm{NiBL}}(t)} \times \{1 + K_{\mathrm{CBL}}(t) \times \{\mathrm{C}^{Z+}\}\} \quad (4.10)$$

where LC50{Ni^{2+}}(t) is the LC50 value after t d of exposure.

4.3 Results

Time-dependent effect of cations on Ni toxicity

The calculated LC50{Ni^{2+}} values for *E. crypticus* exposed in test solutions of different treatments (Control, Ca-set, Mg-set and Na-set) are shown in Table 4.1. Increasing {Ca^{2+}}, {Mg^{2+}} and {Na^{+}} resulted in a significant increase in LC50{Ni^{2+}} at different exposure times ($p<0.001$ for Ca and Mg, $p<0.005$ for Na; R^2 0.93-0.99) (Figure 4.1). The 7, 10 and 14 d LC50{Ni^{2+}} increased up to 4.8-8.4 fold for Ca, up to 4.1-5.3 fold for Mg and up to 2.5-4.5 fold for Na, showing that the protective effects on Ni^{2+} toxicity to *E. crypticus* of Ca^{2+} and Mg^{2+} were similar but stronger than that of Na^{+}. Generally, for the Ca-, Mg- and Na-sets, LC50{Ni^{2+}} values for *E. crypticus* decreased with increasing exposure time. At the highest {Ca^{2+}} treatment, LC50{Ni^{2+}} decreased dramatically from 7 d to 14 d with a 2-fold difference. At the highest {Mg^{2+}} and {Na^{+}} concentrations, LC50{Ni^{2+}} decreased appreciably from 152 μM and 126 μM after 7 d to 129 μM and 68.5 μM after 10 d, respectively, while from 10 d to 14 d, LC50{Ni^{2+}} was further but less pronounced reduced, to 104 μM and 66.3 μM, respectively.

Table 4.1 The 7, 10 and 14 d LC50 {Ni^{2+}} (μM) with 95% confidence intervals (95% CI) for the effect of Ni on the survival of *Enchytraeus crypticus* exposed in test solutions of different ion composition (Control, Ca-set, Mg-set and Na-set). See Table S4.1 for composition of the test solutions.

Treatment		7 day	10 day	14 day
		LC50{Ni^{2+}} (μM) (95% CI)		
Control		28.9 (27.1-30.8)	27.3 (24.2-30.7)	25.4 (23.0-28.0)
Ca-set	Ca-0.5	31.3 (27.8-35.4)	27.3 (24.2-30.8)	27.8 (26.5-29.1)
	Ca-1.0	58.6 (49.7-69.1)	46.3 (39.8-53.8)	32.3 (29.8-35.0)
	Ca-2.0	102 (86.4-121)	77.8 (68.3-88.7)	49.0 (43.6-55.2)
	Ca-4.0	194 (166-226)	135 (120-151)	82.0 (76.5-88.0)
	Ca-8.0	242 (214-273)	200 (170-237)	121 (105-140)
Mg-set	Mg-0.25	35.2 (30.4-40.7)	34.9 (31.6-38.5)	28.4 (25.2-32.1)
	Mg-0.5	60.1 (58.1-62.2)	50.4 (44.7-56.7)	30.9 (27.1-35.2)
	Mg-1.0	66.9 (61.0-73.3)	57.4 (53.0-62.2)	49.7 (44.4-55.5)
	Mg-2.0	84.6 (76.7-93.3)	76.3 (67.4-86.3)	72.3 (66.4-78.8)
	Mg-4.0	152 (134-173)	129 (111-150)	104 (91.7-118)
Na-set	Na-4.0	63.7 (53.9-75.3)	28.8 (25.8-32.1)	25.6 (23.7-27.7)
	Na-8.0	70.4 (61.0-81.4)	41.0 (36.7-45.8)	33.3 (29.5-37.6)
	Na-12	86.0 (77.4-95.4)	48.9 (46.6-51.3)	39.6 (35.4-44.3)
	Na-16	138 (114-169)	64.5 (53.8-77.3)	54.3 (45.4-65.0)
	Na-20	126 (106-150)	68.5 (57.4-81.9)	66.3 (57.2-76.7)

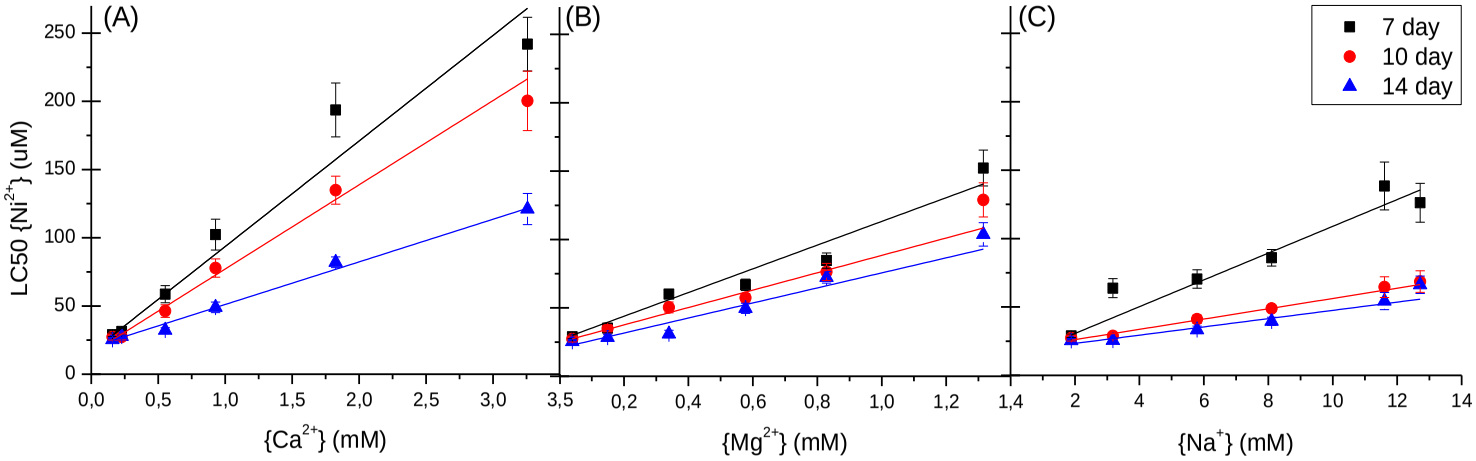

Figure 4.1 The 7, 10 and 14-day LC50{Ni^{2+}} (µM) for the toxicity of Ni to *Enchytraeus crypticus* exposed in test solutions embedded in sand as a function of {Ca^{2+}} (A), {Mg^{2+}} (B) and {Na^{+}} (C) (mM). Data points show the calculated LC50 values, and error bars the 95% confidence intervals. Solid lines are linear regressions (see text for further details).

Quantifying dynamic Ni toxicity with Model (a)

From the relationships between LC50{Ni^{2+}} values and {Ca^{2+}}, {Mg^{2+}} and {Na^{+}} activities at 7 d (Figure 4.1), the binding constants of Ca^{2+}, Mg^{2+} and Na^{+} (denoted as logK_{CaBL}, logK_{MgBL}, and logK_{NaBL}; in L mol^{-1}) were calculated with Equation 4.1, giving values of 4.15, 4.41 and 3.21, respectively. f_{NiBL} was calculated with Equation 4.3 for all treatments at 7 d using the corresponding cation binding constants. LogK_{NiBL} and f_{50} were determined from the best relationships between the logit percentage mortality of *E. crypticus* and f_{NiBL} obtained by changing the value of K_{NiBL}. Fits were best when logK_{NiBL} and f_{50} were 4.00 and 0.0487, respectively (R^2 = 0.66) (Figure S4.1). By incorporating all estimated parameters at 7 d into Equation 4.4, the following equation was obtained to predict LC50{Ni^{2+}} at 7 d:

$$LC50\{Ni^{2+}\} = 5.12\times10^{-6} + 0.072\times\{Ca^{2+}\} + 0.13\times\{Mg^{2+}\} + 0.0082\times\{Na^{+}\} \quad (4.11)$$

With the developed 7-d Ni-BLM, LC50{Ni^{2+}} values at 7, 10 and 14 d were predicted for all treatments, separately, using {Ca^{2+}}, {Mg^{2+}} and {Na^{+}} as inputs. The predicted values did not match well with the observed values for all treatments with R^2=0.76 (Figure 4.2A). Especially for 14 d, most LC50{Ni^{2+}} values were underestimated, indicating that the toxicity of Ni^{2+} was overestimated using the Ni-BLM developed from short-term toxicity data.

Quantifying dynamic Ni toxicity with Model (b)

Relationships between enchytraeid survival and f_{NiBL} at different exposure times are shown in Figure 4.3. The values of f_{50} were estimated by these log-logistic relationships, being 0.043, 0.033 and 0.026 at 7, 10 and 14 d, respectively. The relationship of f_{50} with time, shown in Figure S4.2 and fitted with Equation 4.6, had an R^2 of 0.87. Values of $f_{50}\infty$, a and b were estimated at 0.027 (±0.0026), 0.27 (±0.50) and 2.45 (±1.62), respectively. By incorporating the estimated parameters into Equation 4.6, the f_{50} with time can be expressed as:

$$f_{NiBL}^{50\%}(t) = 0.027 + 0.27\times e^{-\frac{t}{1.62}} \quad (4.12)$$

The time-dependent f_{50} (Equation 4.12) and the estimated K_{NiBL} and K_{CBL} were included into Equation 4.7. The LC50{Ni^{2+}} at different time points with the variation of solution chemistry can be described as:

$$LC50\{Ni^{2+}\}(t) = \frac{0.027 + 0.27\times e^{-\frac{t}{1.62}}}{(1-(0.027 + 0.27\times e^{-\frac{t}{1.62}}))\times 1.00\times10^{4}} \times \{1 + 1.41\times10^{4}\times\{Ca^{2+}\} + 2.60\times10^{4}\times\{Mg^{2+}\} + 1.61\times10^{3}\times\{Na^{+}\}\} \quad (4.13)$$

The 7, 10 and 14 d LC50{Ni^{2+}} values for all treatments were predicted by the developed generic Ni-BLM (Equation 4.13). The predicted and observed pLC50{Ni^{2+}} values correlated well with R^2 of 0.89 (Figure 4.2B), and predicted LC50{Ni^{2+}} were within a factor of 2 of the observed values.

Quantifying dynamic Ni toxicity with Model (c)

Three individual BLMs were developed at different exposure times. The estimated f_{50} were independent of exposure time, being 0.0487, 0.0484 and 0.0489 at 7, 10 and 14 d, respectively. The value of logK_{NiBL}, logK_{CaBL}, logK_{MgBL} and logK_{NaBL} decreased with time from 4.00, 4.15, 4.41 and 3.21 at 7 d, 3.81, 3.85, 4.03 and 2.71 at 10 d, to 3.76, 3.54, 3.95 and 2.65 at 14 d, respectively. The change in time of the binding constants of Ni and the other cations for *E. crypticus* are shown in Figure S4.3 and fitted by Equation 4.8. The model fitted the data very well (R^2 >0.99). By incorporating the estimated parameters, the change with time of the stability binding constants of Ni and the other cations can be expressed as:

$$K_{Ni}(t) = 5.81\times10^{3} + 4.41\times10^{5}\times e^{-\frac{t}{1.50}} \quad K_{Ca}(t) = 1.58\times10^{3} + 8.26\times10^{4}\times e^{-\frac{t}{3.71}}$$

$$K_{Mg}(t) = 8.71\times10^{3} + 2.26\times10^{6}\times e^{-\frac{t}{1.44}} \quad K_{Na}(t) = 456 + 2.26\times10^{6}\times e^{-\frac{t}{0.923}} \qquad (4.14)$$

The estimated K_{final} for Ni^{2+}, Ca^{2+}, Mg^{2+} and Na^{+} were 5.81×10^{3} (±0.257), 1.58×10^{3} (±129), 8.71×10^{3} (±18.6) and 456 (±7.73), respectively. The obtained relationships of K_{NiBL} and K_{CBL} with time (Equation 4.14) were incorporated into Equation 4.10 to yield the following relationship of LC50{Ni^{2+}} with solution chemistry parameters and time:

$$LC50\{Ni^{2+}\}(t) = \frac{0.052}{3.77 + 25.2\times e^{-\frac{t}{1.49}}}\times\{1 + (2.48 + 2.63\times e^{-\frac{t}{15.4}})\times\{Ca^{2+}\} + (3.94 + 19.3\times e^{-\frac{t}{1.89}})\times\{Mg^{2+}\} + (2.61 + 25.2\times e^{-\frac{t}{1.87}})\times\{Na^{+}\}\} \qquad (4.15)$$

With the activities of protective cations and time as inputs, LC50{Ni^{2+}} values were estimated for all test conditions using Equation 4.15. The linear relationship between predicted and observed p(LC50{Ni^{2+}}) values had an R^2 of 0.93, and predicted LC50{Ni^{2+}} never differed by more than a factor of 2 of the observed values (Figure 4.2C).

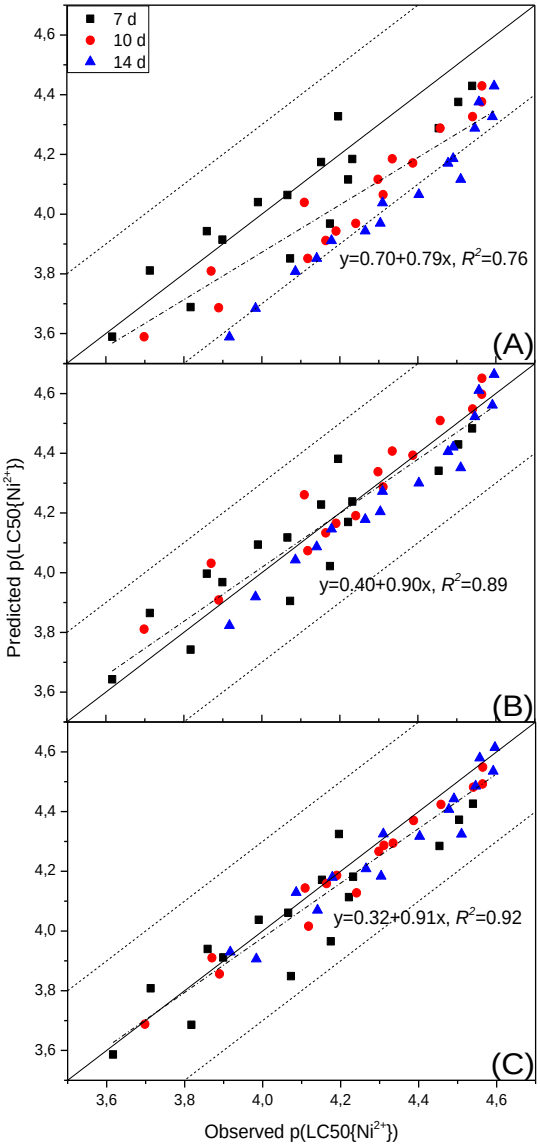

Figure 4.2 Relationships between predicted and observed LC50{Ni^{2+}} for *Enchytraeus crypticus* exposed to Ni solutions with different levels of Ca, Mg and Na embedded in sand at different exposure times (7, 10 and 14 d). The predictions were based on Model (a) (Equation 4.11) (A), Model (b) (Equation 4.13) (B), and Model (c) (Equation 4.15) (C). The solid line represents 1:1 line, the dashed lines represent a factor of 2 differences between predicted and observed values, and the dash-dot line represents the linear relationship between predicted and observed values with corresponding equations and R^2 values.

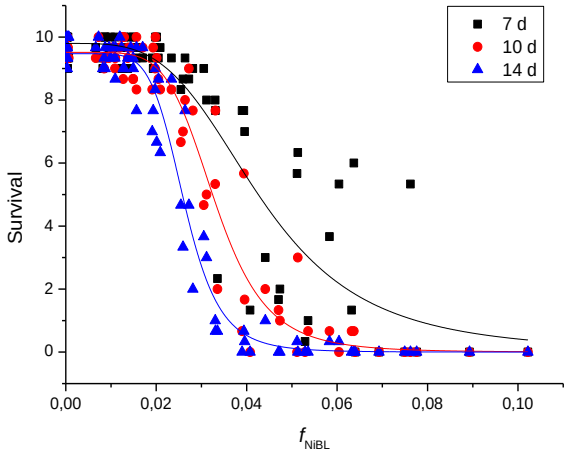

Figure 4.3 Relationship between the survival number of *Enchytraeus crypticus* exposed to Ni solutions with different levels of Ca, Mg and Na embedded in sand and the fraction of biotic ligands occupied by Ni (f_{NiBL}) at different exposure times (7, 10 and 14 d) calculated by Equation 4.3 with the parameters estimated from 7-d toxicity data.

4.4 Discussion

Effect of cations on Ni toxicity with time

Large variations in LC50{Ni^{2+}} between different treatments were observed, suggesting the limitation of using free ion activity for predicting Ni toxicity and emphasizing the importance of taking into account toxicity-modifying factors (i.e., coexisting cations) in estimating Ni toxicity (Thakali et al., 2006b; De Schamphelaere and Janssen, 2002). In the present study, LC50{Ni^{2+}} significantly increased with increasing activities of Ca^{2+}, Mg^{2+} and Na^{+} in test solutions, with the order of importance: Ca^{2+} > Mg^{2+} > Na^{+}. A protective effect of Ca^{2+} and Mg^{2+} was also reported for Ni toxicity to *Hordeum vulgare* and *Daphnia pulex* (Li et al., 2009a; Kozlova et al., 2009). However, the effect of Na^{+} has not been incorporated in the Ni-BLMs developed in previous studies. In *D. magna* Ni had clear effects on Mg homeostasis as the whole-body Mg concentration was significantly decreased, but no impact was observed on the whole body concentration of Ca (Pane et al., 2003). This suggests the existence of a shared uptake pathway for Mg^{2+} and Ni^{2+}. As a result, Mg^{2+} exerts its protective effect mainly through the competition with Ni^{2+} for transporter sites and directly inhibits the uptake of Ni^{2+}. In contrast, the effect of Ca^{2+} cannot be simply explained from the simple competitive equilibrium with the Ni transporter sites on the surface of organisms (Worms and Wilkinson, 2007).

Under the same cation composition of test solutions, LC50{Ni^{2+}} decreased with exposure time, which was also found for the toxicity of Ni to *E. crypticus* and *Folsomia candida* (Broerse and Van Gestel, 2010; He and Van Gestel, 2013). In the present study, Ca^{2+}, Mg^{2+} and Na^{+} exerted significant effects on Ni toxicity, however, with time the magnitude of their effect on Ni toxicity decreased. The BLM assumes there is no significant modification of biological membranes and no obvious biological regulation induced by cations binding to biotic ligands (Hässler et al., 2004). Hence, short-term experiments seem preferred to

increase the likelihood of meeting the assumption. In chronic toxicity tests, however, water hardness (especially Ca) was shown to be an important factor affecting membrane permeability and increasing the stability of membrane proteins under chronic exposure (Taylor et al., 2000; Niyogi and Wood, 2003). This might explain the changes in the protective effects of competitive cations on metal toxicity with time.

Model selection

Model (a) sufficiently described 7-d Ni toxicity but failed in predicting Ni toxicity to *E. crypticus* in time course as shown by the poor relationship between predicted and observed Ni toxicity. The under- or over-estimation of Ni toxicity over time might be attributed to the physiological processes of adaption or acclimation, which are currently not included in the BLM (Heijerick et al., 2005). Initially, BLM was developed for predicting acute toxicity of metals to aquatic organisms (Di Toro et al., 2001; Paquin et al., 2002). Later, attempts were made to extend the time scale of application of the BLM. The developed chronic Zn and Cu BLMs can accurately predict toxicity to *D. magna*, however, the cation binding constants and f_{50} of chronic BLMs differed from those of acute BLMs (Heijerick et al., 2002; Heijerick et al., 2005; De Schamphelaere and Janssen, 2002; De Schamphelaere and Janssen, 2004b). This suggests that the acute BLM cannot simply be used to predict chronic metal toxicity and that some BLM parameters would be altered over the time course. It is therefore important to unite the BLMs developed at different exposure times into a generic model.

In Model (b) the binding constants of metals and cations are considered to be metal- and species-specific, fixed with time. However, f_{50} is assumed to be organism- and time-dependent. In BLM, toxicity is predicted based on the steady-state concentration of the metal at the biotic ligand, however, this may not be the case for acute exposure where no sufficient time for equilibration is allowed (Erickson, 2013). There might be two biological compartments involved in the toxic effects exerted by metal exposure: 1) the binding of metal to biotic ligands on the surface of organisms; 2) the transport of metals bound on the biotic ligands to the target sites inside the organism. In the first step, chemical equilibrium can be reached fast; but the second step, the accumulation of metal, is a rate-limiting step in the causation of metal toxicity (Martin and Boaventura, 2002). Metals accumulate in organisms with time and cause toxic effects when the body metal concentration reaches a critical level (Jager et al., 2011). Consequently, toxicity of metals is time-dependent, with LC50 decreasing with exposure time and reaching equilibrium after certain exposure time (Verma et al., 2012; He and Van Gestel, 2013). When developing BLMs for predicting dynamic metal toxicity, the f_{50} values for Cu, Co and Cd toxicity to *Lemna paucicostata* and As toxicity to *Corbicula fluminea* decreased with exposure time and approached equilibrium after 80 and 150 hours, respectively (Hatano and Shoji, 2010; Chen and Liao, 2010). This is consistent with our findings. In Model (b), the f_{50} of Ni for *E. crypticus* decreased from 0.043 at 7 d to 0.026 at 14 d, with the estimated final f_{50} of 0.027. The 14-d f_{50} was almost the same as the estimated final f_{50}, suggesting that steady state of toxicity was almost reached after 14 d exposure.

In Model (c), the stability binding constants of cations (K_{CBL}) obtained for Ni-BLMs at three different time points were found to decrease with exposure time. In developing a BLM, cation binding constants are derived from the linear relationship between LC50{Ni^{2+}} and

cation activities (De Schamphelaere and Janssen, 2002). Upon long-term exposure the increase in LC50{Ni^{2+}} with increasing cation activity was less strong, leading to lower cation binding constants with time. In our study, logK_{NiBL} reduced from 4.00 after 7 d to 3.81 after 10 d, while after 14 d it was similar (3.76) to 10 d. This indicates that the equilibrium of Ni^{2+} and biotic ligands was reached after approximately 10 d. It was also recognized that the properties of biotic ligands (fish gills) may change during exposure to Zn and Cu because of acclimation, resulting in decreased binding affinities (Taylor et al., 2000; Alsop and Wood, 2000). Hence, the metal binding constants are time-dependent before reaching chemical and biological steady states.

Both Models (b) and (c) well predicted the dynamic toxicity of Ni with the variation of water chemistry, indicating that some BLM parameters need to be adjusted for describing dynamic toxicity. The BLM was developed to predict toxicity based on a sound understanding of the mechanisms involved (Paquin et al., 2002). This mechanistic explanation is generally viewed as one of the strongest attributes of the BLM. A good fit of data to the BLM-based model (such as Models (b) and (c)), however, does not guarantee that the mechanisms usually involved are always properly understood. As found in our study, models with different assumptions yielded similar results in terms of predictive capability. Although direct mechanistic evidence cannot be provided to support model selection, this study does shed light on the importance of toxicokinetics and toxicodynamics in modelling metal toxicity.

4.5 Conclusions

Model (a) based on acute toxicity data was not able to accurately predict the chronic toxicity of Ni, while the adjusted Ni-BLM (Models (b) and (c)) was capable of accurately predicting the toxicity of Ni to *E. crypticus* with the variation of both (pore)water chemistry and time. The adjusted models consider toxicokinetics and toxicodynamics in the assessment of the bioavailability and toxicity of metals. Future research efforts may focus on distinguishing these two time-related processes for the purpose of a mechanistic understanding of dynamic toxicity. The model concept still needs to be validated to see its applicability to other metals and other organisms, and further steps should be made to test its applicability to natural soils.

Supplemental information

Table S4.1 Overview of chemical characteristics of the test solutions in different toxicity tests with *Enchytraeus crypticus* and the measured concentrations of Ni and cations (Ca, Mg and Na) in the test solutions before (initial) and after (porewater) contact with quartz sand.

	Treatment		**Initial concentration**		**Porewater mean measured concentration**	
		Ni (mg/L)	Ni (mg/L)		Ni (mg/L)	
Control		0	0.01		0.01	
		0.8	0.62		0.47	
		1.6	1.27		0.95	
		3.2	2.59		1.94	
		6.4	5.34		4.01	
Ca-set	Ca (mM)	Ni (mg/L)	Ni (mg/L)	Ca (mM)	Ni (mg/L)	Ca (mM)
	Ca-0.5	0	0.02	0.43	0.02	0.29
		1.6	1.44	0.41	0.80	0.28
		3.2	2.86	0.41	1.49	0.29
		6.4	6.15	0.41	3.12	0.29
		12.8	10.3	0.41	6.19	0.30
	Ca-1.0	0	0.02	0.78	0.02	0.73
		1.6	1.34	0.78	1.10	0.72
		3.2	2.72	0.78	1.74	0.73
		6.4	6.20	0.76	3.44	0.74
		12.8	10.7	0.78	6.86	0.75
	Ca-2.0	0	0.02	1.54	0.02	1.28
		1.6	1.40	1.54	1.07	1.28
		3.2	2.82	1.52	2.37	1.30
		6.4	6.19	1.55	4.07	1.26
		12.8	10.7	1.54	7.98	1.28
	Ca-4.0	0	0.02	3.17	0.02	2.73
		3.2	2.87	3.23	2.72	2.72
		6.4	6.29	3.16	5.51	2.73
		12.8	11.0	3.17	9.39	2.76
		25.6	22.0	3.11	18.5	2.72
	Ca-8.0	0	0.02	6.18	0.02	5.41
		6.4	6.34	6.21	5.55	5.41
		12.8	10.9	5.86	9.81	5.41
		25.6	22.1	5.86	20.3	5.31
		51.2	48.0	5.87	43.2	5.53

Table S4.1 continued

	Treatment		Initial concentration		Porewater mean concentration	
	Mg (mM)	Ni (mg/L)	Ni (mg/L)	Mg (mM)	Ni (mg/L)	Mg (mM)
Mg-set	Mg-0.25	0	0.01	0.20	0.01	0.19
		1.6	1.47	0.20	0.95	0.19
		3.2	2.96	0.20	1.52	0.19
		6.4	6.53	0.20	3.55	0.19
		12.8	10.7	0.20	5.77	0.20
	Mg-0.5	0	0.08	0.37	0.08	0.45
		1.6	1.44	0.33	0.96	0.44
		3.2	2.82	0.34	1.49	0.44
		6.4	6.32	0.33	3.37	0.45
		12.8	12.6	0.33	6.57	0.45
	Mg-1.0	0	0.08	0.73	0.08	0.78
		3.2	2.90	0.74	2.01	0.76
		6.4	6.31	0.73	3.52	0.76
		12.8	11.1	0.72	7.19	0.78
		25.6	21.5	0.72	14.3	0.82
	Mg-2.0	0	0.07	1.56	0.07	1.15
		3.2	2.84	1.35	2.37	1.13
		6.4	6.24	1.36	4.74	1.15
		12.8	10.8	1.35	7.83	1.14
		25.6	21.6	1.35	15.90	1.18
	Mg-4.0	0	0.05	2.37	0.05	1.92
		6.4	6.40	2.22	4.99	1.99
		12.8	11.2	2.15	9.08	1.92
		25.6	21.2	2.43	17.6	1.98
		51.2	45.4	2.44	37.4	1.79

Table S4.1 Continued

	Treatment		Initial concentration		Porewater mean concentration	
	Na (mM)	Ni (mg/L)	Ni (mg/L)	Na (mM)	Ni (mg/L)	Na (mM)
Na-set	Na-4.0	0	0.01	3.59	0.01	3.41
		1.6	1.39	3.58	1.02	3.46
		3.2	2.80	3.57	1.45	3.37
		6.4	5.78	3.62	2.98	3.41
		12.8	12.1	3.64	5.98	3.41
	Na-8.0	0	0.03	7.15	0.03	6.34
		1.6	1.38	6.65	1.09	6.44
		3.2	2.76	6.91	1.68	6.35
		6.4	5.58	6.97	2.97	6.34
		12.8	12.2	7.07	5.94	6.25
	Na-12	0	0.06	9.43	0.06	9.01
		1.6	1.38	9.43	0.98	8.90
		3.2	2.74	9.55	1.65	9.06
		6.4	5.66	9.57	3.06	9.01
		12.8	12.3	9.68	6.08	9.06
	Na-16	0	0.07	13.9	0.07	13.1
		3.2	2.68	14.0	1.85	13.2
		6.4	5.68	13.6	3.34	13.1
		12.8	12.4	14.1	6.71	13.0
		25.6	26.7	14.2	13.7	13.2
	Na-20	0	0.08	18.0	0.08	14.5
		3.2	2.77	18.0	2.07	14.8
		6.4	5.81	18.0	3.50	14.5
		12.8	12.5	18.1	7.17	14.2
		25.6	25.8	18.0	14.3	14.4

Table S4.2 Characteristics of the pre-treated quartz sand used for solution only exposure toxicity tests with *Enchytraeus crypticus*.

Texture (µm)	Content (%)
Clay (< 8)	0.63
Silt (8-63)	0.52
Sand (63-2000)	98.85
Very Fine Sand (63-125)	3.65
Fine Sand (125-250)	42.76
Middle Coarse Sand (250-500)	50.36
Coarse Sand (500-1000)	2.07
Very Coarse Sand (1000-2000)	0.00

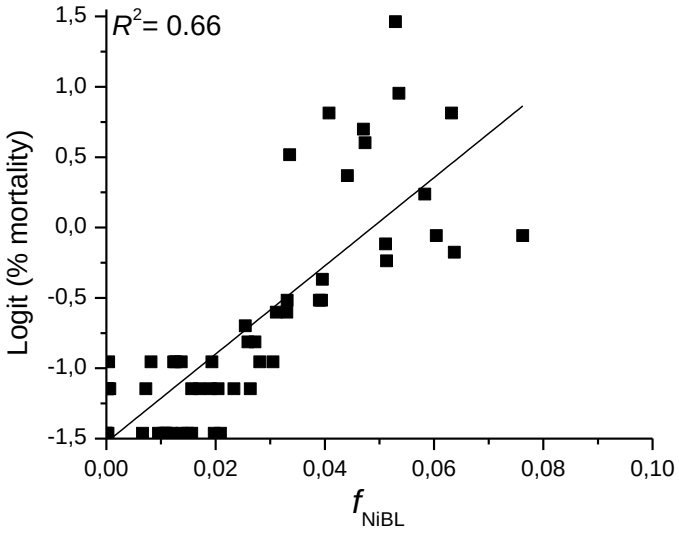

Figure S4.1 Relationship between the logit of the observed percentage mortality of *Enchtraeus crypticus* and the fraction of the biotic ligand sites occupied by Ni (f_{NiBL}) calculated by Equation 4.3 after 7 days exposure to Ni solutions embedded in sand.

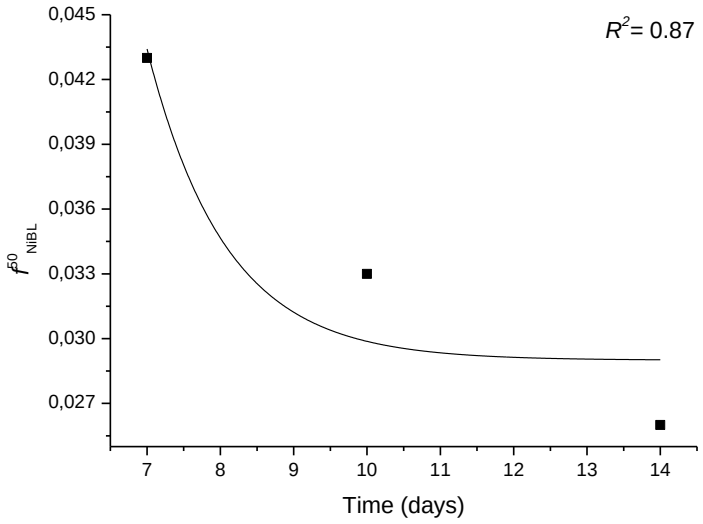

Figure S4.2 Relationship between the fraction of total biotic ligand occupied by Ni^{2+}, where 50% of mortality of *Enchytraeus crypticus* was induced (f_{50}) and exposure time (7, 10 and 14 d). The data points are the values of f_{50} estimated by the log-logistic model (Equation 4.5). The solid line represents the nonlinear fit with Equation 4.6.

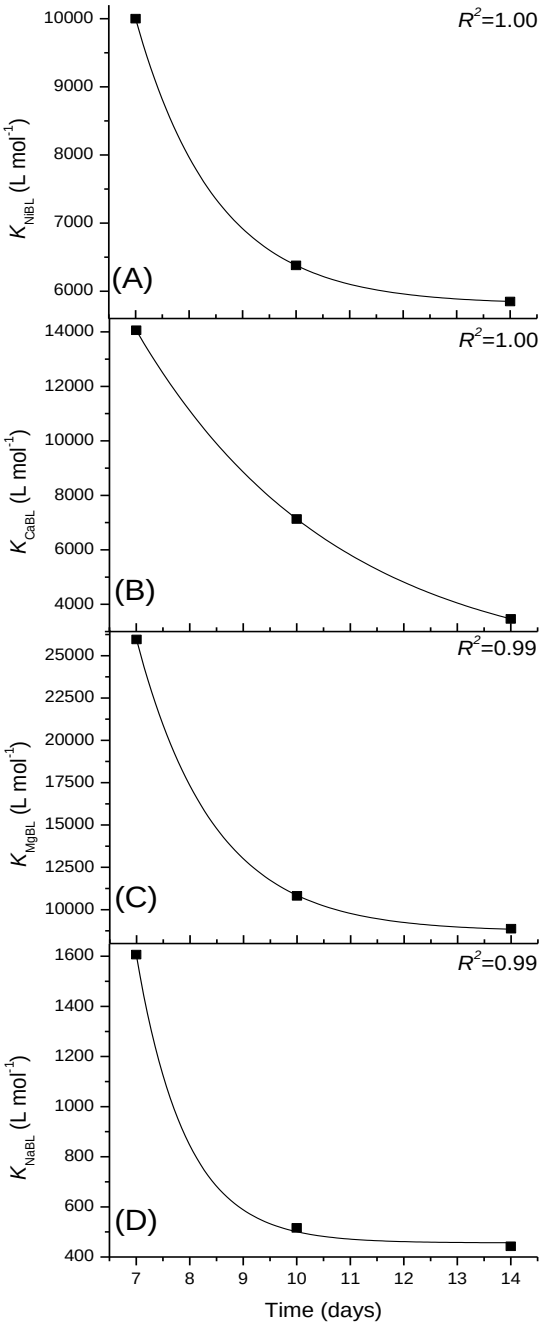

Figure S4.3 The stability binding constants (K_{CBL}) of Ni^{2+} (A), Ca^{2+} (B), Mg^{2+} (C) and Na^{+} (D) as a function of time for *Enchytraeus crypticus* exposed to Ni solutions embedded in sand. The data points show the calculated K_{CBL} of the developed Ni-BLMs at different exposure times. The solid lines represent the relation of K_{CBL} with time estimated with Equation 4.8.

Chapter 5

Interaction between nickel and cobalt toxicity in *Enchytraeus crypticus* is due to competitive uptake

Abstract

Uptake and toxicity of Ni-Co mixtures in *Enchytraeus crypticus* were determined after 4, 7, 10 and 14 d exposure. Generally, body concentrations of Ni and Co increased with increasing exposure concentrations. Ni body concentration was significantly reduced in the presence of Co, while Ni only marginally affected Co uptake. When expressed as free ion activities, individual toxicity of Ni and Co increased with time, with LC50s decreasing from 78.3 and 511 µM at 4 d to 40.4 and 393 µM at 14 d, respectively. When expressed as body concentrations, $LC50_{BodyNi}$ remained constant with time whilst $LC50_{BodyCo}$ increased during the first 7 d but remained stable afterwards. As identified by the MIXTOX model, interactions between Ni and Co were mainly antagonistic when based on free ion activities, however, no interaction was observed when based on body concentrations. A process-based model, incorporating exposure time to analyse the mechanisms underlying the dynamic mixture toxicity confirmed the differences in toxicokinetics of the 2 metals. Our findings suggest that body concentrations, which incorporate bioaccumulation process, are time-independent and can act as a more constant indicator of metal toxicity. The observed antagonism was mainly caused by competition between Co and Ni for binding sites and subsequent inhibition of Ni uptake. This competitive interaction occurred at the uptake level (toxicokinetics), but not at the target level (toxicodynamics).

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Environmental Toxicology and Chemistry, 2015, 34: 328-33

5.1 Introduction

Risk assessment of metals is usually based on toxicity data of single metals. As contamination in the environment rarely occurs as single metals but rather concerns (complex) mixtures of varying composition, this approach may have little environmental relevance (Vijver et al., 2010). Multiple metals may interact with each other, which leads to more-than-additive (synergism) or less-than-additive (antagonism) effects (Van Gestel et al., 2011). Hence, a risk assessment that ignores the possibility of joint action of metals is likely to underestimate or overestimate the actual risks. To meet future regulatory demands and ensuring adequate risk assessment (Kortenkamp et al., 2009), it is necessary to develop simple and efficient approaches for modeling metal mixture toxicity.

There are 2 most widely used models for predicting the effects of mixtures from the individual components: concentration addition (CA) and independent action (IA). The CA model assumes that mixture components have a similar mode of action, while IA assumes that the components have dissimilar modes of action (Van Gestel et al., 2011; Altenburger et al., 2004). The CA model usually estimates a higher combined effect than the IA model and therefore represents the worst-case scenario for mixture response (Boedeker et al., 1993). In a risk assessment context, CA is therefore a more conservative choice when it is difficult to identify the mode of action of mixture components. It should be noted that both the CA model and the IA model in their standard form do not consider mixture interactions in estimating mixture toxicity. However, mixture components may interact at various levels: (1) exposure level, (2) uptake level, (3) target level (Weltje, 1998; Qiu et al., 2011). Identifying interactions at relevant levels will therefore help to explain differences in interaction patterns that occur between different exposure media and between different test organisms.

The investigation of joint effects of mixtures on the basis of both external concentration and body concentration can contribute to a better understanding of the mechanism of interactions at different levels. Body metal concentration has been shown to be a better indicator than external exposure concentration for predicting single metal toxicity to organisms (He and Van Gestel, 2013; McCarty and Mackay,1993; Borgmann et al., 2008). Compared to external exposure concentrations, body concentration avoids the effect of environmental factors on metal accumulation (De Schamphelaere et al., 2005). In the present study, it is envisaged that body concentration of each mixture component may also serve as a useful indicator of mixture effects.

The importance of time in determining uptake and toxicity of single metals in organisms has been widely reported (He and Van Gestel, 2013; Crommentuijn et al., 1994). Generally, with the increase of time the amount of metal accumulated in an organism increases and toxic effects occur when the critical body threshold is reached (McCarty and Mackay,1993; Vijver et al., 2004). Uptake and elimination rates differ for each metal (Spurgeon and Hopkin, 1999; Lock and Janssen, 2001c). The different kinetics of metals cause a time-dependent composition of the internal metal mixtures in exposed organisms, and subsequently the joint toxicities of metal mixtures are also time-related (Broerse and Van Gestel, 2010). Spehar and Fiandt (1986) found that the joint action of metals for fathead minnows was more than additive in an acute toxicity test, but less than additive in a chronic test. Baas et al. (2007) investigated the toxicity of binary metals mixtures to *Folsomia candida*, observing that the

interactions between, for instance, Cu and Cd changed over time when based on the CA (or IA) model. Therefore, the evaluation of mixture toxicity should take time into account. However, at present, most models developed for predicting mixture toxicity of metals are based on a fixed exposure time without considering the impact of time on toxic interactions of metals (Kamo and Nagai, 2008; Le et al., 2013).

Ni and/or Co pollution in the environment mainly resulted from the burning of fossil fuels, spreading of sewage sludge and manure, and mining activities (Liber et al., 2011). Elevated levels of Ni and/or Co can cause harmful effects on the environment and human health. As Ni and Co are frequently encountered together in the environment, assessment of their joint effects is extremely relevant (Gikas, 2007). The toxic effects of single Ni and Co on soil organisms have been well studied (He and Van Gestel, 2013; Lock and Janssen, 2002a; Lock et al., 2006), but the binary mixture toxicity of them has rarely been investigated.

The present study aims to determine time-dependent mixture toxicity of Ni and Co to *Enchytraeus crypticus*, to quantify the mixture interactions at different exposure times with the MIXTOX model, and to describe the dynamics of mixture toxicity with a process-based model. Two research questions will be addressed: 1) do the interaction patterns of Ni and Co vary with time?; and 2) do the interaction patterns differ from each other when exposure is expressed on the basis of free ion activities or body concentrations?

5.2 Materials and methods

Test organism

Enchytraeids play an important role in the functioning of terrestrial ecosystems and are sensitive to chemical stressors (Didden and Römbke, 2001). *Enchytraeus crypticus* (Enchytraeidae; Oligochaeta; Annelida) was used as test organism in the present study. They were cultured in a climate room at 16 °C, with 75% relative humidity and in complete darkness. The animals were fed twice a week with a mixture of oat meal, dried baker's yeast, yolk powder, and fish oil. Adults at 1 cm were used in the present study, which could be distinguished by white spots in the clitellum region.

Test medium

A quartz sand-solution system was used to avoid the disturbance of complex soil processes and to enable better control of metal exposure and speciation in the toxicity tests. The quartz sand was pre-treated following the method of He and Van Gestel (2013) to remove all the organic matter, carbonates, and reactive Fe and Mn components. All used chemicals were of reagent grade (Sigma-Aldrich; > 99%). A basic solution composed of 0.2 mM Ca^{2+}, 0.05 mM Mg^{2+}, 2.0 mM Na^{+} and 0.078 mM K^{+} was used as the control. Stock solutions of $NiCl_2$ and $CoCl_2$ were prepared by adding different amount of $NiCl_2{\cdot}6H_2O$ and $CoCl_2{\cdot}6H_2O$ to the basic solution. Test solutions with Ni alone, Co alone, or a mixture of Ni and Co were prepared by adding different volumes of stock solution to basic solution. All test solutions were adjusted to pH 6.0 (5.95-6.05) by using 0.75 g/L MOPS (3-[N morpholino] propane sulfonic acid) (AppliChem; >99%), 0.75 mg/L MES (2-[N-morpholino] ethane sulfonic acid) (Sigma-Aldrich; >99%) and 0.1 M NaOH when necessary.

Toxicity test

The mixture experiment consisted of 3 simultaneous treatment series (i.e. Ni alone, Co alone, and mixtures of Ni and Co). The concentration of added Ni ranged from 0.2 to 12.8 mg/L, and the concentration of added Co ranged from 3 to 96 mg/L. The detailed design of concentrations of mixture combinations: can be seen from Figure S5.1 (Supplemental Data). Some combinations of the 2 metals at their highest concentrations were excluded because in our preliminary studies the test animals never survived combined exposure to those high concentrations. Toxicity tests with *E. crypticus* were conducted with 4 exposure times (4, 7, 10 and 14 d) and 3 replicates for each treatment and exposure time. As metal toxicity varies with time, the test concentrations of mixtures were slightly different at different time intervals. Ten adults were exposed in 100 mL glass jars filled with 20.0 g pre-treated quartz sand and 5.4 mL test solution. The sand and the test solution were equilibrated for 1 d before introducing the animals. The experiments were incubated at 20 °C with a cycle of 12h light: 12h dark. The jars were weighted twice a wk and water evaporation was compensated by adding deionized water. Animals were not fed during the experiment. Mortality was checked after different exposure times. Surviving animals were collected, washed with deionized water and frozen at -18°C for further analysis of body metal concentrations.

Physical and chemical analysis

The initial concentrations of Ni and Co in the test solutions were analyzed by flame atomic absorption spectrophotometry (AAS; Perkin Elmer AAnalyst 100). The frozen animals were freeze dried for at least 24 h, weighed individually on a microbalance, and digested in a 7:1 mixture of concentrated HNO_3 (Mallbaker Ultrex Ultra Pure, 65%) and $HClO_4$ (Mallbaker Ultrex Ultra Pure, 70%). Body concentrations of Ni and Co were measured by graphite furnace AAS (Perkin Elmer 1100B). DOLT-4 was used as certified reference material for quality control; the measured Ni and Co concentrations were always within 15% of the certified values.

Data analysis

Free ion activities of Ni and Co in the test solutions were calculated using the Windermere Humic Aqueous Model (WHAM VII) (Lofts and Tipping, 2011). The measured pH values and concentrations of Ni, Co, Ca, Mg, Na and K were used as input parameters. The median lethal concentrations (LC50) with 95% confidence intervals (95% CI) of single Ni and Co based on free ion activity and body metal concentration were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977).

Model description

Concentration-addition model (CA)

Both Ni and Co are divalent cations in solutions with almost the same molecular weight. It is assumed that they may have a similar mode of action because of structural electronic similarities. The CA model was therefore used to predict the toxicity of Ni and Co mixtures.

$$\mathrm{MT} = \sum \mathrm{TU}_{Xi} = \sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}_{Xi}} \qquad (5.1)$$

where c_i is the concentration of component *i* in the mixture, EC_{Xi} is the concentration of component *i* causing a certain effect X when applied alone, TU_{Xi} is the dimensionless toxic unit that quantifies the relative contribution of the individual component *i* to the toxicity of the mixtures; Mixture toxicity (MT) is regarded as the sum of the toxic units of the individual metals. In the present study, binary mixtures of Ni and Co were investigated and LC50 for mortality was selected as the endpoint, so Equation 5.1 can be rewritten as:

$$\mathrm{MT} = \frac{c_{\mathrm{Ni}}}{\mathrm{LC50\ of\ single\ Ni}} + \frac{c_{\mathrm{Co}}}{\mathrm{LC50\ of\ single\ Co}} \quad (5.2)$$

When using free metal ion activity as the expression of exposure, c_{Ni} and c_{Co} are the free ion activities of Ni and Co in the test solutions (μM), LC50 (μM) is free ion activity of Ni and Co causing 50% mortality of *E. crypticus* when applied singly (denoted as LC50{Ni^{2+}} and LC50{Co^{2+}}, respectively). When using body metal concentration as the expression of exposure, c_{Ni} and c_{Co} are the body concentrations of Ni and Co in the organism (μM/kg dry body weight), LC50 (μM/kg) is the dry body concentration of Ni and Co causing 50% mortality of *E. crypticus* when applied singly (denoted as $LC50_{BodyNi}$ and $LC50_{BodyCo}$, respectively).

A logistic dose-response model was used to correlate the survival of *E. crypticus* to the calculated MT after different exposure times.

$$S = \frac{S_{\max}}{1+(\frac{\mathrm{MT}}{\mathrm{MT_{50}}})^b} \quad (5.3)$$

where *S* is the number of surviving *E. crypticus*, S_{max} the number of survivors in the control, *b* the slope parameter, and MT_{50} the MT level causing 50% mortality. The parameters were estimated by fitting Equation 5.3 to data at each exposure time separately, using the nonlinear regression module in SPSS 19.0.

MIXTOX model

The MIXTOX model is basically a more elaborate version of the standard CA model that allows using additional parameters to quantify possible deviations (i.e, synergism, antagonism, dose-ratio or dose-level dependent synergism/antagonism) from the standard model, following the method of Jonker et al. (2005). Extra parameters are introduced into the model using a stepwise approach to describe deviations (see Jonker et al. (2005) for details). The model was fitted to the data using the method of maximum likelihood while minimizing the sum of the squared residuals. The statistical significance of the improvement in fit from the extended parameters was obtained through chi-square (χ^2) tests. The interpretations of the extra parameters can be found in Jonker et al. (2005).

Process-based model

A process-based model was used for better understanding the dynamics of the effects of the mixture by taking into account the processes of uptake and elimination (Baas et al., 2007). The main difference with the CA (or IA model) is that the whole time course of the toxic effects of the mixture is incorporated within 1 model. In the process-based model, it was assumed that when the internal concentration exceeds a certain threshold, the probability to die starts to deviate from that of the control. For both metals in single and mixture exposures, 3 time-independent parameters were estimated to describe the dynamic effect: a toxicological

threshold below which no effects occur, no effect concentration (NEC) (mM), and which is a measure for the toxic potency of the compounds, the killing rate (mM^{-1} d^{-1}) and a kinetic parameter, the elimination rate (d^{-1}). In addition there is the control or blank mortality rate (d^{-1}) to correct for control mortality, and an interaction parameter for the mixtures.

5.3 Results

Body concentrations of mixtures of Ni and Co

Body concentrations of 1 metal in the presence of the other metal after different exposure times are shown in Figures 5.1 and 5.2. Generally, as exposure concentrations of Ni and Co increased, so did their uptake by *E. crypticus*. At different time points, the body concentration of Ni was significantly and negatively correlated with the increase of Co concentrations (p<0.01) (Figure 5.1). For example, at a Ni exposure concentration of 27.3 µM, body Ni concentration decreased from 177 to 51.6, 147 to 92.8, 153 to 80.8 and 151 to 55.3 µM/kg at 4, 7, 10 and 14 d, respectively, when Co concentration increased from 0 to 814 µM. In contrast, the addition of Ni did not significantly affect the uptake of Co at the different exposure times (p>0.05) (Figure 5.2). These findings suggest a strong interaction effect of Co on Ni during the uptake phase.

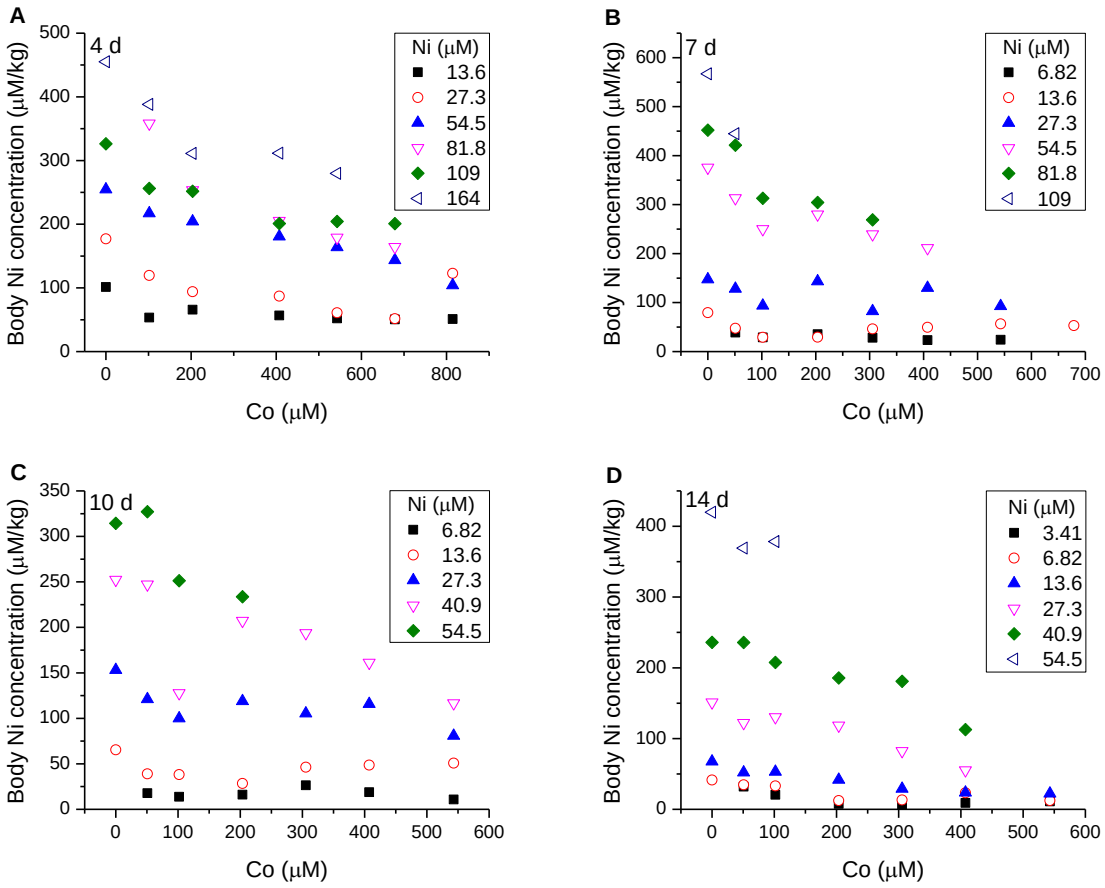

Figure 5.1 Body Ni concentrations in *Enchytraeus crypticus* under the influence of Co after different times of exposure to different Ni concentrations in solutions embedded in an inert sand matrix. See Supplemental Data, Figure S5.1, for test design.

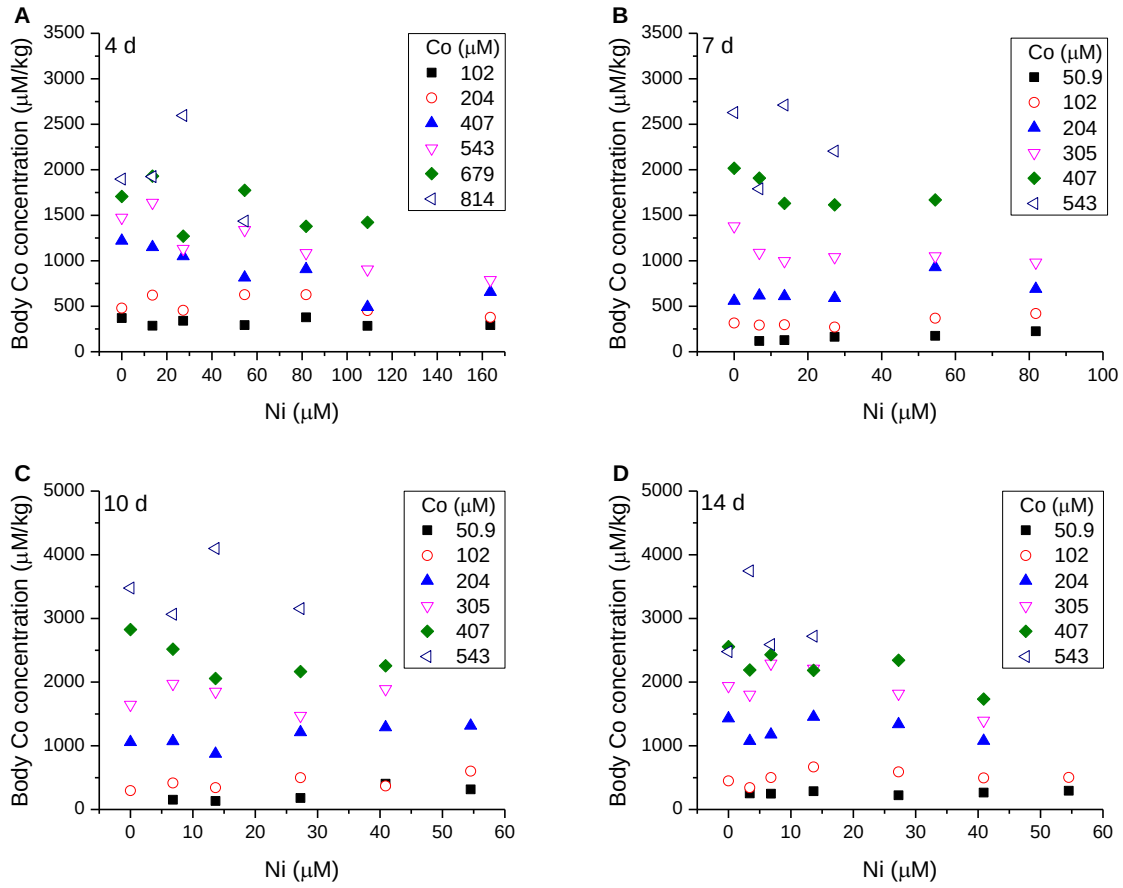

Figure 5.2 Body Co concentrations in *Enchytraeus crypticus* under the influence of Ni after different times of exposure to different Co concentrations in solutions embedded in an inert sand matrix. See Supplemental Data, Figure S5.1, for test design.

Individual toxicity of Ni and Co after different exposure times

The dose response relationships for the effects of Ni and Co on the survival of *E. crypticus* after different exposure times are shown in Figure 5.3, using 2 expressions of exposure (free ion activities and body concentrations). Generally, mortality increased with increasing free ion activity and body concentration of each metal tested. When expressed as free ion activities, LC50{Ni^{2+}} decreased gradually from 78.3 at 4 d to 40.4 µM at 14 d, and the final LC50{Ni^{2+}} was not reached during 14 d exposure. LC50{Co^{2+}} were reduced from 511 at 4 d to 393 µM at 14 d, the toxicity of Co almost reached steady state after 10 d exposure, with an LC50{Co^{2+}} of 401 µM (Table 5.1). Interestingly, the slope of the dose-response curve for Ni became much steeper after 14 d compared to 4 d. When expressed as body concentrations, $LC50_{BodyNi}$ remained almost constant with the increase of time, being 341, 383, 330 and 341 µM/kg at 4, 7, 10 and 14 d, respectively, and slope of the dose-response curves showed little difference for different exposure times. The $LC50_{BodyCo}$ increased from 2155 after 4 d to 3184 µM/kg after 7 d, and then levelled off to values of 3840 and 3591 µM/kg after 10 and 14 d, respectively (Table 5.1). Ni was more toxic than Co to *E. crypticus*, with LC50s after different exposure times differing a factor of 7 to 10 when based

on free ion activities and a factor of 6 to 10 when expressed on the basis of body concentrations.

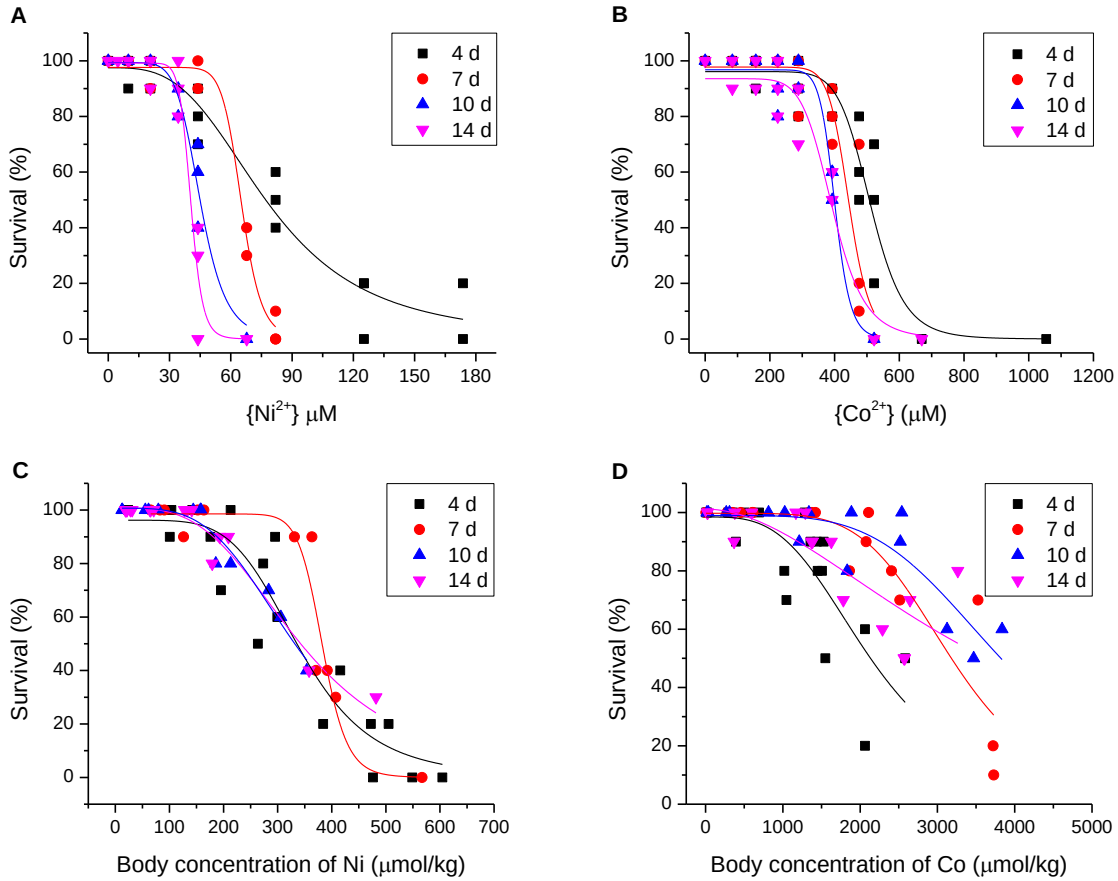

Figure 5.3 Effects of Ni and Co on the survival of *Enchytraeus crypticus* exposed for 4, 7, 10 and 14 d to solutions embedded in an inert sand matrix. Ni and Co exposure levels are expressed as free ion activities (A, B) and body metal concentrations in the surviving enchytraeids (C, D).

Table 5.1 Median lethal concentrations (LC50) for toxicity of single Ni and Co to *Enchytraeus crypticus* at different exposure times in solutions embedded in an inert quartz sand matrix[a].

Time (d)	LC50{Ni^{2+}} (µM)	LC50{Co^{2+}} (µM)	$LC50_{BodyNi}$ (µM/kg)	$LC50_{BodyCo}$ (µM/kg)
4	78.3 (68.8-87.9)	511 (489-534)	341 (301-381)	2155 (1718-2592)
7	65.2 (63.1-67.3)	444 (423-465)	383 (371-394)	3184 (2842-3527)
10	45.0 (42.9-47.2)	401 (389-413)	330 (305-356)	3840 (3337-4342)
14	40.4 (38.7-42.1)	393 (370-416)	341 (313-368)	3591 (3162-4091)

[a] Exposures were expressed as free ion activities and body metal concentrations in surviving animals, respectively.
The 95% confidence intervals are given in between parentheses.

Toxicity of mixtures of Ni and Co after different exposure time

In the mixture solutions of Ni and Co, the free ion activities of 1 metal were not significantly affected by the presence of the other metal (Supplemental Data, Figure S5.2). The observed toxic effects of Ni-Co mixtures after different exposure times are plotted against MT based on both free ion activities (Figure 5.4) and body metal concentrations (Figure 5.5). Generally, the survival rate of *E. crypticus* significantly decreased with increasing MT (p<0.01). On the basis of free ion activity, the model fit improved significantly from 4 d to 7 d, with R^2 values increasing from 0.69 to 0.84. After 7 d no further improvement of the fits was observed, with R^2 of 0.82 at 10 d and 0.81 at 14 d (Table 5.2). The estimated MT_{50} (95% CI) were 1.19 (1.09-1.29), 1.13 (1.07-1.19), 1.18 (1.13-1.22) and 1.16 (1.11-1.20) at 4, 7, 10 and 14 d, respectively (Figure 5.4). With the increase of exposure time, no significant changes in model fit were observed when based on body metal concentration, with R^2 varying between 0.50 and 0.58 at the different exposure times (Table 5.2). The estimated MT_{50} (95% CI) were 1.02 (0.893-1.15), 0.845 (0.729-0.929), 0.980 (0.880-1.08), 0.880 (0.744-1.02) at 4, 7, 10 and 14 d, respectively (Figure 5.5).

The mixture data were further analysed using the MIXTOX model to quantify the deviation from concentration addition. The estimated value of the interaction parameters (a and b), the goodness of fitting (R^2), and significance test results ($p(\chi^2)$) are shown in Table 5.2. When based on free metal ion activity, the MIXTOX model showed that the interaction between Ni and Co after different exposure times was mainly antagonistic, with the value of parameter a being positive. The inclusion of parameter a (2.46) at 4 d significantly improved the model fit, with $p(\chi^2)$<0.05. For 7 and 10 d, the best model fit was obtained when including a second parameter b_{DR} to describe a dose ratio-dependent deviation. The estimated values of b_{DR} were positive, being 1.32 at 7 d and 2.12 at 10 d, indicating that a decreased joint effect was due mostly to Co. For 14 d exposure, the extension of CA model with a second parameter b_{DL} (-5.86) to describe dose level-dependent dependence provided the best description of the data. This revealed the interaction pattern was antagonism and the magnitude of antagonism was dose-level dependent. However, on the basis of body metal concentrations, for the treatments at all 4 exposure times, the deviation from additivity was not significant ($p(\chi^2)$ >0.05).

The survival data of *E. crypticus* exposed to Ni and Co at different time points were fitted using the process-based model for both single and mixture exposure. The estimated parameters are shown in Table 5.3. For the single metal exposures, control mortality rate was rather low, 8.0×10^{-4} and 6.5×10^{-3} d^{-1} for Ni and Co, respectively. The estimated NEC for Ni and Co were 0.036 and 0.37 mM, respectively, indicating that *E. crypticus* is approximately 10 times more sensitive to Ni than to Co. The killing rate of Ni (10.2 $mM^{-1}d^{-1}$) was almost 3 times higher than that of Co (3.2 $mM^{-1}d^{-1}$). The elimination rates of Ni and Co were similar, with values of 1.66 d^{-1} for Ni and 1.20 d^{-1} for Co. For the mixtures of Ni and Co, the killing rate of each metal was lower than that for the single exposures, being 3.63 $mM^{-1}d^{-1}$ for Ni and 1.43 $mM^{-1}d^{-1}$ for Co (i.e. ≈ 3-fold differences). The elimination rate of Ni decreased from 1.66 to 0.70 d^{-1} in the presence of Co, while the elimination rate of Co was not affected by the presence of Ni. No significant interaction was found in the mixtures.

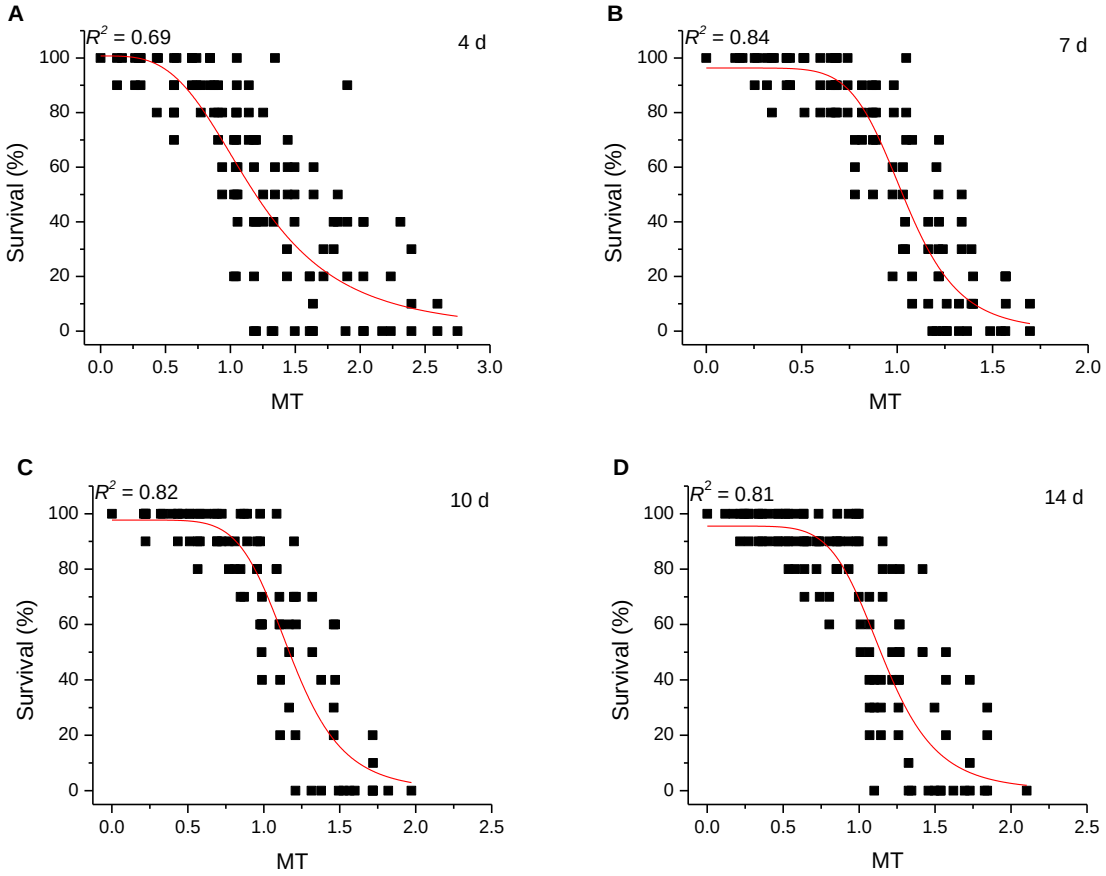

Figure 5.4 The relationship between the survival of *Enchytraeus crypticus* after 4 (A), 7 (B), 10 (C) and 14 (D) d exposure and binary mixture concentrations of Ni and Co expressed as the sum of toxic units (MT) based on metal free ion activities. The data points represent the observed values; the solid line shows the fit of a logistic dose-response model. See Supplemental Data, Figure S5.1, for test design.

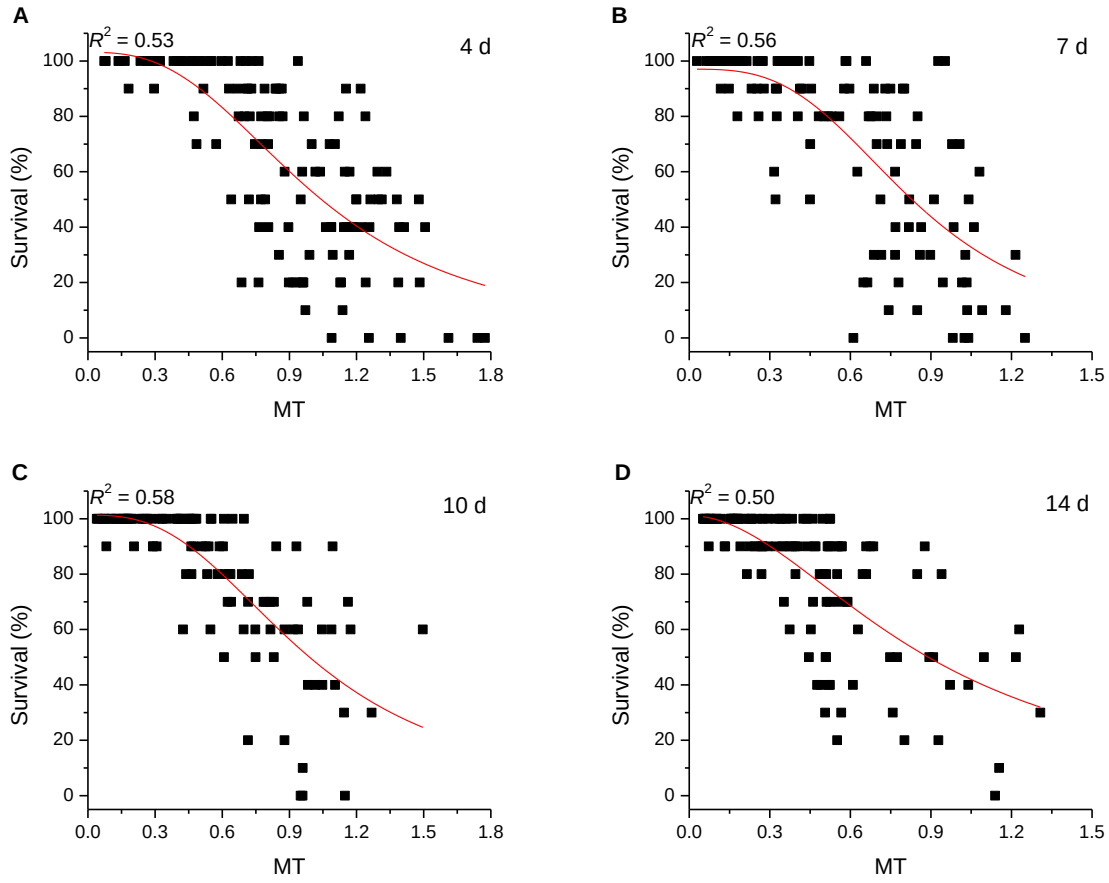

Figure 5.5 The relationship between the survival of *Enchytraeus crypticus* after 44 (A), 7 (B), 10 (C) and 14 (D) d exposure and binary mixture concentrations of Ni and Co expressed as the sum of toxic units (MT) based on body metal concentrations. The data points represent the observed values; the solid line shows the fit of a logistic dose-response model. See Supplemental Data, Figure S5.1, for test design.

Table 5.2 Mixture toxicity of Ni and Co to *Enchytraeus crypticus* after different times of exposure to test solutions embedded in an inert sand matrix[a].

Time (d)		Free ion activity based					Body concentration based	
		R^2 [b]	a [c]	b_{DR}/b_{DL} [c]	p (χ^2) [d]	pattern	R^2 [b]	pattern
4	CA	0.69					0.53	
	Deviation	0.86	2.46		2.1×10^{-18}	Antagonism		No deviation
7	CA	0.84					0.56	
	Deviation	0.90	0.16	1.32	0.027	DR		No deviation
10	CA	0.82					0.58	
	Deviation	0.91	0.18	2.12	1.5×10^{-5}	DR		No deviation
14	CA	0.81					0.50	
	Deviation	0.90	0.14	-5.86	0.033	DL		No deviation

[a] See Supplemental Data, Figure S5.1, for test design. The table summarizes the parameter values obtained by fitting the Concentration Addition (CA) module of the MIXTOX model (Jonker et al., 2005).
[b] The R^2 value indicates the goodness of fit.
[c] a and b are the parameters of the deviation functions.
[d] $p(\chi^2)$ is the statistic outcome of the χ^2 test ($p<0.05$, significant difference).

Table 5.3 The estimated parameters for the blank mortality rate, no-effect concentration (NEC), killing rate, and elimination rate for the mixture toxicity of Ni and Co to *Enchytraeus crypticus* after different exposure time exposed to test solutions embedded in an inert sand matrix, using the mixture toxicity model of Baas et al. (2007). SD = standard deviation.

	Single		Mixture	
	Ni (±SD)	Co (±SD)	Ni	Co
Blank mortality rate (d^{-1})	8.0×10^{-4} ($\pm8\times10^{-4}$)	6.5×10^{-3} ($\pm2\times10^{-3}$)	0.004	
NEC (mM)	0.036 (±0.003)	0.37 (±0.007)	0.043	0.40
Killing rate ($mM^{-1}d^{-1}$)	10.2 (±2.3)	3.20 (±0.85)	3.63	1.43
Elimination rate (d^{-1})	1.66 (±0.98)	1.20 (±0.34)	0.70	1.10

5.4 Discussion

Single toxicity

When applied singly, Ni was more toxic to *E. crypticus* than Co, with the individual LC50 and NEC of Ni approximately 10 times lower than that of Co. The slope of the dose-response curve of single Ni after 14 d exposure was much steeper than that after 4 d exposure, while the slope of Co was rather constant (Figure 5.3). In addition, it took a longer time for Ni toxicity to reach steady state than for Co toxicity. This indicates that Co is a faster acting toxicant than Ni. A number of studies have investigated the single toxicity of Ni and Co to aquatic and soil organisms. For instance, Griffitt et al. (2008) found that 48h LC50 values of Ni and Co were 1.48 and 9.72 mg/L, respectively, for *Daphnia pulex*, and 19.6 and 94.7 mg/L, respectively, for *Ceriodaphnia dubia*. Their study showed that Ni is approximately 5 times more toxic than Co at 48h. These results are consistent with our findings. The present study showed that the toxicity of Ni and Co increased with exposure time when based on free ion activities. Previous studies reported that toxicity of Ni to *F. candida* and *E. crypticus* increased over time and almost reached steady states after 49 d and 21 d exposure, respectively (He and Van Gestel, 2013; Broerse and Van Gestel, 2010). Bioaccumulation of a metal is the net result of uptake, distribution and elimination processes in an organism during exposure. The body concentrations of metals increase over time until steady state is reached between influx and efflux (Harrahy and Clements, 1997). Toxic effects are induced when body metal concentration exceeds a critical level. Noteworthy, when single toxicity of Ni and Co was based on body concentrations, the $LC50_{BodyNi}$ was almost constant with time and the $LC50_{BodyCo}$ also remained constant from 7 d exposure onwards. He and Van Gestel (2013) reported that the LC50 for the toxicity of Ni to *E. crypticus* expressed as body concentrations was approximately constant and independent of exposure time, with a value of 285 µM/kg, which was comparable with the result obtained in the present study (330-383 µM/kg). This suggests that body concentration is a better indicator of toxicity than free ion activity and that both Ni and Co in *E. crypticus* comply with the concept of Critical Body Residues (McCarty and Mackay, 1993).

Mixture toxicity

In general, the CA model well predicted the mixture toxicity of Ni and Co on the basis of free ion activities. The explained variation increased from 69% after 4 d to 84% after 7 d, and then remained constant. This suggests that the steady state of Ni and Co accumulation in the mixtures was not yet reached after 4 d exposure. Accumulation (uptake and elimination) rates vary according to the chemical nature of the compound, and also the size and type of organisms tested (Slaveykova and Wilkinson, 2005). The accumulation rate of Co was found to be higher than that of Ni in bivalve species (Markich et al., 2001). The differences in toxicokinetics of each mixture component may become an uncertainty factor when considering mixture interactions at a fixed time point during the uptake phase. When using body metal concentration as the metric of exposure, the toxicokinetic process is included (Ashauer and Escher, 2010). So the fit of the CA model did not change with time when mixture toxicity was related to body concentrations, even when interactions occurred in the uptake phase. However, only approximately 50% of the variation in the data was explained

by the CA model when using body concentrations. Within an organism, metal exposure can be regulated by storing in inert forms and detoxification, suggesting that body concentration cannot fully represent the concentration at target sites (Vijver et al., 2004). Hence, the use of internal concentrations as indicator of toxicity still does not incorporate the toxicodynamic processes that quantitatively link the body concentration to the effect at the level of the individual organism over time (Jager et al., 2011). In addition, unlike plants, the body concentration of metals can only be analysed in surviving animals, which may not directly reflect the concentration in the dead animals and subsequently reduce the model performance. One evidence is that the tissue concentration showed to be the best predictor of toxic effects of As to plants, but was not predictive of the toxicity to earthworm (García-Gómez et al., 2014).

Interaction patterns at different levels

In the present study, the existence of 1 metal cannot significantly affect the free ion activities of the other metal in the mixture solutions, indicating that no interaction occurred at the exposure level. The joint effect of Ni and Co after different exposure times was mainly antagonistic on the basis of free ion activities, and the fit of the MIXTOX model to the data was significantly improved when considering the deviations from concentration addition. Gikas (2007) investigated the effects of Ni and Co on the microbial growth rate of activated sludge and found that mixture interactions shifted from synergism at relative low concentrations to antagonism at relative high concentrations. Whether mixture interactions are synergistic or antagonistic depends on whether 1 metal facilitates the uptake of the other or whether they compete for the same transport sites (Franklin et al., 2002). A possible explanation of the antagonistic interaction is that Ni and Co can compete with each other for uptake, thus resulting in less accumulation of either 1 or both metals. According to the concept of the Biotic Ligand Model (BLM), the coexisting cations can exert a protective effect by competing with metal ions for the binding sites on the surface of organisms and inhibiting the uptake of metals (Di Toro et al., 2001). He et al. (2014) provided evidence that Mg reduced the uptake of Ni through competition with Ni for the binding sites of *E. crypticus*. Lock et al. (2006) reported that Mg also has a significant protective effect on the toxicity of Co to *Enchytraeus albidus*. Ni and Co are both divalent in solution and belong to the VIIIB group of the periodic table, having similar physicochemical properties. The competition for membrane binding sites and intracellular binding sites can occur for metals with similar ionic radii and coordination geometry (Sunda and Huntsman, 1998). So, it is likely that Ni and Co share some common transport or target sites on the surface or inside the organisms.

There was no deviation from additivity on the basis of body concentrations. Weltje (1998) reported that the toxic effects of Cd, Cu, Pb and Zn mixtures were mainly antagonistic using total soil concentrations and concentration additive behaviour was found when using metal concentrations in earthworm tissues. This finding is consistent with the result of the present study. The deviation from CA models can result from toxicokinetic and toxicodynamic interactions among components in the mixture. Metals in solutions may interact at various levels including during uptake (toxicokinetics) and at target sites within an organism (toxicodynamics) (Vijver et al., 2010; Weltje, 1998). Different conclusions on the interaction of metals can be drawn when using different expressions of exposure. The

activities of Ni and Co in the exposure solution were not affected by the presence of each other, ruling out the interactions at the exposure level. The difference of the interaction patterns based on free ion activities and body concentrations suggests that the competitive interaction between Ni and Co mainly occurred during uptake, which affected toxicokinetics and subsequently the quantity available at the binding sites on the surface of the organisms. No competition at the target level, which may affect toxicodynamics and the concentration of metals on the target sites inside the organism, was observed.

In the presence of Co, the uptake of Ni was significantly reduced by 20-70%, while Ni did not exert appreciable effect on the uptake of Co (Figure 5.1 and 5.2), suggesting that Co acted as an antagonist and modified the bioaccumulation of Ni. In agreement with our results, Wang et al. (2013) showed that the uptake of Zn and Co by plant roots was reduced in the presence of each other through a site competition mechanism. Franklin et al. (2002) found that Cu inhibited the binding and cellular uptake of Zn, which resulted in decreased mixture toxicity to freshwater alga (*Chlorella* sp.). These findings support our hypothesis that the antagonistic interaction between Ni and Co is caused by competition and subsequently reduced metal uptake. In the mixture solutions, Co concentrations were many-fold higher than Ni concentrations, this may explain why the uptake of Ni was reduced in the presence of Co and not vice-versa. Ni and Co interact with each other during the uptake process. This competition suggests a similar mode of action, providing a basis for assuming concentration-additive effects and supporting the selection of the CA model as a conservative choice for estimating the mixture toxicity of Ni and Co.

5.5 Conclusions

The present study focused on the joint toxicity of Ni and Co to *Enchytraeus crypticus* and used 3 models (CA, MIXTOX and process-based model) to evaluate the toxic effects of Ni-Co mixtures at different intervals. It was found that body concentration was a time-independent measure of metal toxicity and that a certain exposure time is needed to reach steady state. Interaction between Ni and Co was mainly antagonistic on the basis of free ion activities, and concentration additive on the basis of body concentrations. Toxicity of the Ni and Co mixture was dominantly determined by interactions at the uptake level (toxicokinetics), but not at the target level (toxicodynamics). The present study provided insight into the mechanism of the interactive effect of binary Ni and Co mixtures on the mortality of *E. crypticus* at different interaction levels. Further research is needed to obtain more insight into the mechanisms of the interaction between metals by applying more advanced metal speciation models (e.g. WHAM-F_{TOX}) and to investigate the mixture toxicity of metals in the context of soil ecosystems with more complex interactions.

Supplemental information

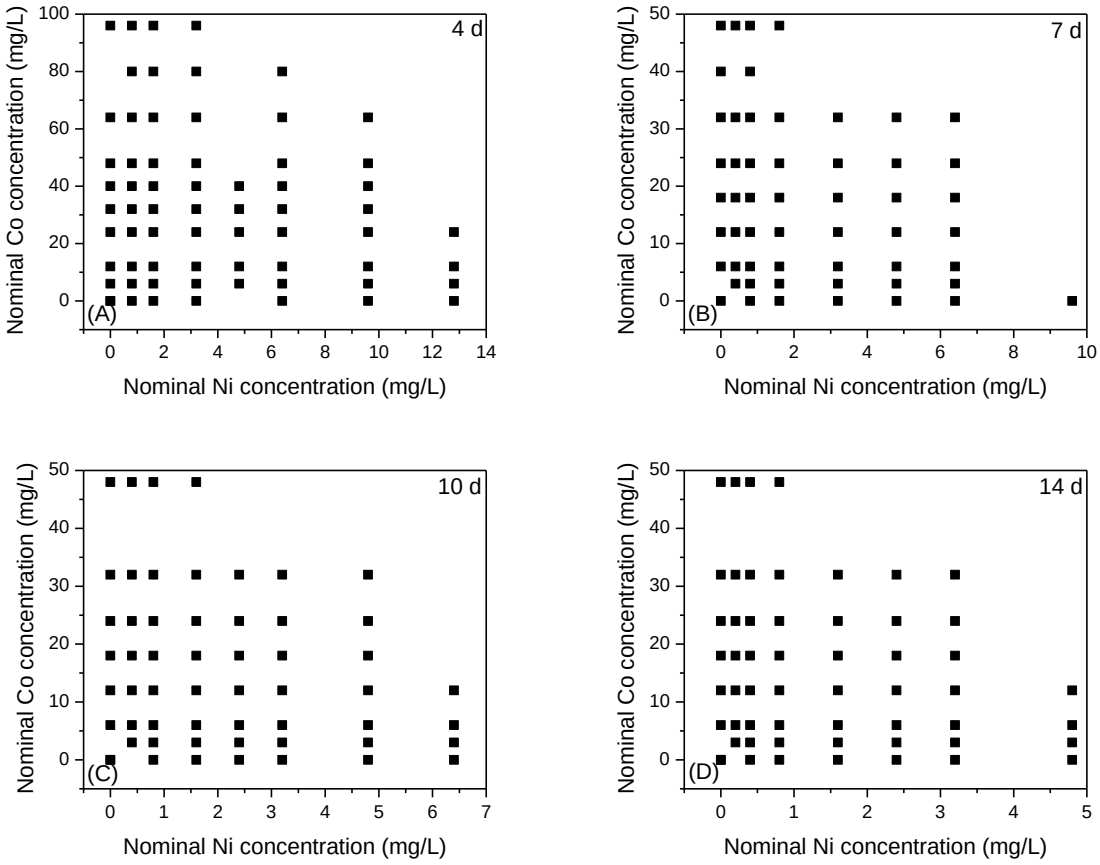

Figure S5.1 Test designs of the Ni and Co mixture toxicity tests with *Enchytraeus crypticus*, using exposures in solutions embedded in an inert sand matrix. Shown are the Ni and Co concentrations for the 4 (A), 7 (B), 10 (C) and 14 (D) d toxicity tests.

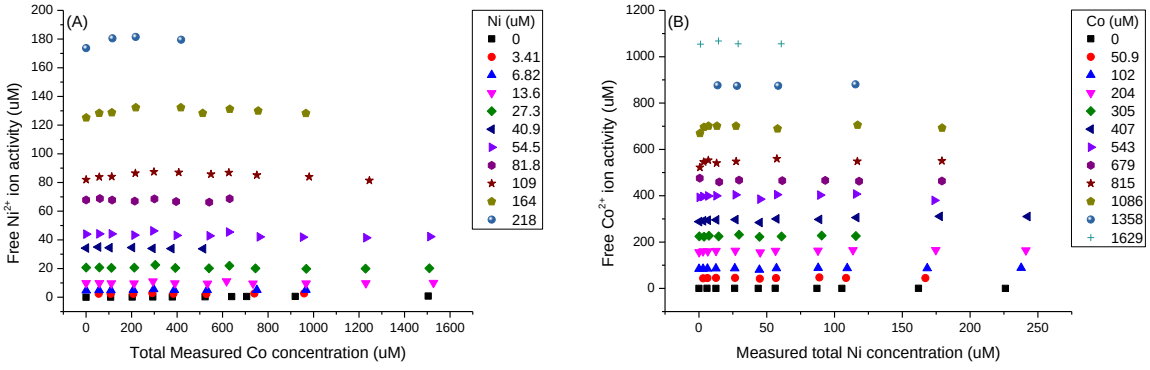

Figure S5.2 The influence of the presence of Co and Ni on the free ion activities of Ni (A) and Co (B) at different exposure levels of Ni and Co in the test solutions used for mixture toxicity tests with *Enchytraeus crypticus*.

Chapter 6

Delineating the dynamic uptake and toxicity of Ni and Co mixtures in *Enchytraeus crypticus* using a WHAM-F_{TOX} approach

Abstract

Uptake and toxicity of Ni, Co and their mixtures in *Enchytraeus crypticus* after different exposure times (4, 7, 10 and 14 d) were predicted using the WHAM-F_{TOX} model, which incorporates the effects of metal speciation, affinity and competition of metals for binding sites. The combined toxicity of metals was quantified by the toxicity function (F_{TOX}), a linear combination of the amount of metal binding to non-specific ligand sites (ν_i) and a toxicity coefficient (α_i). Observed body concentrations of Ni and Co in the animals only slightly deviated from the WHAM-calculated amounts binding to humic acid, supporting the use of humic acid as a surrogate for metal binding sites of *E. crypticus*. The toxicity of metals at different exposure times was well predicted by the WHAM-F_{TOX} model. The derived α_{Ni} increased with time and reached equilibrium after approximately 14 d, while α_{Co} remained almost independent of time. This suggests for Ni more time is needed than for Co to reach equilibrium of body concentrations, so the toxicity of Ni is much more time-dependent. The WHAM-F_{TOX} model provides a new tool for evaluating the potential mixture toxicity of metals to soil organism in a dynamic environment. However, as α_i varied with exposure time, caution is warranted when using the parameters estimated from acute toxicity experiments for predicting the chronic toxicity of metal mixtures.

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Submitted

6.1 Introduction

Metal contamination in the aquatic and terrestrial environment has received increasing attention due to its wide existence and adverse ecological effects. As metals are often introduced into the environment as mixtures, assessment of mixture effects is extremely relevant (Vijver et al., 2010). However, ecological risk assessment and environmental quality standard derivation are predominantly based on laboratory toxicity data with single-metal exposure (Lock and Janssen, 2002b). Hence, it is urgent to develop predictive models for effective and accurate risk assessment of metals in mixtures.

The biotic ligand model (BLM) was initially developed to predict single metal toxicity by taking into account the effect of water chemistry on metal bioavailability and toxicity (Di Toro et al., 2001). The extension of the use of BLM for metal mixtures is feasible based on the assumption that different toxic metals share the same biotic ligands and one metal may exert its protective effects to the coexisting metals through competition for binding sites (Jho et al., 2011; Le et al., 2013). Jho et al. (2011) successfully predicted the toxicity of Cd and Pb with different concentrations of Ca^{2+} using a BLM with parameters derived from single metal toxicity data and considering the competition between Ca and Cd or Pb and between Cd and Pb. According to the concept of BLM, toxicity depends upon the extent of occupation of biotic ligands by metal ions, hence the model requires specific equilibrium constants for metals and competing cations, which are derived from single metal toxicity data (Steenbergen et al., 2005; He et al., 2014). Subsequently, the extension of BLMs to predict mixture toxicity for the full range of conditions, mixtures and organisms in the environment would require a major research effort to gather the necessary data (Stockdale et al., 2010). Thus, the application of BLMs for predicting the toxicity of mixtures might be hampered if the conditional stability constants describing metal binding affinities are still unknown.

The problem encountered when extending BLMs for predicting mixture toxicity can be avoided by using a newly developed approach, the WHAM-F_{TOX} model (Tipping and Lofts, 2013). Both the BLM and WHAM-F_{TOX} approaches take the organism as a reactant in a conventional system that is in chemical equilibrium. In a BLM, biotic ligands are considered to be independent and homogeneously distributed, most often represented by a single ligand-binding constant (Slaveykova and Wilkinson, 2005). However, WHAM-F_{TOX} assumes that organisms accumulate metals by binding at non-specific ligand sites. These sites are represented by the array of binding sites postulated for humic acid in the Windermere Humic Aqueous Model (WHAM) (Stockdale et al., 2010). The competitive binding of toxic metals and cations to biotic ligands is proposed to be represented by competitive binding to particulate humic acid. The WHAM-predicted binding of metals to humic acid has been shown to be a good guide to their accumulation in living organisms, and therefore a measure of metal exposure. The accumulation of a number of major and trace metals by aquatic bryophytes linearly correlated with the metal load bound to humic acid as calculated with WHAM (Tipping et al., 2008). The amount of Cu bound to humic acid estimated with WHAM well predicted the toxic effect of Cu on the root elongation of *Lemna minor* (Antunes et al., 2012). The WHAM-F_{TOX} model has been applied successfully for the prediction of the accumulation and toxicity of metal mixtures, it provided a good estimation of the impact of metal mixtures (Zn, Cd and Cu) on macroinvertebrates at varying water chemistries (Iwasaki et al., 2013). However, most of the previous work on WHAM-F_{TOX} has

focused on aquatic organisms in field situations (Tipping et al., 2008; Stockdale et al., 2010; Antunes et al., 2012). To our knowledge, the applicability of WHAM-F_{TOX} to terrestrial organisms in laboratory-controlled conditions has never been investigated.

Traditional ecotoxicological studies were often performed for a certain endpoint after a fixed exposure time (Lock and Janssen, 2002a; Qiu et al., 2011), while toxicity may vary with time. For instance, Utgikar et al. (2004) and Baas et al. (2007), investigating the mixture toxicity of Zn and Cu to *Vibrio fischeri* and of Cu and Cd to *Folsomia candida*, respectively, found that the toxicity of these metal mixtures increased with exposure time. Neglecting the nature of the response with time may have little utility in predicting effects of mixtures in the dynamic environment. Mixture effects determined at only one fixed time point may under or over estimate the actual risk. In addition, the WHAM-F_{TOX} approach is developed for situations in which equilibrium is reached (Tipping and Lofts, 2015). Its applicability for dynamic situations is still unknown. For better understanding of the dynamic effects of mixtures and the predicative ability of the WHAM-F_{TOX} approach, measurement at different time points are needed.

In the present study, time-varying accumulation and toxicity of Ni and Co mixtures were investigated using the soil organism *Enchytraeus crypticus*. We aimed at determining the relationship between body metal concentrations and the amount of metals bound by humic acid as estimated from WHAM; and to examine the applicability of the WHAM-F_{TOX} approach for predicting the toxicity of Ni, Co and their mixtures to *E. crypticus* in the course of time. The parameters estimated with the WHAM-F_{TOX} model at different exposure times were compared to explore the underlying mechanisms of the dynamic mixture toxicity.

6.2 Materials and methods

Test organism

Enchytraeus crypticus (class Oligochaeta, family Enchytraeidae) are ecologically relevant soil-dwelling annelids and were chosen as test organisms. *E. crypticus* were cultured in agar prepared with soil extracts, kept in a climate chamber at 16℃, 75% relative humidity and without illumination, and fed twice a week. Experiments used adult *E. crypticus* with a well-developed clitellum and a length of approximately 1 cm.

Preparation of test medium

A simplified exposure system, which consists of modified soil solutions and inert quartz sand, was used in the present study. The quartz sand was pretreated by combustion and acid washing; see He et al. (2013) for the pretreatment method and the particle size distribution of pretreated quartz sand. All test media contained 0.2 mM $CaCl_2$, 0.05 mM $MgSO_4$, 2.0 mM NaCl and 0.078 mM KCl. For each treatment, different concentrations of $NiCl_2{\cdot}6H_2O$ and/or $CoCl_2{\cdot}6H_2O$ were added to the test media. The concentrations of added Ni and Co ranged from 0.2 to 12.8 mg/L and from 3 to 96 mg/L, respectively. Solution pH were adjusted to 6.0 ± 0.05 with 0.75 g/L MOPS (AppliChem; >99%), 0.75 mg/L MES (Sigma-Aldrich; >99%) and 0.1 M NaOH when necessary.

Toxicity tests

The toxicity tests included three series: Ni alone, Co alone, and Ni-Co mixtures. For each treatment, 5.4 mL of test solution was added to 20.0 g of pretreated quartz sand in a 100 mL glass jar and left for 1 day to obtain near-equilibrium solutions. Thereafter, 10 adults of *E. crypticus* were exposed for 4, 7, 10 and 14 d, with three replicates for each treatment and exposure time. The toxicity tests were performed at 20 °C with a cycle of 12h light: 12h dark. The jars were weighted twice a week and deionized water was added when necessary to compensate for water evaporation. During the experiment animals were not fed. The survival of the animals was recorded after different exposure times. Surviving animals were collected, washed with deionized water and frozen at -18°C for further analysis.

Chemical analysis

Solution pH was measured using a pH meter (691, Metrohm AG). Before commencing the toxicity tests, dissolved Ni and Co concentrations in the test solutions were measured by flame atomic absorption spectrophotometry (AAS, Perkin Elmer AAnalyst 100). At the end of the experiment, the frozen animals were freeze dried for 48 h, weighed with a microbalance, and digested using 7:1 (v/v) HNO_3 (65%; Mallbaker Ultrex Ultra Pure) and $HClO_4$ (70%; Mallbaker Ultrex Ultra Pure). Total Ni and Co concentrations were analyzed using graphite furnace AAS (Perkin Elmer 5100). Standard reference material (NRC DOLT-4) was used for analytical quality control. Measured Co and Ni concentrations generally were in good agreement (±10%) with the certified reference values.

Data analysis

Free metal ion activities in the test solutions were calculated using the Windermere Humic Aqueous Model (WHAM VII) (Lofts and Tipping, 2011). The input parameters included solution pH, temperature (293K), pCO_2 and concentrations of Ni, Co, Ca, Mg, Na, K, Cl^-, and SO_4^{2-}. For all calculations, measured values were used unless otherwise stated. The median lethal concentrations (LC50) with 95% confidence intervals (95% CI) of single Ni and Co were calculated using the trimmed Spearman-Karber method based on free ion activities (Hamilton et al., 1977). All data were statistically analyzed using one way analysis of variance (ANOVA) at a significance level of $p < 0.05$ with SPSS 19.0. Duncan's posthoc test was used to detect significant differences.

WHAM-F_{TOX} model

It is assumed that the combined toxicity of cations can be quantified by a linear toxicity function (Tipping and Lofts, 2013):

$$F_{TOX} = \sum \alpha_i v_i \quad (6.1)$$

α_i is the toxicity coefficient of cation i (i.e., Ni^{2+} Co^{2+}, and H^+, in this study); v_i is the amount of metal bound to particulate humic acid. The toxicity coefficients relate the toxicity of the individual metal to its amount bound metals to humic acid. The value of v_i for each metal is calculated by WHAM. When performing solution speciation calculations, particulate humic acid (HA) was included as solution component at a concentration of 5×10^{-5} g/L, which is sufficiently low and has no impact on metal speciation in solution (Iwasaki et al., 2013). The WHAM-F_{TOX} model does not depend on the absolute values of v_i, but is using their relative

values. Thus, any given concentration of particulate humic acid can be used as long as it does not significantly affect specication calculation (Stockdale et al., 2010).

A threshold model was used to link the toxic response (i.e. survival rate of *E. crypticus*) to F_{TOX}. When F_{TOX} is less than a lower threshold, $F_{TOX\text{-}LT}$, no toxic effect will be observed and the survival rate is 100%. When F_{TOX} exceeds an upper threshold, $F_{TOX\text{-}UT}$, maximum toxic response will occur and the survival rate is 0%. In between, it is assumed that the survival rate changes linearly with F_{TOX}.

$$\text{Survival rate (\%)} = \begin{cases} 100\%, & F_{TOX} \leq F_{TOX-LT} \\ \frac{F_{TOX}-F_{TOX-LT}}{F_{TOX-UT}-F_{TOX-LT}} \times 100\%, & F_{TOX-LT} < F_{TOX} < F_{TOX-UT} \\ 0\%, & F_{TOX} \geq F_{TOX-UT} \end{cases} \quad (6.2)$$

By substituting, Equation 6.1 into Equation 6.2, the model parameters to be estimated are α_i, $F_{TOX\text{-}LT}$ and $F_{TOX\text{-}UT}$. When applying WHAM-F_{TOX} model to each date set at different exposure times, these parameters were estimated by minimizing the sum of the squared differences between the observed and calculated survival rates using the SOLVER function in Excel 2007. In practice the value of α_H for all mixture combinations was set to 1, since the toxicity coefficients are only relative numbers.

To generalize the WHAM-F_{TOX} model within a regulatory framework, its applicability for different species and metals with different exposure times needs to be investigated. Therefore, two toxicity data sets reported in the literature were collected. Data on the toxicity of binary mixtures of Pb and Hg to *Lemna minor* after 7 days exposure were compiled from hydroponic experiments (Dirilgen, 2011). Raw data on the toxicity of Cu and Ni mixtures to *Spirodela polyrrhiza* after 14 days exposure were collected from Montvydienė and Marčiulionienė (2007).

6.3 Results

Modelling metal accumulation

Figure 6.1 shows the relationships between metal binding to humic acid as calculated by WHAM and the body concentrations of Ni and Co measured in *E. crypticus* sampled after different exposure times (4, 7, 10 and 14 d). Generally, the calculated metal binding levels correlated well with measured body concentrations regardless of exposure time. The model performance slightly improved from acute (4 d) to chronic (14 d) exposure, with R^2 values increasing from 0.79 at 4 d to 0.93 at 14 d. For Ni, the calculated amounts of metal binding to humic acid were lower than the measured body metal concentrations in enchytraeids. At relatively low Co concentrations, the estimated humic acid bound concentrations were higher than the measured concentrations in *E. crypticus*.

Time-varying toxicity of individual metals

The single toxicity of Ni and Co to *E. crypticus* after different exposure times (4, 7, 10 and 14 d) is shown in Figure 6.2. Generally, Ni was more toxic than Co to *E. crypticus*, with the LC50 values of Co being >6 times higher than the ones for Ni. With the increase of exposure time, toxicity of both single metals increased. LC50{Ni^{2+}} decreased significantly from 78.3 μM at 4 d to 40.4 μM at 14 d (p<0.05), while LC50{Co^{2+}} did not decrease

significantly with time (p=0.08). LC50{Co^{2+}} decreased from 511 µM at 4 d to 401 µM at 10 d, afterwards equilibrium was almost reached with a 14 d LC50{Co^{2+}} of 393 µM.

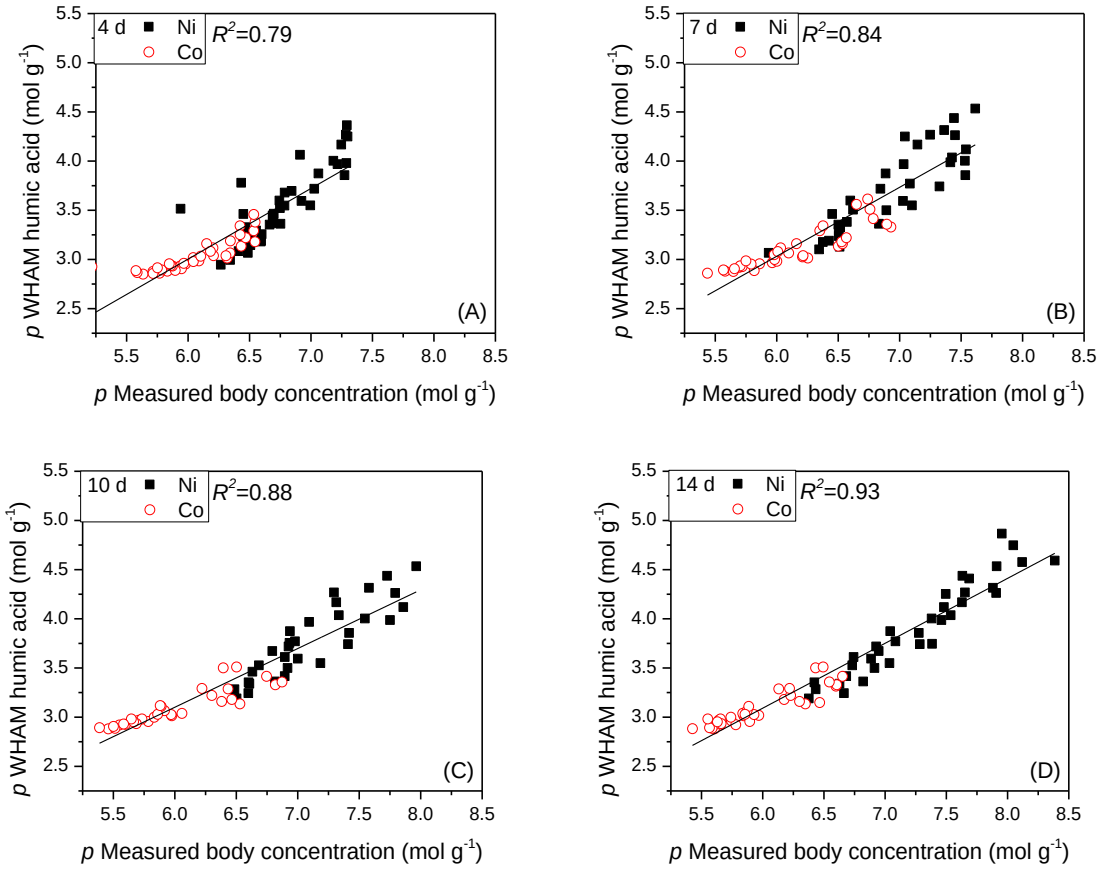

Figure 6.1 Comparison of the concentrations of Ni and Co bound to humic acid calculated using WHAM and the measured body concentrations of Ni and Co in *Enchytraeus crypticus* after different exposure times (4, 7, 10 and 14 d) in solutions embedded in an inert sand matrix. The solid lines represent the linear regression.

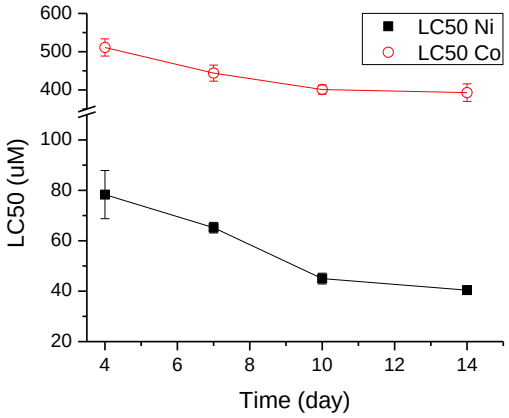

Figure 6.2 Media lethal concentration (LC50) with 95% confidential intervals based on free ion activities after different exposure times for the toxicity of single Ni and Co to *Enchytraeus crypticus* exposed in solutions embedded in quartz sand.

Fitting of toxicity data using WHAM-F_{TOX}

The parameters F_{TOX-LT}, F_{TOX-UT}, α_{Ni} and α_{Co} of the WHAM-F_{TOX} model were estimated separately for each data set at different exposure times (4, 7, 10 and 14 d) using Equations 6.1 and 6.2 and are shown in Table 6.1. The resulting values of F_{TOX-LT} and F_{TOX-UT} were almost constant with the variation of exposure time, and ranged from 3.73 to 3.92 and from 4.32 to 4.51, respectively. The estimated values of α_i for Ni and Co at different exposure time were obviously greater than zero, α_{Ni} was appreciably higher than α_{Co}, suggesting that both Ni and Co contribute significantly to the overall toxic effects. With the increase of time, α_{Ni} increased significantly from 2.56 at 4 d to 3.50 at 10 d ($p < 0.05$), and almost reached equilibrium after 14 d exposure. However, the value of α_{Co} remained approximately constant at different exposure times with values ranging from 1.85 after 4 d to 1.95 after 14 d.

In Figure 6.3 the observed survival rate of *E. crypticus* is plotted against the calculated F_{TOX} using the parameters estimated at different exposure times. Generally, the WHAM-F_{TOX} model well predicted time-varying mixture toxicity of Ni and Co, with 73% to 89% variation explained. The model fit was improved from 4 d (R^2=0.73) to 7 d (R^2=0.89), and reached equilibrium afterwards at 10 and 14 d. Toxicity data points of single Ni at 4 d slightly deviated from the fitted line, but this was not observed upon longer exposure. The survival predicted with the WHAM-F_{TOX} model at different exposure times is plotted against the observed values (Figure S6.1). The predicted survival correlated very well with the observed one, with R^2 value of 0.83.

Application of the WHAM-F_{TOX} approach to Pb-Hg and Cu-Ni toxicity data

The 7 d toxicity data of Pb and Hg to *L. minor* (Dirilgen, 2011) and 14 d toxicity data of Cu and Ni to *S. polyrrhiza* (Montvydienė and Marčiulionienė, 2007) were tested. The relationships between the observed relative growth rates of *L. minor* and *S. polyrrhiza* and the calculated F_{TOX} are shown in Figures S6.2. For *L. minor*, the estimated toxicity coefficient α_{Pb} (2.11) was greater than α_{Hg} (1.40). The obtained values of α_{Cu} and α_{Ni} for the toxicity of Cu and Ni to *S. polyrrhiza* were 4.73 and 5.60, respectively. For both toxicity tests with different exposure times (7 and 14 d), the observed values were in good agreement with the predicted values, with R^2 of 0.88 and 0.90, respectively.

Table 6.1 Results of fitting the WHAM-F_{TOX} model (Equations 6.1 and 6.2) to data on the toxicity of Ni and Co to *Enchytraeus crypticus* after different exposure times (4, 7, 10 and 14 d) in solutions embedded in an inert sand matrix. See text for an explanation of the different parameters.

Time	F_{TOX-LT}	F_{TOX-UT}	α_{Ni}	α_{Co}	R^2
4 d	3.73	4.51	2.56	1.85	0.73
7 d	3.92	4.32	3.09	1.91	0.89
10 d	3.88	4.36	3.50	1.91	0.85
14 d	3.86	4.38	3.57	1.95	0.84

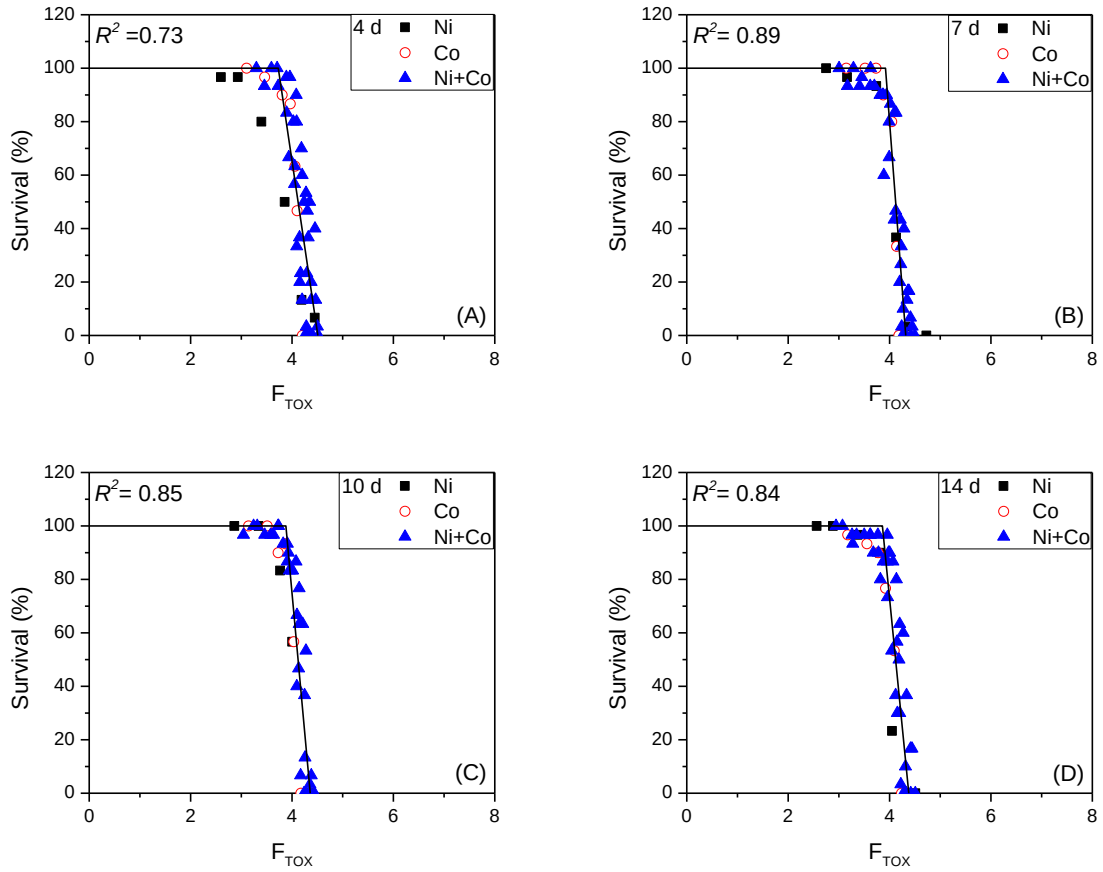

Figure 6.3 Toxicity of Ni, Co and their mixtures to the survival of *Enchytraeus crypticus* after different exposure times (4, 7, 10 and 14 d) in solutions embedded in an inert sand matrix. The solid lines represent the fit with WHAM-F_{TOX}.

6.4 Discussion

Modelling uptake

In the WHAM-F_{TOX} model, the existence of non-specific binding sites is assumed because of the presence of weak acid groups in different biomolecules, e.g. proteins, polysaccharides, lipids, nucleic acid and fatty acid (Tipping and Lofts, 2013). In the present study, the measured body concentrations of Ni and Co in *E. crypticus* after different exposure times were well described by the calculated amounts of these metals binding to humic acid, with 79% to 93% of the variations explained. This suggests that humic acid can be a surrogate for the binding surface of *E. crypticus*, and the accumulation of metals by organisms seems to follow the same mechanisms as metal binding to humic acid, that is by competitive interactions of toxic and non-toxic metals and protons for the binding to weakly acidic sites (Stockdale et al., 2010).

WHAM, however, slightly underestimated the accumulation of Ni in *E. crypticus* while the body concentrations of Co were overestimated by the model at relatively low Co exposure concentrations. Over\under estimation of metal accumulation by organisms using WHAM was also observed by Tipping et al. (2008) and Stockdale et al. (2010). The amount of HA-bound metals generally exceeded the measured concentrations of Al, Ni, Cu, Zn, Cd and Pb

in aquatic insects (*Rhithrogena*, *Leuctra*, *Nemoridae*). However, the model severely underestimated the body concentrations of K, Al, Mn, Fe and Co in stream bryophytes. The different physiology and membrane structure of different species may explain the large differences in metal uptake and the predictability from cation binding to humic acid. The predictions of WHAM just follow the chemistry of the exposure solutions. However, metal accumulation is not simply controlled by the chemical reaction; organisms themselves have the ability of regulating metal accumulation either by limiting metal uptake and in that way regulating body concentration or by involving organism-specific accumulation strategies with active excretion and\or storage in an inert form to minimize the damage of metals (Vijver et al., 2004). Subsequently, caution is warranted when assuming that metal body concentration is simply controlled by quasi-equilibrium chemical reactions (Tipping and Lofts, 2013). The use of humic acid as a surrogate of metal binding sites on organisms may over-simplify the relationship between toxic responses and organism-accumulated cation levels (Tipping and Lofts, 2015). In the present study, the linear relationships between the calculated and measured body concentrations gradually improved with time, suggesting that a certain time is needed for metal ions to react with binding sites and to reach equilibrium.

Modelling toxicity

In the WHAM-F_{TOX} model, the toxicity coefficient α_i represents the toxic strength, so the greater the value of α_i the greater the toxicity (Stockdale et al., 2010). In the present study, the estimated α_{Ni} was greater than α_{Co}, indicating that Ni was more toxic to *E. crypticus* than Co. This was also confirmed by our finding that the LC50s for Co were approximately 6 to 10 times higher than those for Ni across the different exposure times. Griffitt et al. (2008) reported that the LC50 of Co is higher than that for Ni toxicity to *Daphnia pulex* and *Ceriodaphnia dubia*, with 7 and 5 folds difference, respectively, which is consistent with our finding.

The parameters of WHAM-F_{TOX} model were estimated separately for the different exposure times to optimize the fitting. It appeared that α_{Ni} increased with exposure time and almost reached steady state after 14 d. It has been well recognized that toxic effects of metals are dependent on both intensity (dose/concentration) and duration of exposure (Newman and McCloskey, 1996). This phenomenon was also observed in our study where single toxicity of Ni to *E. crypticus* increased significantly with time. F_{TOX}, which quantifies the toxicity of the metals, should be correlated with time. F_{TOX} is determined by the toxic coefficient (α_i) and the amount of metals bound to the organism (v_i). As v_i is calculated based on the water chemistry of the test solutions, it is supposed to be time-independent as long as the organism does not affect its immediate environment. Hence, α_i should be time-dependent. When exposing *Folsomia candida* and *E. crypticus* to Ni contaminated soil and sand-solution system, respectively, the toxicity of Ni increased with time until steady state was reached (Broerse and Van Gestel, 2010; He and Van Gestel, 2013). This may explain why α_{Ni} increased with time and leveled off after a certain period of exposure. However, the estimated α_{Co} was almost constant with time. In the present study, LC50{Co^{2+}} slightly but not significantly decreased with time. This suggests unlike Ni, which exerted effects gradually and reached equilibration of toxicity after a certain time, Co induced acute toxicity. Spurgeon and Hopkin (1999) investigated the accumulation and excretion patterns of metals in the

earthworm *Eisenia fetida*. For Cd and Pb, equilibrium was not reached during 21 d uptake period due to slow excretion rate, and body concentration was concentration- and time-dependent. However, for Cu and Zn, body concentrations rapidly reached equilibrium and were mainly determined by exposure concentration. So we could conclude that Ni and Co have different accumulation and excretion patterns in *E. crypticus*. For Co, the time needed to reach steady state of body concentration was much shorter than for Ni, meaning that the toxicity of Ni was more time-dependent.

Generally, the WHAM-F_{TOX} model well predicted the dynamic toxicity of Ni and Co to *E. crypticus* using the parameters estimated at different exposure times. By applying the WHAM-F_{TOX} model, interactions of mixture components at the exposure and uptake level were included, while internal interactions cannot be ruled out. This may explain why still some of the variation in toxicity remained unexplained. Furthermore, WHAM-F_{TOX} approach assumes that interactions occur through cation competition and strictly additive toxicity when exposure is expressed in terms of accumulated or bound metal (Tipping and Lofts, 2015). In reality, other mechanisms such as electrostatic interactions may also affect the observed mixture toxicity (Wang et al., 2013). Ni^{2+} and Co^{2+} both have two valence electrons and their molecular weights are similar, suggesting they may share same ion channel or bind to the same transport or uptake sites (Niyogi and Wood, 2004). Therefore the assumption of cation competition is most likely to be valid. For combinations of other ions, such as Cu^{2+} and Ag^{+}, this assumption may not hold. Le et al. (2013) developed a multi-metal BLM, which is based on cation competition, for predicting Cu^{2+} and Ag^{+} toxicity to lettuce; with this model only 64% of the variation in toxicity of the mixture was explained. This may be attributed to Cu^{2+} and Ag^{+} binding to different transporters, which means that in addition to cation competition, other interaction mechanisms may also play a role in determining the mixture toxicity of Cu^{2+} and Ag^{+}. To generalize WHAM-F_{TOX} model within a regulatory framework, it has to be investigated whether this model can be used for other species and other mixture combinations. Two data sets were selected because of the availability of raw data and because they include different metal combinations, species, and exposure times. WHAM-F_{TOX} provided good predictions for the toxicity of Hg-Pb and Cu-Ni mixtures to two plant species ($R^2 \geqslant 0.88$), showing its suitability to predicting the acute and chronic toxicity of single metals and mixtures.

Our study showed that WHAM-F_{TOX} is an alternative option for delineating uptake and toxicity of metal mixtures in organisms. It complies with the basic assumption of BLM (i.e. cation competition), while requiring fewer parameters (Tipping and Lofts, 2013). More importantly, WHAM-F_{TOX} differs from the BLM in including an array of non-specific binding sites for cations, and a data base of metals binding to these sites is already available in WHAM. It should be noted that the WHAM-F_{TOX} approach gives best predictions when applied to steady state situations. As found in the present study, the estimated α_i varied with exposure time. Cautions should therefore be taken when applying the parameters of WHAM-F_{TOX} model estimated from an acute toxicity experiment for predicting the chronic toxicity of metal mixtures. In the future, other possible interaction mechanisms should be explored and incorporated into the developed models for evaluating mixture metal toxicity in dynamic situations.

6.5 Conclusions

The WHAM-F_{TOX} well predicted individual and mixture toxicity of Ni and Co to *E. crypticus* at different exposure times. External validation using toxicity data from literatures showed the applicability of the model for other metal mixtures and plants. Generally, the WHAM-F_{TOX} model, which is based on chemical speciation concepts and includes the competition effects, is able to adequately delineate the mixture toxicity data of different species (aquatic and terrestrial organisms), metals and exposure durations (acute and chronic test).

Supplemental information

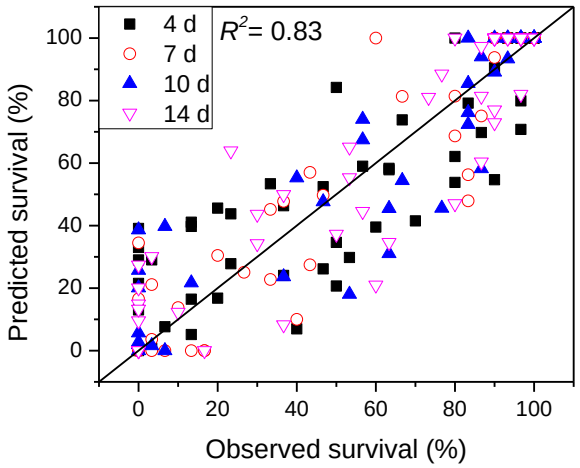

Figure S6.1 Relationship between the WHAM-F_{TOX} predicted and observed survival of *Enchytraeus crypticus* after different exposure times (4, 7, 10 and 14 d) in solutions embedded in an inert sand matrix. The solid line represents the 1:1 line.

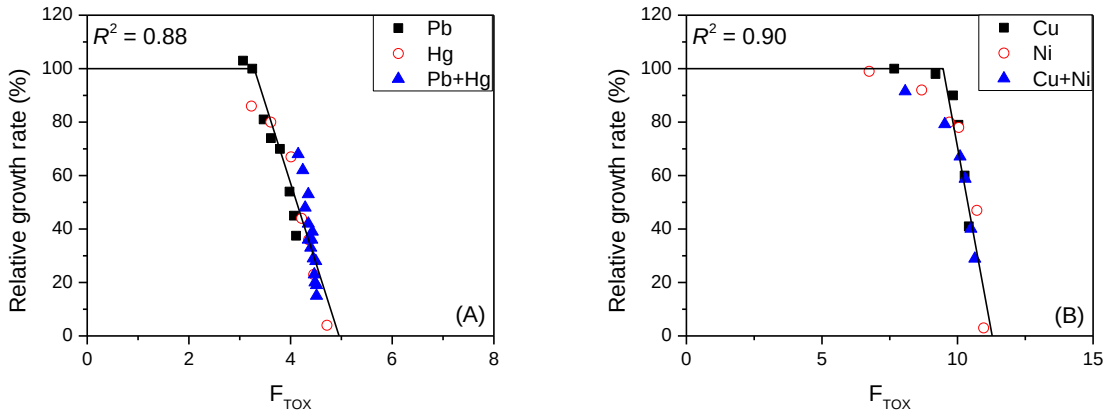

Figure S6.2 Toxicity of Pb, Hg and their mixtures to the growth of *Lemna minor* (Dirilgen, 2011) after 7 d and toxicity of Cu, Ni and their mixtures to the growth of *Spirodela polyrrhiza* after 14 d (Montvydienė and Marčiulionienė, 2007) fitted with WHAM-F_{TOX} model. The solid lines represent the best model fit.

Chapter 7

General discussion

Accurate environmental risk assessment of metals for aquatic and terrestrial organisms is hampered by a lack of knowledge on the mechanisms underlying their bioavailability and toxicity. A comprehensive understanding of the environmental or biological factors affecting bioavailability and toxicity of metals is needed to bridge the gap between metal exposure and the resulting adverse effects.

This thesis aims at building a unified framework that is generally applicable for predicting metal uptake and toxicity, and their dynamic changes. The biological response of a soil-living reference species, the potworm *Enchytraeus crypticus*, was investigated when exposed to metals and their mixtures (single Ni, single Co, and Ni-Co mixtures). Due attention has been paid to exposure time and chemical compositions of the media (e.g. variation in levels of Ca, Mg, Na, K and pH). By accounting for these modifying factors, mechanistically underpinned models, a biotic ligand model (BLM) based model and WHAM-F_{TOX} have been successfully developed to estimate bioaccumulation and toxicity of metals under different conditions.

Referring to the research questions listed in the Introduction Chapter (section 1.8), the discussion below aims to integrate the results presented in the five research **chapters (2-6)** and aims to provide answers to these questions.

Research question 1: Uptake and toxicity of Ni in *E. crypticus* exposed to a solution-sand system were time-dependent. At each exposure concentration (ranging from 0.053 to 0.94 mg/L), body Ni concentrations in the animals increased with increasing exposure time and reached a steady state after approximately 14 d. LC50 values of for the toxicity of Ni expressed as dissolved concentrations in solution decreased with increasing exposure time. However, when expressed as body concentrations, LC50s of Ni remained constant (~16.7 mg/kg dry body wt) and were independent of exposure time. Our findings therefore show that body concentration is a more reliable indicator of Ni toxicity in a dynamic environment. A one-compartment model was further used to quantify Ni uptake and elimination kinetics. The elimination rates estimated from body concentration and from survival data were different, suggesting that not all internal Ni contribute to toxicity.

Research question 2: Ca^{2+}, Mg^{2+}, and Na^{+} but no other cations (K^{+} and H^{+}) exerted a significant role in inhibiting Ni uptake and alleviating Ni toxicity to *E. crypticus*. An extended Langmuir model, which complies with the basic assumption of the BLM concept (i.e., cation competition), described Ni uptake well. A newly developed BLM in which the protective effects of Ca^{2+}, Mg^{2+}, and Na^{+} were incorporated, provided good predictions of Ni toxicity with the predicted LC50{Ni^{2+}} never differing by more than a factor of 2 from the observed values. This illustrates the applicability of the BLM concept for estimating Ni uptake and toxicity not only for aquatic, but also for terrestrial organisms. It is interesting to note that the derived binding constants of Ni^{2+}, Mg^{2+}, and Na^{+}, showing their affinity for the uptake and toxic action sites on the animals, were found to be similar, while for Ca^{2+} they differed. This suggests that for Ca^{2+} other mechanisms (e.g., regulating membrane permeability) than competition are involved to affect Ni toxicity.

Research question 3: Since Ca^{2+}, Mg^{2+}, and Na^{+} were identified as competing cations that have significant effects on Ni uptake and toxicity (Chapter 3), they were expected to be incorporated into the anticipated generic BLM. We further investigated the effects of these cations on Ni toxicity at three different exposure times (7, 10, and 14 d). To extend the static

BLM for predicting Ni toxicity to *E. crypticus* in the course of time, three different hypotheses were proposed: 1) the BLM parameters are independent of time; 2) the binding constants are fixed but the f_{50} value (indicating the fraction of biotic ligand sites occupied at which 50% effect occurs) varies with time; 3) all the BLM parameters are time-dependent. The derived BLM model (a) based on acute toxicity data, failed to predict the chronic toxicity of Ni, falsifying the first hypothesis. By adjusting f_{50} values via a toxicokinetic equation, model (b) successfully described dynamic Ni toxicity, supporting the second hypothesis. According to the third hypothesis, three individual BLMs were developed at 7, 10, and 14 d following the standard BLM parameter estimation procedure. The f_{50} was almost constant and independent of exposure time, however, the binding constants of Ni and the competing cations (Ca^{2+}, Mg^{2+}, Na^{+}) decreased with exposure time. A model (c) was developed by incorporating the time-dependent factors and showed a similar level of precision in predicting the dynamic toxicity of Ni. Apparently, some BLM parameters need to be adjusted to take toxicokinetics and toxicodynamics into account in estimating Ni toxicity in a dynamic environment.

Research question 4: The body concentration of Ni was significantly reduced in the presence of Co, whereas Ni only slightly affected Co uptake. Toxicological interactions between Ni and Co were mainly antagonistic when using free ion activity as the expression of exposure. The interaction pattern shifted from pure antagonism at 4 d to dose-ratio dependent antagonism at 7 and 10 d, and then to dose-level dependent antagonism at 14 d. However, no interaction was found when based on body concentrations at different exposure times. This shows that the use of body concentration, which incorporates bioaccumulation processes, may remove at least some interactions (if not all because internal interactions may still be present) and act as a more constant indicator of metal mixture toxicity. In our study, interactions between metals and environmental compartments were already considered in the speciation calculations using the WHAM VI model. The observed antagonism was most probably due to competitive binding of Ni and Co for active sites and subsequent inhibition of uptake. It therefore was concluded that mixture interactions occurred at the uptake level, but not at the target level.

Research question 5: Dynamic uptake and toxicity of Ni-Co mixtures in *E. crypticus* were modeled with the WHAM-F_{TOX} approach. There were good matches between the observed body concentrations of Ni and Co in the enchytraeids and the WHAM-calculated amounts of these metals binding to humic acid, supporting the use of humic acid as a surrogate for the metal-binding sites of *E. crypticus*. The WHAM-F_{TOX} approach, postulating that competitive chemical reactions at one or more biotic ligands of the organism can be represented by the competitive binding to particulate humic acid, well predicted mixture toxicity in time course. The derived α_{Ni} increased with time and reached equilibrium after approximately 14 d exposure, while α_{Co} remained almost independent of time. This suggests a difference in toxicokinetics and toxicodynamics between Ni and Co. It should be noted that the WHAM-F_{TOX} approach would give best predictions when applied to steady state situations. External validation using toxicity data from the literature showed the applicability of the model for other metal mixtures and other species. Generally, the WHAM-F_{TOX} model, which is based on chemical speciation concepts and includes the competition effects, is able

to adequately delineate mixture toxicity data for different species, different metals and different exposure durations.

In summary, findings in this PhD thesis open the perspective of incorporating toxicokinetics and toxicodynamics into bioavailability models for effectively delineating metal (mixtures) effects.

7.1 Development of a generic model framework for accurate risk assessment of metals

Generalization of BLM concept

In this thesis, the BLM-based Langmuir model and a BLM were successfully expanded to describe Ni uptake and toxicity in a representative soil species (*E. crypticus*), exposed in media with varying solution chemistry (**Chapter 3**). This provides direct evidence in favor of the applicability of the BLM concept to soil animals. We combined uptake and toxicity by means of biotic ligand theory involving cation competition, which enabled us to gain insights into different processes determining metal uptake and toxicity. The present research therefore improved our understanding with regard to the explanation and interpretation of the variation in the toxicity of metals in different exposure media.

The BLM was originally developed for fish and the sodium or calcium channel proteins in the gill surface that regulate the ionic composition of the blood were assumed as the sites of toxic action (Di Toro et al., 2001). This theoretical basis of the BLM invokes a site-specific competition between metal ions and protective cations for binding to the sites of toxic action. This means that toxicity is caused by the metal ions bound to the active sites and that alleviation of the toxicity is caused by the protective cations bound to the same active sites (see Figure 7.1). For fish, the amount of metal bound to the active sites (i.e., gill) can be directly determined (Meyer et al., 1999). The accumulation of Cu on the gill of fathead minnows was reduced in the presence of Ca^{2+} and H^{+} and metal-complexing ligands (Playle et al., 1992). The amount of metal bound to fish gills was shown to be a constant predictor of metal toxicity (Meyer et al., 1999). These findings provide direct evidence to support the statements that the fish gill serves as the site of toxic action and that competitive interactions for binding sites do exist between major cations and metal ions. For other species, the direct measurement of the sites of toxic action is still not possible. Therefore, a hypothetical biotic ligand is used to facilitate the applicability of the BLM concept to other aquatic organisms (Figure 7.1). Assuming the similarity of mechanisms of toxicity between aquatic and terrestrial organisms, the development of BLMs for soil organisms is feasible by hypothesizing that a biotic ligand exists and that mortality can be modeled in a similar way (Van Gestel and Koolhaas, 2004; Steenbergen et al., 2005; Thakali et al., 2006 a,b). Our study with *E. crypticus* provided an experimental validation of this statement.

Many empirical models have been developed to relate metal toxicity to soil properties, such as pH, organic matter content, and cation exchange capacity (CEC) (e.g., Rooney et al., 2007; Smolders et al. 2009). The predictive ability of these models seems to be reasonably well. However, the mechanisms underlying such empirical relationships are not fully understood. In real soil, it is difficult to manipulate soil porewater properties. Furthermore, many soil properties are more or less correlated with each other (Wang et al., 2010), making it difficult to distinguish the real source of an effect from one or more interdependent factors.

For the purpose of mechanistic studies, a solution-only system was therefore used in the present research as it facilitates univariately control over the solution composition. In the present study we found that the effect of Mg^{2+} and Na^{+} on Ni toxicity is mainly through competition with Ni^{2+} on the surface of the organism and inhibiting the uptake of Ni^{2+}. However, the effect of Ca^{2+} cannot simply be explained by competition, suggesting different interactions at both the uptake level and the level of internal processing. These interactions are also expected to be of importance upon soil exposures, but given the complexity of the soil matrix, they will be very hard to show in soil tests. We are the opinion that by revealing these interactions, our results can have a large value for environmental risk assessment purposes.

A fundamental assumption of the BLM is that toxicity is driven by exposure to the dissolved metal alone, rather than by a combination of toxic effects from dermal and dietary exposures (Niyogi and Wood, 2004). Unlike aquatic organisms in water, soil organism could take up metals from both pore water and soil particles, invalidating a strict application of an aquatic BLM and complicating the development of a terrestrial BLM. Some authors have reported that diffusion of metal ions from soil pore water through the skin is the major exposure route for several soil organisms, while the uptake via ingestion contributes little to metal accumulation (Vijver et al., 2003; Saxe et al., 2001). Hence, the BLM concept may be assumed to hold for soft-body soil invertebrates (e.g., earthworms, potworms) as they are in close contact with soil pore water and their exposure is predominantly via the dermal route. For organisms exposed through both pathways a more complex approach is necessary.

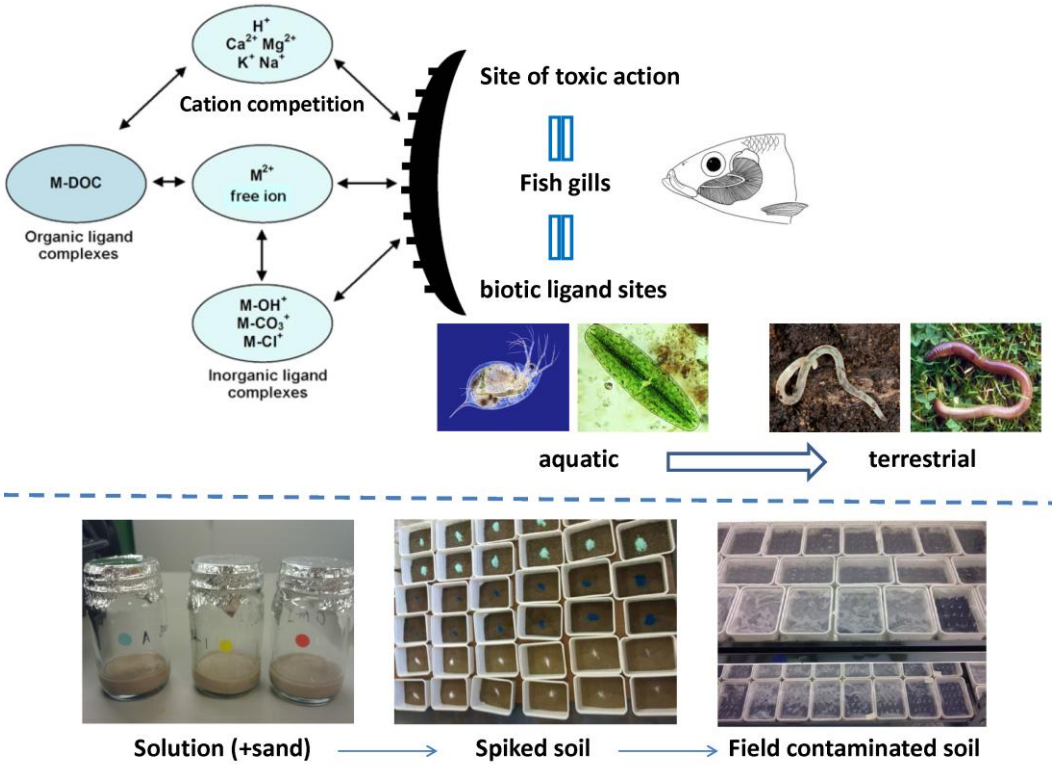

Figure 7.1 Schematic overview of the (anticipated) application of the BLM concept from aquatic to terrestrial species and from solution (sand) systems to contaminated soils in field settings.

To our knowledge, experimental validation of the applicability of the BLM theory to metal toxicity in environmentally contaminated soils (not metal-spiked soils) has rarely been done. Two steps are needed to go from the BLM developed in a solution (sand) system to real soils (Figure 7.1). A first step is to extend the toxic effects observed in solutions to a soil environment (spiked soil). Assuming that soil porewater is the main route of exposure and that the metal transport into the organism is not limited by diffusional control, the extrapolation of BLMs developed in solution to soil is then possible. The free ion activities of the metal and the competing cations in soil porewater can be calculated with a speciation model (e.g. WHAM VI), with the properties of the porewater as inputs. The BLM obtained in solution can be validated qualitatively by comparing whether competing cations are the same for the target metal and can be validated quantitatively by comparing whether the binding constants of cations and metal are the same when using soil exposure. The second step is to extend the soil toxicity effects in laboratory spiked soils to field contaminated soil, in which the effects of metal aging/leaching on metal bioavailability and toxicity should be considered. These effects can be conservatively estimated by introducing a bioavailability correction factor (Smolders et al., 2009). By doing so, the mechanistic information obtained in this study will be truly meaningful and the models obtained can be extrapolated to natural soils.

Incorporation of temporal effects

An important issue regarding the development of bioavailability models that has been raised concerns the effects of time. Most of the current toxicity data has been generated using standardized guidelines which tend to focus on short-term integral adverse effects determined at fixed exposure times. The predictive ability of the currently used toxicity models for metals with long-term mixture effects, that trigger adverse effects through various different toxicity pathways, remains unresolved (Altenburger et al., 2012). As found in our study, accumulation and toxicity of metals (Ni, Co) are both concentration- and time-dependent (**Chapter 2 and 5**). The Ni-BLM developed at 7 d could not be used to estimate Ni toxicity after 10 d or 14 d exposure (model (a) in **Chapter 4**). De Schamphelaere and Janssen (2004b) reported that the acute BLM for Cu toxicity to *Daphnia magna* could not serve as a reliable basis for predicting chronic Cu toxicity, which is consistent with our findings. These results stress the importance of incorporating toxicokinetics and toxicodynamics in developing models for assessing the toxicity of metals and metal mixtures.

Our study showed that the standard one-compartment bioaccumulation model well predicted Ni body concentrations in *Enchytraeus crypticus* based on a set of first order uptake and elimination rate constants (**Chapter 2**). For measuring bioavailability, one important way is to follow the changes in body concentration of metals with time as well as their uptake rates (Ardestani et al., 2014b). A current limitation of bioaccumulation models is that metal speciation and competitive binding are not considered (Veltman et al., 2010). Attempts were made to incorporate the aspects of toxicokinetics and toxicodynamics into the state-of-the-art modeling approach (i.e., BLM) on the basis of experiments of dynamic Ni toxicity tests applying different exposure scenarios (**Chapter 4**).

In the conventional BLM, several assumptions are required, including that no significant modification of the plasma membrane occurs, and no significant biological regulation is induced by metals binding to sensitive sites (Hässler et al., 2004). In order to increase the

likelihood of these assumptions, previously the development of BLMs was mainly based on short-term toxicity tests. Regardless of the progress made in estimating acute toxicity, chronic toxicity should be the regulatory criteria for defining environmental quality criteria. For instance, chronic toxicity data has been used as the basis for environmental risk assessment in the European Union (Villavicencio et al., 2011).

The BLM in its original form ignores time-dependent toxicity because the binding constants of the metal-BL interaction can only be derived under equilibrium condition (Hatano and Shoji, 2010), which might not be reached in the short-term toxicity tests. According to the BLM concept, the binding constants are assumed to be metal- and species-specific and time invariant (Niyogi and Wood, 2004). Indeed, current efforts on the development of a dynamic BLM are also based on the assumption that the affinity constants are fixed values and that f_{50} should be time-dependent (Chen and Liao, 2010; Hatano and Shoji, 2010). The successful development of model (b) in Chapter 4 seemingly confirmed the above assumption. On the other hand, many researchers have found that these assumptions may not be true in long-term toxicity tests (Heijerick et al., 2002; 2005). The binding constants of cations in chronic toxicity tests were always lower than those from acute toxicity tests (see model (c) in **Chapter 4**). The presence of major cations (e,g. Ca^{2+}) can affect membrane permeability and increase the stability of membrane proteins under chronic exposure (Taylor et al., 2000; Niyogi and Wood, 2003). As a consequence, the mechanisms of acute and chronic toxicity of metals like Ni were found to be different (Pane et al., 2003). These reasons may explain why binding constants are not fixed with time like normal chemical parameters, but prone to variation in time as biological factors do affect the constants as well. This probably also explains why both model (b) and model (c) provided similar results in terms of predictive capability, even though they are based on different assumptions (**Chapter 4**). We therefore have two options to predict dynamic Ni toxicity at varying solution composition by incorporating the time-dependent model parameters: allow variation of binding constant or variation of toxicity thresholds. To support model selection, we still need more knowledge on the underlying mechanisms that accounts for different binding constants and\or *f*50 values in acute and chronic BLMs. Even so, our study nevertheless sheds a new light on the importance of time as a modulating factor for toxicity.

In **Chapter 6**, the WHAM-F_{TOX} approach offered a new option to predict dynamic toxicity of metal mixtures. With this model, it is hypothesized that the competition of metal with cations for binding to biotic ligand sites can be represented by competitive binding to the functional groups of natural organic matter (humic acid), being a proxy of non-specific biotic ligand sites (Iwasaki et al. 2013). The derived toxicity coefficient α_{Ni} increased with exposure time and reached equilibrium after approximately 14 d exposure, while α_{Co} remained almost independent of time. The WHAM-Ftox approach would give best predictions when applied to steady state situations. This is because the kinetics of the complexation of metals by humic acid can drastically affect the interpretation of toxicity tests when insufficient equilibration time is allowed (Ma et al., 1999). The application of a chemical speciation model (e.g., WHAM) for calculating free metal ion activity should take into account the kinetics of the reactions of metals with DOM. Care should therefore be taken when extrapolating the parameters of WHAM-F_{TOX} from acute toxicity data to chronic toxicity data.

Insights into mechanisms of mixture interactions

Mixture interactions have been evaluated in several studies using the concentration addition (CA) and independent action (IA) as the reference models (Norwood et al., 2003; Fortunati et al., 2005). The choice of a reference model for prediction of metal mixture toxicity is dependent on the similarity/dissimilarity of the mixture components, which is often unknown for mixtures in the environment (Backhaus et al., 2000). In this thesis, CA was chosen as the reference model for predicting Ni-Co mixture toxicity (**Chapter 5**), as it generally predicts a higher response compared to the IA model, and therefore is more conservative (Altenburger et al., 2000). Deviations from CA suggested that Ni-Co interactions did occur. Previously, interpretation of the interaction observed was usually limited to overall synergism or antagonism. Here, the MIXTOX model was used to determine specific dose level- and dose ratio-dependent interaction patterns. The joint effect of metal mixtures is the result of interactions at various levels, inside and outside the organism. The contribution of each interaction level could be assessed by expressing exposure in different ways (e.g. total and extractable soil metal concentration, porewater concentration, unidirectional fluxes, body metal concentration) (Calamari and Alabaster, 1980; Posthuma et al., 1997; Komjarova et al., 2009). In our study, interaction patterns of Ni and Co were determined and quantified using two different expressions of exposure: external concentration (free ion activity in solution) and body metal concentration, respectively. Antagonistic interaction (shifting from pure antagonism to dose-level dependent antagonism with time) was observed when judged by the external concentration, while concentration addition was the dominant interaction when based on the body metal concentration (**Chapter 5**). This difference indicated that the interaction mainly happened during uptake (toxicokinetics). As metal coordination sites are never entirely specific for a single metal, the antagonistic interaction between Ni and Co was most likely caused by competing for binding to the membrane transport sites or intracellular metabolic binding sites as they have similar ionic radii and coordination geometry (Sunda and Huntsman, 1998). Toxicity is based on the effect that a metal produces at the target sites in an organism (Norwood et al., 2013). Measurement of the metal at the target site is hardly possible. Instead, body metal concentration is used as a surrogate measure in predictive models. Therefore, we do not pretend that our analysis has removed all interactions, because internal interactions may still be present but cannot be identified by the MIXTOX model. This is supported by the fact that the CA model using body metal concentrations explained 50-58% of the variation in toxicity of Ni-Co mixtures at different exposure times. Results from this study provide mechanistic insights into the role of mixture interactions in determining uptake and toxicity, and justify the need to develop a mechanistic bioavailability based model for interpreting mixture toxicity.

The BLM has successfully been applied for interpreting toxicity of single metals to various organisms under different exposure conditions (De Schamphelaere and Janssen 2002; Lock et al. 2007; Steenbergen et al. 2005). However, only limited efforts have been directed towards extension of BLMs for mixture exposure (Jho et al. 2011; Le et al. 2013). The development of a multi-metal BLM for predicting the toxicity of Ni-Co mixtures is possible since we found that competition (antagonism) acts as a mechanism of Ni-Co interactions (Niyogi and Wood 2004). If stability binding constants of metal ions and other coexisting

cations for the biotic ligands are known, it is possible to predict mixture toxicity by combining BLMs with the CA model. However, until now BLM constants have only been determined for a limited number of metals (metal combinations) and organisms (Antunes et al. 2012; Niyogi and Wood 2004). Since the binding affinity of Co to *E. crypticus* remained unknown in previous studies, an alternative WHAM-F_{TOX} approach was therefore used in the present study to model Ni-Co toxicity (**Chapter 6**). Good correlations were found between the observed body concentration of Ni and Co and the WHAM-calculated amount of metals bound to humic acid, supporting the use of humic acid as a surrogate for the binding sites of *E. crypticus*. The toxicity of Ni-Co mixtures at different exposure times was well predicted by the WHAM-F_{TOX} model. Our study thus provides a new option for delineating mixture toxicity.

The WHAM-F_{TOX} approach assumes that interactions occur through cation competition and are strictly additive toxicity when exposure is expressed in terms of accumulated or bound metal (Tipping and Lofts, 2013). This competition assumption may not hold when mixture components bind to different transporters (Niyogi and Wood, 2004, Le et al., 2013), such as for a combination of Cu and Ag. Other interaction patterns than competition (e.g., electrostatic interactions) may dominate the mixture patterns observed (Wang et al., 2013) and synergism by definition cannot be modeled by either the extended BLM or the WHAM-F_{TOX} approach. By applying the WHAM-F_{TOX} model, interactions of mixture components at the exposure and uptake level were included, while internal interactions cannot be ruled out. This may explain why still some of the variation in toxicity remained unexplained (**Chapter 6**). It should be noted that all of the above assumptions of interaction at various levels or bindings sites only serve as examples of possible mechanisms in which interaction of the metals of interest could occur.

Research efforts are needed to gain more insight into the mechanisms of the interaction between metals by applying more advanced tools (e.g., patch clamp or Adverse Outcome Pathways (AOPs)) and to investigate the mixture toxicity of metals in the context of soil ecosystems with more complex interactions. In soil, the interactions at the environmental-chemical level should also be considered, which deal with competitive sorption interactions and result in a specific distribution of the interacting metals over the solid and liquid phase of the soil.

To sum up, any progresses regarding the above-mentioned aspects towards a generic model framework will ultimately contribute to better risk assessment of metals and metal mixtures.

7.2 Application of bioavailability models in risk assessment and regulatory practice

There is an increasing insight into the concept of bioavailability in ecotoxicology: only a fraction of the total amount of metal present in the aquatic and terrestrial environment is actually available for uptake by organisms and subsequently for inducing toxicity. However, more than two decades after its inception and numerous successful experiments, bioavailability analysis is not yet sufficiently adopted in the current framework of risk assessment. Until now, risk assessment of metals is still predominantly based on their total concentrations in the environment. A major obstruction for this is the lack of simple and

efficient models that precisely define bioavailability processes. In this thesis, the developed bioavailability models (the extended Langmuir and the BLM in **Chapter 3**, dynamic BLMs in **Chapter 4**, and the WHAM-F_{TOX} in **Chapter 6** provide a mechanistic framework to normalize Ni and Co toxicity to soil organisms in different exposure media and at different time scales. These models directly link the toxicological endpoints to porewater chemistry, showing their potential for future use in risk assessment and for deriving more appropriate environmental quality criteria.

BLM is a state-of-the-art approach to remove the influence of a wide variety of bioavailability factors in the aquatic ecotoxicity database. It was originally developed for copper in aquatic species, and the above-mentioned benefits of this model triggered the US EPA to incorporate it in the most recent water quality criteria for copper (US-EPA, 2007). The BLM approach has also been proposed for use in European Union risk assessments (Ahlf et al., 2009). Given a target metal, the general practice is to assess the effects on single species of selected test organisms under standardized conditions following OECD\ISO guidelines and to extrapolate the obtained effect concentrations to critical limits for populations and communities (Van Gestel, 2012). In the context of risk assessment, such critical limits are then compared with the measured or predicted exposure levels to determine the potential risk for the exposed ecosystems. However, the derived critical values do not consider site-specific conditions. Exceeding the critical values does not necessarily imply a risk. We therefore propose the use of bioavailability-based models because it can overcome this disadvantage by relating critical values to test-specific abiotic conditions (e.g., hardness, pH, and dissolved organic matter).

Another important issue regarding the implementation of bioavailability in risk assessment involves regulatory acceptance (Ehlers and Luthy, 2003). Legislators and regulators are prone to an operational and user-friendly approach instead of bothering with complex concepts and calculation procedures. The new Guidance for deriving Environmental Quality Standards under the European Water Framework Directive supports the use of a tiered compliance assessment regime for metals that incorporates both correction for natural background concentrations and bioavailability. However, the guidance does not provide practical tools to implement a bioavailability-based compliance regime. The BLMs for calculating water type-specific no effect concentrations represent a major improvement in risk assessment of metals in surface waters. However, regulatory use is hampered by the model complexity and data requirement. In the study of Verschoor et al. (2012), BLMs for the calculation of Ni, Cu, and Zn HC5 values (hazard concentrations for 5% of species) were simplified to linear equations with an acceptable level of accuracy, requiring a maximum of 3 measured water chemistry parameters. Based on this, a quick, user-friendly and unified chronic BLM normalization tool (PNEC.pro; http://www.pnec-pro.com/) has been developed for several metals (i.e. Cu, Ni and Zn). The tool requires the input of the following abiotic water parameters: dissolved organic carbon (DOC), pH and hardness concentration. A SOIL PNEC CALCULATOR with bioavailability correction was also developed to calculate the predicted ecological risks of metals (Cu, Ni, Zn, Pb, Cd, Mo and Co), based on their PNEC to soil organisms (http://www.arche-consulting.be/en/our-tools/soil-pnec-calculator/). This calculator allows the easy derivation of site-specific ecological soil quality standards. Actions in this respect are therefore encouraged. These user-friendly tools, when used as part of a

tiered approach, will provide water\soil managers with an opportunity to efficiently account for metal bioavailability in a transparent way and deliver a robust metric of potential environmental risk.

7.3 Outlook and recommendations

In this thesis, a sand-solution system was used for toxicity tests without the addition of dissolved organic matter. The influence of DOC on metal bioavailability and toxicity was not investigated. The potential role of DOC should be addressed in the future, as DOC concentrations in natural soil pore waters can be so high as to dominate metal speciation and toxicity. The role of DOC in modifying toxicity is supposed to be based on its capacity to form metal-DOC complexes and thus reduce free metal ion activity (i.e., alleviating metal toxicity) (Doig and Liber, 2006). On the other hand, certain fractions of the metal-DOC complex might also be available for organisms, for example by phagocytosis. In addition the formation of metal-DOC complexes may facilitate metal uptake by organisms and thus increase metal toxicity (Erickson et al., 1996; Kozlova et al., 2009). It is still unclear which statement is true as experimental evidence is lacking. We are currently performing studies on the potential effect of concentration and composition of DOC on Ni bioavailability and toxicity to *E. crypticus* using the solution-sand system. When applying the BLM approach to real soils, the general practice is to first conduct speciation calculations for soil pore water. As this step already takes into account the effect of DOC on metal speciation, extrapolation of study results obtained from our solution-sand system to real soils should be possible. For a terrestrial BLM, a speciation model (WHAM) is needed in order to account for the partitioning of the metals between the soil and the solution phase, and to determine its speciation in the solution (Thakali et al., 2006a). In WHAM, the soil organic matter (SOM) is often assumed to be composed of particulate humic acid (HA) and fulvic acid (FA) in a ratio of 84: 16 (Tipping, 1994). Hence, application of the partitioning model to soils for which a larger proportion of the SOM is not humic substances would require adjustment of the fraction of active organic matter.

As it is unrealistic to perform toxicity tests with all species, it is worthwhile to investigate whether or not the bioavailability models developed for *E. crypticus* in this study can be extrapolated to other species. This question can be addressed by qualitatively comparing trends (to see whether the toxicity-modifying factors are the same for different species) and\or quantitatively comparing stability constants. Assuming that the parameters which describe interactions between major cations and metal ions are constant for taxonomically closely related species, and that only intrinsic sensitivity varies among species (Di Toro et al., 2001; Van Sprang et al., 2009), quantitative extrapolation of BLMs would be possible. It becomes even more challenging to utilize the existing BLMs to predict toxicity for taxonomically dissimilar species with reasonable accuracy, because the mechanism of toxicity and the influence of water chemistry on metal toxicity may differ across species (De Schamphelaere and Janssen, 2004a; Schlekat et al., 2010). When a sufficient number of species-specific BLMs are available, it is suggested to combing the BLMs with Species Sensitivity Distributions (SSDs) for the purpose of cross-species extrapolation. Alternatively, rather than modeling detailed mechanisms using bioavailability models, we might pay

attention to overarching principles to which most species obey. One possible option is the use of a traits-based approach for interpreting differences in the accumulation and toxicity of metals between species.

Another issue requiring attention is the multiple effects of some cations. For example, the effects of Ca on metal toxicity may be partly attributable to effects on physiology (e.g., membrane permeability), in addition to competition with the metal. Further investigation into the mechanisms of interactions between major cations and toxic metal ions in soil organisms is clearly needed. When interpreting metal toxicity, the aspects of toxicokinetics and toxicodynamics need to be understood as well. The advent of new genomic techniques (i.e., transcriptome, proteome, metabolome) has raised the expectation that core questions on metals (mixtures) toxicology, such as for mechanisms of internal\physiological interactions, can be answered in the near future.

In this thesis, we systematically investigated the effect of solution composition, time, and mixture interactions on metal bioavailability and successfully developed several mechanistically underpinned models for predicting uptake and toxicity in a soil organism. Our findings serve the preparation for future regulatory demands and to ensure adequate environmental protection.

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Summary

Contamination of soil with metals may pose a serious threat to environmental and human health. Since bioavailability is a key factor in determining metal toxicity, the factors affecting metal bioavailability should be qualitatively and quantitatively determined. Many of these factors are related to the chemical aspects of metal speciation, and the interactions of the metal with other metals and ions present in the soil solution. Another important factor greatly affecting toxicity is time. Metal bioaccumulation and subsequent toxicity are highly determined by the exposure time, involving toxicokinetic and toxicodynamic processes.

The main goal of this thesis is therefore to take into account these modifying factors (varying soil solution chemistry, interactions of metals in mixtures, metal toxicokinetics and toxicodynamics) in developing a unified framework to predict toxicity of metals.

All experiments described in this thesis were performed with the terrestrial oligochaete worm *Enchytraeus crypticus*, using a substrate of aqueous solutions embedded in inert quartz sand. In this way, solution chemistry and therefore metal speciation could be well controlled, while at the same time allowing the worms to behave naturally

In **Chapters 2 and 3**, experiments were performed to determine environmental factors (Na^+, Mg^{2+}, K^+, Ca^{2+}, and H^+) that affect bioavailability and toxicity of nickel (Ni) to *E. crypticus* in the course of time during an exposure period of 3 weeks.

The uptake and toxicity of Ni in *E. crypticus* after different exposure times were investigated in **Chapter 2**. The body concentration and toxicity of Ni gradually increased with time and tended to reach equilibrium at longer exposure times. The dynamic accumulation process of Ni was well described by a one-compartment model using constant rate parameters for uptake and elimination. When expressed as free ion activity in the test solution, LC50 of Ni decreased approximately by a factor of 3 during an exposure period running from 4 to 21 d. However, when based on Ni concentrations in the test animals, LC50 was almost constant and independent of exposure time. Since bioavailability of metals is determined by the characteristics of the organism, the environment and the toxicant together, body concentration rather than external concentration could be a more reliable indicator of metal toxicity as it incorporates toxicokinetic processes. This study revealed that time is an important factor which should be taken into account when modeling metal toxicity. In reality, soil organisms are usually exposed to metals for much longer times than the duration of standard laboratory ecotoxicological tests. Hence, the use of chronic toxicity data to conduct risk assessment and derive environmental quality criteria is preferred.

The influence of porewater chemistry (Ca^{2+}, Mg^{2+}, Na^+, K^+ and H^+) on Ni uptake and toxicity in *E. crypticus* was determined in **Chapter 3**. The same factors (Ca^{2+}, Mg^{2+}, Na^+) were found to modify both Ni uptake and toxicity, suggesting that the way by which these cations exert their protective effects is mainly through competing with metal ions for binding sites on the surface of the organisms, leading to an inhibition of the uptake of metal ions. Ni free ion activity was calculated with the model WHAMVI to take into account the influence of water chemistry (e.g. pH, DOC) on metal speciation. By incorporating the effect of metal speciation and competitive cations, extended Langmuir and Biotic Ligand models were developed, which well described the uptake and toxicity of Ni, respectively. The ligand binding constants of Mg^{2+} and Na^+ derived from uptake and toxicity data were similar.

However, for Ca^{2+}, the binding constants derived from uptake data differed considerably from the toxicity-derived estimates. This may be explained by the possible influence of Ca^{2+} on membrane permeability, or from an interaction between Ni^{2+} and Ca^{2+} at the target sites inside the organism. Our findings show that the biotic ligand model (BLM), which was originally developed for aquatic organisms, is also applicable to terrestrial organisms, with the body surface representing the biotic ligand.

As shown in **Chapters 2** and **3**, bioavailability and toxicity of metals are dependent on both exposure time and characteristics of the exposure solution. Existing BLMs were often developed for equilibrium conditions with fixed exposure times, neglecting the effect of time on metal uptake or toxicity. In **Chapter 4**, we show that the developed acute BLM cannot be used for accurately predicting chronic metal toxicity; the acute BLM underestimates chronic Ni toxicity to *E. crypticus*. In this chapter therefore two generic bioavailability models were developed based on a static BLM to take into account the influence of time by incorporating time-dependency in the parameters. In model b the fraction of binding sites occupied causing 50% mortality (f_{50}) was allowed to vary; in model c the binding 'constants' of the metal and competitive cations (K_{MeBL} and K_{CBL}) were variable. With these generic models, toxicity of Ni to *E. crypticus* was successfully described for varying exposure times and water chemistries. In previous studies, the stability binding constants were supposed to be metal- and species-specific and can only be derived under equilibrium conditions. As such chemical and biological equilibrium might not be reached upon short exposure times, the future development of BLMs should be cautious when using binding constants obtained in acute toxicity tests to predict chronic metal toxicity.

Metals are commonly present in mixtures in mining, industrial and domestic effluents, hence, risk assessment should take into account mixture toxicity. For this purpose, in **Chapters 5 and 6**, the dynamic toxicity of Ni and Co mixtures to *E. crypticus* was evaluated, using the same exposure media as described above and again following a toxicokinetics and toxicodynamics approach.

In **Chapter 5**, the Concentration Addition (CA) model was used to predict the toxic effect of metal mixtures. Deviations from concentration addition indicated that interactions occurred between Ni and Co. In order to better understand the underlying mechanism causing this finding, the interactions were determined at different levels. The interaction patterns at different exposure times were determined with the MIXTOX model, relating toxicity to metal free ion activities in solution and body concentrations in the test animals, respectively. When based on free ion activity, the interaction was mainly antagonistic and varied with exposure time, while when based on body concentrations no deviation from concentration addition was found. This suggests that interaction between Ni and Co mainly occurred during uptake, probably through competition for binding or uptake sites on the biotic ligand. This hypothesis is further supported by reduced Ni body concentrations in the animals in the presence of Co. Moreover, the use of body concentrations for predicting toxicity of metal mixtures could reduce the time dependence of the interaction pattern by taking into account toxicokinetic processes. The variation in toxicodynamic processes should be considered as well in future studies for establishing more accurate environmental quality criteria.

In **Chapter 6**, a newly developed WHAM-F_{TOX} model, assuming humic acid as a surrogate of non-specific biotic ligand sites, was applied to test its feasibility for delineating

the dynamic uptake and toxicity of metal mixtures. This assumption was confirmed by our findings that uptake of Ni and Co in *E. crypticus* linearly correlated with the amount of metal binding to humic acid, which was calculated with the WHAMVI speciation model taking into account the chemical composition of the test solutions. Single and mixture toxicity at different exposure times was well fitted by the WHAM-F_{TOX} model. Metal-specific accumulation kinetics (slow/fast) led to more/less time dependence of metal toxicity. This could explain why the estimated toxicity coefficient of Ni was time-dependent, while this was not the case for Co. The WHAM-F_{TOX} model shows to be a promising tool for predicting metal toxicity, which is mainly based on pure chemistry. Nevertheless, the biological aspect plays an important role in regulating metal uptake and metal detoxification, which cannot be ignored in the development of a mechanistic model.

In summary, our research showed that kinetic and dynamic processes must be included in the analysis of bioaccumulation and toxicity of metals. Traditional toxicological studies with fixed exposure times are of limited meaning for the risk assessment of toxicants with long-term exposure. In our research, the toxicity of metals was always related to two exposure levels: environmental concentrations and body concentrations in the test organisms. In this way it was shown that metal bioavailability and toxicity are affected mainly through the competitive interactions between ions during metal uptake. The developed bioavailability models, incorporating the effect of exposure time and exposure conditions, successfully predicted toxicity of metals, both single and in mixtures. The results of this thesis contribute to improving our mechanistic understanding of the uptake and toxicity of metals and to better define their risks in the environment.

Samenvatting

Verontreiniging van de bodem met metalen kan een bedreiging vormen voor de gezondheid van mens en milieu. De giftigheid van metalen in de bodem wordt echter in belangrijke mate bepaald door hun biologische beschikbaarheid. Het is dus van groot belang om de factoren die de biologische beschikbaarheid van metalen bepalen zowel kwalitatief als kwantitatief vast te stellen. Veel van deze factoren zijn gerelateerd aan de chemische aspecten van metaalspeciatie, en de interactie van het metaal met andere metalen en ionen in de bodemoplossing. Een andere factor die de toxiciteit van metalen beïnvloedt is tijd: de mate van opname van metalen in organismen en de daaruit voortvloeiende effecten zijn afhankelijk van de duur van de blootstelling, ofwel van de processen van toxicokinetiek respectievelijk toxicodynamiek.

Het voornaamste doel van dit proefschrift is te komen tot een eenduidig raamwerk voor het voorspellen van de giftigheid van metalen, met name nikkel (Ni), voor bodemorganismen, dat rekening houdt met factoren als variatie in de chemie van de bodemoplossing, interacties tussen metalen in mengsels, toxicokinetiek en toxicodynamiek.

Alle in dit proefschrift beschreven experimenten zijn uitgevoerd met de potworm *Enchytraeus crypticus,* en maakten gebruik van waterige oplossingen van de te onderzoeken metalen in een medium van inert kwartszand. Het gebruik van dit medium maakte het mogelijk om de chemie van de oplossing en daarmee ook de speciatie van de metalen goed te controleren, terwijl het dier zich toch natuurlijk kan gedragen; dit is in een echte bodem niet mogelijk.

In de Hoofdstukken 2 en 3 worden experimenten beschreven waarin is onderzocht in welke mate verschillende kationen (Na^+, Mg^{2+}, K^+, Ca^{2+}, en H^+) de biologische beschikbaarheid en de toxiciteit van nikkel voor *E. crypticus* beïnvloeden bij verschillende tijden van blootstelling.

De opname en giftigheid van Ni in *E. crypticus* na verschillende tijden van blootstelling zijn onderzocht in Hoofdstuk 2. De Ni concentratie in de dieren en de giftigheid van het metaal namen toe bij toename van de blootstellingduur, en leken een evenwicht te bereiken bij langere blootstellingtijden. De kinetiek van Ni-opname inde dieren kon goed worden beschreven met een zogenaamd eerste-orde één-compartiment model, gebruikmakend van constante snelheidsparameters voor opname en eliminatie. Wanneer de toxiciteit van nikkel werd uitgedrukt op basis van de vrije Ni^{2+} ion activiteit in de testoplossing, nam de LC50 (concentratie waarbij 50% van de dieren sterft) af met een factor 3 in de periode tussen 4 en 21 dagen van blootstelling. Werd de toxiciteit echter gebaseerd op interne nikkelgehalten in de dieren, dan was de LC50 bijna constant en nauwelijks afhankelijk van de blootstellingduur. Omdat de biologische beschikbaarheid van metalen wordt bepaald door de combinatie van de eigenschappen van het organisme, het milieu en het metaal, zou de concentratie in het testorganisme een betere indicator kunnen zijn voor de giftigheid van het metaal, omdat hiermee impliciet rekening wordt gehouden met de toxicokinetiek. Het onderzoek laat zien dat tijd een belangrijke factor is, waarmee rekening moet worden gehouden bij het voorspellen van de giftigheid van metalen. In het milieu worden organismen veel langer aan metalen blootgesteld dan in standaard laboratoriumtesten, die meestal een relatief korte blootstellingtijd kennen. Het gebruik van chronische toxiciteitgegevens is daarom aan te

bevelen voor de risicobeoordeling en het afleiden van risicogrenzen voor metalen in het milieu.

De invloed van de chemie van de testoplossing (met name concentraties van de kationen Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and H^{+}) op de opname en toxiciteit van Ni voor *E. crypticus* is bepaald in Hoofdstuk 3. Dezelfde factoren (Ca^{2+}, Mg^{2+}, Na^{+}) hadden invloed op zowel de opname in de dieren als de toxiciteit van Ni; bij hogere concentraties van deze kationen waren zowel de opname als de toxiciteit van Ni lager. Dit geeft aan dat het gelijktijdig aanwezig zijn van deze kationen beschermend werkt door competitie met het metaal voor bindingsplaatsen aan het oppervlak van het organisme, waardoor de opname van metaalionen wordt tegengegaan. De activiteit van het vrij Ni^{2+} ion werd berekend met het speciatiemodel WHAMVI om rekening te houden met de chemie van de testoplossingen (o.a. pH, gehalte aan opgelost organisch koolstof) op de metaalspeciatie. Rekening houdend met de metaalspeciatie werden uitgebreide Langmuir sorptiemodellen en biotische ligandmodellen (BLM) ontwikkeld, die de opname respectievelijk toxiciteit van Ni voor *E. crypticus* goed konden beschrijven. De constanten voor de binding van Mg^{2+} en Na^{+} aan liganden, die werden afgeleid uit de opname- en uit de toxiciteitsgegevens waren vrijwel hetzelfde. Voor Ca^{2+} waren de bindingsconstanten verkregen op basis van bioaccumulatie- en toxiciteitsgegevens echter nogal verschillend. Dit kan enerzijds worden verklaard uit het feit dat Ca^{2+} invloed kan hebben op de doorlaatbaarheid van de membraan voor metalen. Anderzijds zou in het organisme sprake kunnen zijn van competitie tussen Ni^{2+} en Ca^{2+} voor binding aan de target sites. Deze resultaten tonen aan dat het biotische ligand model, dat oorspronkelijk is ontwikkeld voor waterorganismen, ook toepasbaar is voor bodemorganismen, waarbij het lichaamsoppervlak een surrogaat is voor de biotische liganden waaraan het metaal kan binden. Toxische effecten zijn het gevolg van een overmatige bezetting van deze liganden met het metaal.

Zoals aangetoond in de Hoofdstukken 2 en 3, zijn de biologische beschikbaarheid en de toxiciteit van metalen afhankelijk van de blootstellingsduur en de eigenschappen van het blootstellingsmedium. De bestaande BLMs zijn meestal ontwikkeld voor evenwichtstoestanden bij één vaste blootstellingstijd, en gaan daarmee voorbij aan de invloed van de tijd op de processen van metaal-opname en -toxiciteit. In Hoofdstuk 4 wordt aangetoond dat een BLM ontwikkeld op basis van acute toxiciteitsgegevens niet kan worden gebruikt om de chronische toxiciteit van metalen te voorspellen. De acute BLM bleek de chronische toxiciteit van nikkel voor *E. crypticus* te onderschatten. In dit hoofdstuk zijn daarom twee algemene biologische beschikbaarheidsmodellen ontwikkeld, die zijn gebaseerd op een statische BLM maar rekening houden met het effect van de blootstellingstijd. Dit is gedaan door op twee manieren een van de parameters in het model tijdsafhankelijk te maken: (b). de fractie van de bindingsplaatsen die bezet is met het metaal en 50% sterfte van de testorganismen veroorzaakt (f_{50}) en (c). de bindingsconstanten van het metaal en de concurrerende kationen (K_{MeBL} respectievelijk K_{CBL}). Met deze generieke modellen kon de toxiciteit van Ni voor *E. crypticus* goed worden beschreven bij verschillende blootstellingstijden en bij verschillen in de chemische samenstelling van de testoplossingen. In eerdere studies is aangenomen dat de bindingsconstanten metaal- en soort-specifiek zijn en alleen kunnen worden afgeleid bij evenwicht. Als een dergelijk chemisch en biologisch evenwicht niet is bereikt, wat zeker het geval kan zijn bij een korte blootstellingsduur,

kunnen de afgeleide bindingsconstanten niet gebruikt worden voor het voorspellen van chronische toxiciteit. Het is belangrijk dat hiermee in de toekomst rekening wordt gehouden bij het ontwikkelen van BLMs voor chronische toxiciteit.

Metalen zijn in het milieu meestal aanwezig in mengsels, bijvoorbeeld in mijnafval en in industrieel of huishoudelijk afvalwater. De risicobeoordeling voor metalen moet daarom ook rekening houden met mengseltoxiciteit. Om die reden is in de Hoofdstukken 5 en 6 de toxiciteit van mengsels van nikkel en kobalt voor *E. crypticus* onderzocht bij verschillende blootstellingstijden. Ook hierbij is gebruik gemaakt van blootstelling in het eerder beschreven medium van een waterige oplossing in inert kwartszand, onder toepassing van een combinatie van toxicokinetische en toxicodynamische benaderingen.

In Hoofdstuk 5 is het Concentratie Additie (CA) model gebruikt om de toxiciteit van de metaalmengsels te voorspellen. Afwijkingen van concentratie additie gaven aan dat er sprake was van een interactie tussen Ni en Co. Om het achterliggende mechanisme te begrijpen, zijn de interacties tussen beide metalen bepaald op verschillende niveaus. Daartoe zijn de interacties bij verschillende blootstellingstijden geanalyseerd met het zogenaamde MIXTOX model, en werd de toxiciteit gerelateerd aan de vrije ion-activiteiten in de testoplossingen en aan de concentraties van de metalen in de testorganismen. Wanneer de toxiciteit werd gebaseerd op vrije ion-activiteiten was de interactie voornamelijk antagonistisch en variërend in de tijd. Maar wanneer de toxiciteit werd uitgedrukt op basis van interne metaalconcentraties in de dieren werd geen afwijking van concentratie additie gevonden. Dit suggereert dat de interactie tussen Ni en Co voornamelijk plaatsvindt bij opname in de dieren, waarschijnlijk door concurrentie om dezelfde bindings- of opnameplaatsen. Deze hypothese wordt ondersteund door het feit dat de nikkelgehalten in de dieren lager waren bij aanwezigheid van kobalt in het medium. Deze resultaten suggereren ook dat het gebruik van lichaamsconcentraties voor het voorspellen van de toxiciteit van metaalmengsels de invloed van de factor tijd op de interactie van de metalen in het mengsel kan reduceren, omdat hiermee impliciet rekening wordt gehouden met de toxicokinetiek. De variatie in de toxicodynamiek moet worden meegenomen in toekomstige studies die gericht zijn op het vaststellen van milieukwaliteitscriteria.

In Hoofdstuk 6 is het recent ontwikkelde WHAM-F_{TOX} model, dat aanneemt dat humuszuur model kan staan voor niet-specifieke biotisch liganden, toegepast om na te gaan of het geschikt is voor het beschrijven van de dynamische opname en toxiciteit van metaalmengsels. Deze aanname werd bevestigd door het feit dat de opname van Ni en Co in *E. crypticus* lineair gerelateerd aan de mate van binding aan humuszuur, berekend met het WHAMVI speciatiemodel en rekening houdend met de chemische samenstelling van de testoplossing. De toxiciteit van zowel de metalen apart als van de mengsels bij verschillende blootstellingstijden kon goed beschreven worden met het WHAM-F_{TOX} model. Metaal-specifieke verschillen in de opnamekinetiek (langzaam/ snel) van de metalen leiden tot een grotere of kleinere tijdafhankelijkheid van de toxiciteit. Dit kan verklaren waarom de geschatte toxiciteitscoëfficiënt voor Ni tijdsafhankelijk was, terwijl die voor Co niet afhankelijk was van de tijd. Het WHAM-F_{TOX} model lijkt dus een veelbelovend instrument voor het voorspellen van de toxiciteit van metalen gebaseerd op louter chemische parameters. Desondanks moet er rekening mee worden gehouden dat ook biologische factoren een belangrijke rol spelen bij de regulatie van de opname en de detoxificatie van metalen. Deze

processen kunnen niet worden genegeerd bij het ontwikkelen van een mechanistisch model voor metaaltoxiciteit.

Samenvattend laat dit onderzoek zien dat kinetische en dynamische processen betrokken moeten worden bij de analyse van zowel de opname als de toxiciteit van metalen. Traditionele toxiciteitstesten met een vaste tijdsduur zijn van beperkte waarde voor de risicobeoordeling van stoffen met een langdurige blootstelling. In dit onderzoek werd de toxiciteit van metalen altijd gerelateerd aan twee blootstellingsniveaus: concentraties in het milieu en in de testorganismen. Zodoende kon worden aangetoond dat de biologische beschikbaarheid en toxiciteit van metalen vooral worden beïnvloed door competitieve interacties tussen ionen bij de opname van metalen in organismen. De ontwikkelde biologische beschikbaarheidsmodellen, die rekening houden met de blootstellingstijd en de –condities, bleken goed in staat de toxiciteit te voorspellen van zowel de metalen apart als in mengsels. De resultaten van dit proefschrift kunnen bijdragen aan verbetering van het begrip van de mechanismen van opname en toxiciteit van metalen en daarmee aan het beter karakteriseren van hun mogelijke risico’s in het milieu.

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愿有岁月可回首，且以深情共白头~

Erkai He
June 2015

Curriculum vitae

Erkai He was born in Nanyang, Henan, China, on June 12, 1987. Since 2002, she studied at Nanyang High School in her hometown. From 2005 to 2009, she was an undergraduate student at the department of Chemistry and Environmental Science, Nanjing Normal University. During the last year of her bachelor study, she did a half-year internship with Dr. Shiyin Li, exploring the effect of the extracts of aquatic plants on the growth and metabolism of cyanobacteria. She obtained her bachelor degree in the subject of Environmental Science in June, 2009. She did her master study at the department of Environmental Science and Engineering in Sun Yat-Sen University. Under the supervision of Prof. Rongliang Qiu, she worked on the development of joint strategies (with hyperaccumulating plants and sludge-derived biochar) for the remediation of metal-contaminated soils in mining areas. After graduation with a master degree in June, 2011, she secured a PhD grant from China Scholarship Council. From September of 2011 until 2015, she carried out her PhD research at the department of Ecological Science in VU University, Amsterdam, supervised by Prof. Nico Van Straalen and Dr. Kees Van Gestel. Her research mainly focused on investigating the ecotoxicological effects on soil invertebrates and modelling the dynamic bioavailability and toxicity of metals. After the completion of her PhD thesis, she would like to continue her scientific career in a university or a research institute.

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