Quantitative modelling of the response of earthworms to metals

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Chapter I

General Introduction

1.1 Soil system and metals

Soil is a crucial component of the earth's ecosystem. As the largest biodiversity reservoir on our planet, soil provides habitat for billions of organisms, including bacteria, fungi, protozoa, nematodes, earthworms, arthropods, plants, et cetera (Baskin, 2005). Soil biodiversity underpins various ecosystem processes and functions which deliver benefits to mankind (ecosystem services) (Blouin et al., 2013). Human activities are increasingly causing degradation and impoverishment of soil and decline in biodiversity which in turn threatens to diminish the capacity of the earth to sustain us. This creates a sense of urgency for us to prevent biodiversity loss, to protect soil functioning and to maintain sustainability of ecosystems. Soil is a dynamic and heterogeneous environment consisting of solid, liquid, and gas phases. The solid phase includes organic material and mineral particles, the liquid phase contains dissolved organic matter and dissolved nutrients, and the gas phase is composed of volatile organic substances and various gases. Soil textures are classified according to the relative proportions of sand (0.05mm - 2mm), silt (0.002mm - 0.05mm) and clay (< 0.002mm) present in a soil (Davis and Bennett, 1927). Various soil constituents show a great capacity to adsorb and retain metals (Van Leeuwen and Hermens, 2007). As a result, soil may act as a sink for the metals released into the environment.

Sources of metals

Metals are naturally occurring components in the environment, with their occurrence primarily in rocks. The release of metals to soil is facilitated by weathering of parent rock and atmospheric deposition (Reeder et al., 2006). Consequently, natural concentrations of metals in soils are strongly correlated to the varied distribution of rock types and vary on a large scale between different geographic regions (Alloway, 1995). Elevated concentrations of metals in the soil environment mainly result from anthropogenic activities such as the extraction, smelting, and processing of metal-bearing ores, the distribution and use of metal-containing products, and the return of concentrated metals through the disposal of processing wastes and the discard of spent products (D'Amore, et al., 2005). These human activities have interfered with the biogeochemical cycles of metals that occur slowly in the natural environment.

Metal behavior

When metals are introduced into soils, a series of complex physical, chemical, and biological reactions (e.g., complexation/dissociation, oxidation/reduction, sorption/ desorption, and precipitation/dissolution) can take place. As a result, metals are distributed differently among fractions of the soil solid and solution phases (Roberts et al., 2005). Speciation is defined as the distribution of a metal over various chemical forms in the soil solid phases and soil solution (Peijnenburg et al., 2007). Metal can be bound in the soil solid phase by three types of reactions: (i) precipitation with anions such as hydroxides, carbonate, sulphate and phosphate, (ii) adsorption due to ion exchange, chemical bond formation, and complexation, and (iii) occlusion into the crystal structure of minerals (Levy et al., 1992). The soil solution phase may host the metal as free ion or complexed with inorganic (OH^-, Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, etc.) and organic ligands (dissolved organic carbon like humic and fulvic acids) (Almas et al., 2007; Roberts et al., 2005; Ure and Davidson, 1995). Solid-solution partitioning of metals reflects the differences in chemical behavior and mobility of metals in different soils, to a large extent determining availability of metals to organisms and effects.

Metal effects

Concerns about the input of metals to soil are related to their ecotoxicological impact on organisms living in the soil. Next to effects on soil organisms, metal may be transferred via the food chain, resulting in health effects on animals and humans. Several metals (e.g., Cu, Ni, and Zn) are essential for living organisms because they play an important role in various biochemical and physiological processes, while some (e.g., Cd, Pb, and Hg) are highly toxic and have no known physiological function (nonessential) (Peijnenburg et al., 2007). There is an optimal level of essential metals for maximum benefit. Below this level symptoms of metal deficiency may occur whereas above this level the metal may become less beneficial and eventually toxic with increasing levels of availability (Hopkin, 1989). Toxic effects of metals on soil invertebrates (e.g., earthworms, enchytraeids, and nematodes) and microorganisms are reduced species diversity, abundance, and biomass (Bengtsson and Tranvik, 1989; Santorufo et al., 2012) and changes in microbial processes (e.g., glucose induced respiration and potential nitrification rate) (Oorts et al., 2006; Vig et al., 2003). Effects of metals on vascular plants can be reflected in the form of toxicity symptoms (reduced development and growth of shoots and roots) (Le et al., 2012), physiological symptoms (decreased nutrient contents in leaf tissues, elevated concentrations of total sugar and starch) (Prasad 1995), and biochemical symptoms (decreased enzymatic activity) (Das et al. 1997; De Vries et al., 2007). These effects could have serious consequences for soil biodiversity, and in turn soil functioning and ecosystem sustainability. Therefore, an understanding of the actual risks posed by metals in soil is needed.

Metal risk assessment

Ecological risk assessment for metals in soil is routinely conducted based on laboratory data from standardized ecotoxicity tests using selected terrestrial species (e.g., earthworms, Enchytraeids and Collembola) (Løkke and Van Gestel, 1998). These ecotoxicity tests focus on establishing quantitative concentration-effect relationships so that toxicity thresholds and therefore risk limits can be derived. This information is required in the modern regulatory system of the European Union - REACH (Registration, Evaluation and Authorization of Chemicals). REACH makes industry responsible for assessing and managing the risks posed by metals and other chemicals. Recently, there are also new moves toward establishing a Chinese REACH system: two guidelines concerning risk assessment under the order No.7 of the Ministry of Environmental Protection of China are pending (MEP, 2011). The original *Guideline for the Hazard Evaluation of New Chemical Substances* (HJ/T 154-2004) is revised into two separate documents, *the Guideline for Risk Assessment of Chemicals* and *the Guideline for Hazard Identification of New Chemical Substances*. The (draft) Guideline for Risk Assessment of Chemicals specifies the technical methods and procedures for the environmental and health risk assessment of chemical substances. The hazardous new chemical substances of priority environmental concern are screened out based on the outcome of risk assessment.

To date, soil quality criteria and risk assessment of metals are still predominantly based on total concentrations (Janssen et al., 2003). However, efforts to relate the total concentration of a metal to toxic effects have proven difficult (Reeder et al., 2006). Metal toxicity, even to the same organisms, can vary largely in different soils because of the impact of soil properties and metal bioavailability, which affects the relevant exposure concentration for organisms (Rooney et al., 2006; Criel et al., 2008; Santorufo et al., 2012). Improving the accuracy of the risk assessment and decision making requires an explicit consideration of bioavailability and other ecological knowledge.

1.2 Bioavailability

The concept of bioavailability was introduced to consider the fraction of a contaminant that will actually have an effect on organisms (Patrick et al., 1977; Mayer, 2002). However, bioavailability cannot be directly related to toxic effects as the latter follow from the amount of metals that react with the target sites of cells (Peijnenburg et al., 2007). As such, bioavailability can be considered a three-step approach, in which the available metal causes exposure (environmental availability), exposure leads to dynamic or passive uptake (environmental bioavailability), and subsequent effects result from reaction with a biological target (toxicological bioavailability) (Figure 1.1) (Dickson et al., 1994; Landrum et al., 1994; Peijnenburg et al., 1997).

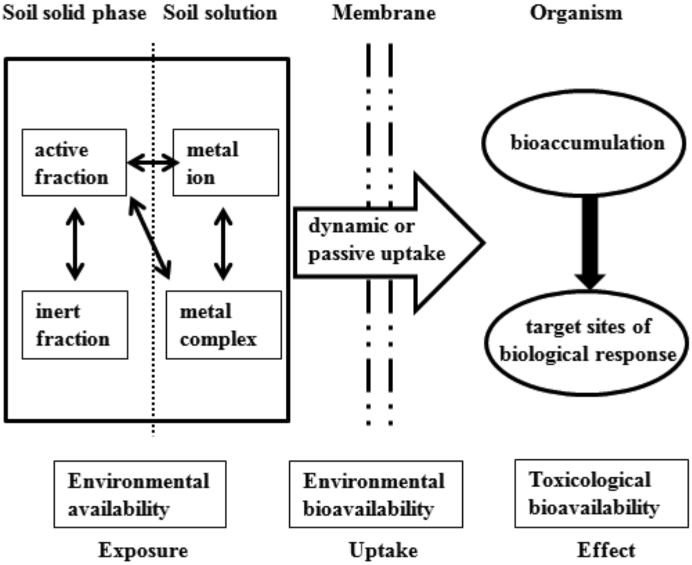

Figure 1.1 Schematic generalized depiction of the concept of bioavailability in soil systems. The active fraction is the metal that is available for exchange with the soil solution. The inert fraction is the metal that is occluded in mineral particles (clay, oxides) and organic matter. In soil solution, metals are present in dissolved species as metal ions and complexes. Metal interactions with various soil components affect actual exposure (environmental availability). Exposure leads to dynamic or passive uptake of

the metal across the membrane of orgamism (environmental bioavailability). The amount of accumlated metal that reacts with the target sites determines the subsequent effects (toxicoligical bioavailability).

Metals exist in different solid-phase and solution-phase forms that can vary greatly in terms of their bioavailability. Although the majority of metals are in the soil solid phase, metals in the solution phase pose the largest risks for soil dwelling organisms (Groenenberg, 2010). Metals in the soil solution are far more mobile and available than those in the solid phase (De Vries et al., 2005). Uptake by soil organisms via soil solution is supposed to be the most relevant pathway for metal exposure (Van Gestel and Koolhaas, 2004; Vijver et al., 2003). When a metal is supplied from the solid phase, it must first be transferred to solution before it can be taken up. Furthermore, not all dissolved metal species in the solution phase are readily available for uptake by organisms (Zhang et al., 2004). Therefore, the metal concentration and speciation in the soil solution are the major aspects that govern bioavailability. Extensive studies have reported that soil properties which control metal partitioning and speciation, such as pH, organic matter content, clay content, cation exchange capacity and the concentration of Fe- and Al-oxyhydroxides, have a significant influence on bioavailability (Janssen et al., 1997; Peijnenburg et al., 1999; Oorts et al., 2006; Smolders et al., 2009).

Besides physiochemical properties of the soil, biological processes may also play an important role in controlling bioavailability. For example, earthworms appear to affect metal bioavailability through stimulating soil microbial population, altering pH and DOC of the soil solution (Sizmur and Hodson, 2009). Plants can change metal bioavailability through root activities and related rhizosphere processes (rhizosphere acidification or root proliferation and secretion of organic acids). Mench and Martin (1991) reported that soil Cu, Cd, Ni, Zn, Fe, and Mn were dissolved by root exudates of corn.

A variety of chemical extraction methods are available for determining bioavailable fractions of metals in soil (Peijnenburg et al., 2007). These methods are usually operationally defined and are based on the correlation between water-soluble or exchangeable metal and biological effects (internal concentration or other endpoints) (Houba et al., 1996; Menzies et al., 2007). Chemical extraction methods are convenient, rapid, and cost-effective, and they thus provide data of significance for risk assessment practices. The limitations lie in that the extractant is often non-selective and non-exclusive, hindering the comparison between different soils. For different soil types, selection of a reasonable extractant and procedure is difficult. Several devices and methods have already been proposed to mimic bioavailability, such as the Donnan membrane technique (DMT) (Temminghoff et al., 2000), the device of diffusion gradients in thin films (DGT) (Zhang et al., 2001), physiologically based extraction tests (PBET) (Ruby et al., 1993), and simplified bioaccessibility extraction tests (SBET) (Medlin, 1997). Although the correlations between the results of these approaches and the effects of the specific metals seem reasonably good, these approaches do not provide any mechanistic information on bioavailability and toxicity. It is therefore difficult to develop an integrated approach to predict metal effects on organisms. To provide a mechanistic framework for site-specific risk assessment of metals, bioavailability models, which combine chemical insights in metal speciation with biological insights regarding effects, have been developed. These models will be introduced below.

1.3 Effect modelling

Already for a long time researchers attempt to understand the exact mechanisms that induce differences in bioavailability and subsequent toxicity across water and soils. It has been recognized that metal bioavailability and toxicity are to a large extent controlled by the free ion activity in (soil) solution (Morel, 1983; Campbell, 1995; Batley et al., 2004; Van Gestel and Koolhaas, 2004). Based on these assumptions, mechanistically underpinned models such as the free ion activity model (Morel, 1983), the biotic ligand model (Di Toro et al., 2001) and the electrostatic model (Wang et al., 2008) were developed. Given the global desire of minimizing animal testing and reducing costs of regulatory testing of chemicals (Höfer et al., 2004), these modelling approaches are favorable for conducting ecological risk assessment of metals.

Free ion activity model

The bioavailability models for predicting metal toxicity were initially developed for the aquatic environment. In 1983, François Morel published a book "Principles of Aquatic Chemistry", which led to the development of the conceptual free ion activity model (Morel, 1983). The model describes how variations in the effect levels of metals can be related to their aqueous speciation and interactions with the organism (Morel, 1983; Paquin et al., 2002). It is assumed that among the various metal species, only the free metal ion can bind to the active sites (carrier, channel or toxic action sites) of the cell surface membrane, and subsequently be transported across the membrane to induce toxic effects (Morel, 1983; Campbell, 1995; Brown and Markich, 2000). Interactions between a metal ion and an organism generally involve three steps: (1) diffusion of the metal ion in the bulk solution to the surface of the cell membrane; (2) sorption or surface complexation of the metal ion at the active sites of the cell membrane; (3) uptake or transport of the metal ion across the cell membrane of the organism (Campbell, 1995). The key assumption which underpins the free ion activity model is that there is a rapid equilibrium between metal ions in the solution and those at the cell membrane (as follows):

$$\{\mathrm{M}^{z+}\} + \{\text{-}R_{\mathrm{cell}}\} \overset{k_1}{\leftrightarrow} \{\mathrm{M}\text{-}R_{\mathrm{cell}}\} \qquad (1\text{-}1)$$

that is,

$$\{\mathrm{M}\text{-}R_{\mathrm{cell}}\} = k_1 \{\mathrm{M}^{z+}\} \{\text{-}R_{\mathrm{cell}}\} \qquad (1\text{-}2)$$

where $\{\mathrm{M}^{z+}\}$ is the free metal ion activity, $\{\text{-}R_{\mathrm{cell}}\}$ is the concentration of free surface sites on the cell membrane, $\{\mathrm{M}\text{-}R_{\mathrm{cell}}\}$ is the activity of the surface complex, k_1 is the conditional stability constant. The biological response is assumed to be directly proportional to $\{\mathrm{M}\text{-}R_{\mathrm{cell}}\}$. The nature of the cell surface is assumed to remain unchanged during exposure (Hassler et al., 2004; Tessier and Turner, 1995). Therefore the biological response is determined by $\{\mathrm{M}^{z+}\}$. Later studies found that even in exposure media with the same free metal ion activity, other factors such as hardness, DOC, and pH can affect metal toxicity (Allen and Hansen, 1996; Campbell, 1995). Based on these findings, the concept of the biotic ligand model has been proposed and developed to consider both chemical speciation and ion competition in estimating metal toxicity (Di Toro et al., 2001; Paquin et al., 2000; Santore et al., 2002).

Biotic ligand model in the aquatic environment

The biotic ligand model (BLM) is a theoretical framework in which toxicity is related to the binding of metal ions to the sites of toxic action on an aquatic organism. For modelling purposes, the sites of toxic action are treated as a biotic ligand, that is, the biological counterpart of chemical ligand to which metals can bind. Toxic effects occur when the concentration of metal-biotic ligand complex reaches a certain critical level (Di Toro et al., 2001). The model also considers that other dissolved constituents that are present in the aquatic environment can influence the extent of metal binding to the biotic ligand. These constituents may either reduce the concentration of metal-biotic ligand complexes by competing with the metal for binding at the biotic ligand (e.g., Ca^{2+}, Mg^{2+}, Na^+ and K^+) or by the formation of dissolved complexes with the metal (e.g., dissolved organic carbon, inorganic ligands) (Figure 1.2) which reduce the free metal ion activity. The principal feature in the BLM is the competition of the free metal ion with other cations for binding at the biotic ligand. This feature distinguishes the BLM from earlier concepts that considered only the free metal ion as the toxic species. The metal-organism interaction part of the biotic ligand model is based on the free ion activity model.

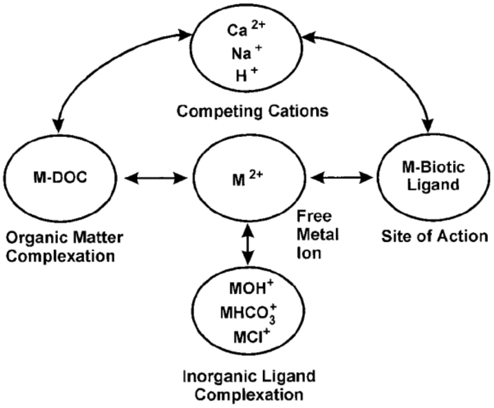

Figure 1.2 Schematic diagram of the framework of Biotic Ligand Model. For further information see the text and Di Toro et al. (2001).

For fish, the primary sites of toxic action for most metals is either known or suggested to be the Ca^{2+} or Na^+ channel proteins in the gill surface (Pagenkopf, 1983; Di Toro et al., 2001). The amount of metal bound to the fish gill can be experimentally determined (Meyer et al., 1999). For the other species, the sites of toxic action are not readily accessible to direct measurement. Therefore, the term *biotic ligand* is adopted to facilitate the applicability of the BLM concept to other aquatic organisms. A detailed historical overview of the development of the aquatic biotic ligand model is given by Paquin et al. (2002), including an account of the primary sites of toxic action in aquatic organisms (i.e., the gill) and the associated physiological processes involved in ion-regulation between the organism and the external environment. The most important adverse effects of metals on aquatic organisms have been demonstrated to involve the disturbance of the organisms' ability to regulate their internal ion balance. Three principal categories of physiological mechanisms of toxicity are distinguished: monovalent metals affecting Na^+ transport, divalent metals affecting Ca^{2+} metabolism, and metals that affect the organism centrally after passing the gill. Paquin et al. (2002) refer to various experimental studies demonstrating that metal accumulation at the site of toxic action is truly a function of metal complexation and competitive interactions, as conceptualized in the earlier models such as the gill surface interaction model (Pagenkopf, 1983) and the free ion activity model (Morel, 1983).

According to the assumption of the BLM, metal ions (M^{z+}) and other cations (H^+, K^+, Ca^{2+}, Na^+, and Mg^{2+}) can bind to the theoretical biotic ligand (BL) sites (De Schamphelaere and Janssen, 2002). The interaction between cations and BL is treated as a surface complexation reaction. At equilibrium, for example, the stability constant for M^{z+} binding to biotic ligands K_{MBL} (L/mol) can be expressed as a function of the concentrations of cation-biotic ligand complexes [MBL] (mol/L) and unoccupied biotic ligand sites [BL] (mol/L):

$$K_{\mathrm{MBL}} = \frac{[\mathrm{MBL}]}{\{\mathrm{M}^{z+}\}\times[\mathrm{BL}]} \tag{1-3}$$

where $\{M^{z+}\}$ is the free metal ion activity (mol/L).

Metal toxicity is assumed to be proportional to the fraction (f) of the total biotic ligand sites $[\mathrm{BL}]_\mathrm{T}$ occupied by the toxic metal. The f value depends on the binding affinity of M^{2+} to the BL and the presence and binding affinity of the competing cations (De Schamphelaere and Janssen, 2002):

$$f = \frac{[\mathrm{MBL}]}{[\mathrm{BL}]_\mathrm{T}} = \frac{K_{\mathrm{MBL}}\times\{\mathrm{M}^{z+}\}}{1+K_{\mathrm{MBL}}\times\{\mathrm{M}^{z+}\}+\sum K_{\mathrm{XBL}}\times\{\mathrm{X}^{z+}\}} \tag{1-4}$$

where $\{X^{Z+}\}$ is the activity of major cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) in the solution, K_{XBL} is the binding constant of cations X^{Z+} binding to the BL.

The biological response is related to f using the logistic dose-response relationship:

$$R = \frac{R_0}{1+\left(\frac{f}{f_{50}}\right)^{\beta}} \tag{1-5}$$

where R is the survival rate, R_0 is the control survival rate, f_{50} is the fraction of the total BL sites occupied by M^{2+} at which the survival rate is reduced by 50%, β is the slope parameter.

The value of f at the 50% effect level is assumed to be constant according to the BLM theory. Equation 2 then can be rewritten as:

$$\mathrm{EC50}\{\mathrm{M}^{z+}\} = \frac{f_{50}}{(1-f_{50})\times K_{\mathrm{MBL}}}\times\{1+\sum K_{\mathrm{XBL}}\times(\mathrm{X}^{z+})\} \tag{1-6}$$

where $\mathrm{EC50}\{M^{z+}\}$ is the free metal ion activity inducing 50% effect. The BLM parameters required for predicting the effects are f_{50} and the binding constants of each cation. To date, the biotic ligand sites have been analyzed only for fish gills and f_{50} and binding constants have been experimentally determined for a number of metals (Playle et al., 1993). For the other species, these BLM parameters are generally obtained by fitting the model to observed measurements of toxic effects such as mortality. In other words, toxicity data are often used as the empirical basis for determining f_{50} and binding constants of metals to the biotic ligand

(De Schamphelaere and Janssen, 2002; Deleebeeck et al., 2009). The validity of the BLM can be established only if the critical concentration is the same over the entire range of environmental conditions that have been tested. A speciation model (e.g., WHAM) is needed for the necessary calculations. A major strength of the BLM is that it provides a mechanistic and operational framework for interpreting the effects of exposure water chemistry on metal bioavailability and toxicity. Many aquatic BLMs have been successfully developed and applied to predict metal toxicity to fish, algae, and water fleas in different exposure conditions (Alsop et al., 2000; De Schamphelaere and Janssen, 2004; Heijerick et al., 2002; Santore et al., 2002). The toxic effects estimated by these BLMs are generally within a factor of two of the observed toxic effects. Some exceptions are also reported especially in alkaline exposure conditions (De Schamphelaere and Janssen, 2002; Li et al., 2009a, Wang et al., 2009). Metal speciation changes substantially over the alkaline pH range and species other than the metal ion dominate in the solution (Wang et al., 2012). It has been suggested that the effect of pH on metal toxicity at a relatively high pH (>8) is a speciation effect rather than significant competition effects between protons and metal ions (De Schamphelaere and Janssen, 2002; Markich et al., 2003; Wang et al., 2010). In addition to free metal ions (e.g., Cu^{2+}), other metal forms (e.g., $CuOH^{+}$ and $CuHCO_3^{+}$) may also contribute to toxicity at pH-values exceeding 8. There is also the possibility of physiologically initiated modification of the structure of the membranes at high pH levels (Lavoie et al., 2012). Therefore, the applicability of BLM in high-pH media needs to be investigated further.

Biotic ligand model in the terrestrial environment

According to the BLM theory, toxicity occurs as a consequence of free metal ions reacting with the biotic ligand at the interface of solution and organism (Di Toro et al., 2001). It is assumed that the toxicity principles for fish and other aquatic species are also applicable to terrestrial species such as earthworms and plants. In case of these specific terrestrial organisms, the sites of toxic action are in direct contact with the external (soil) solution. This is an evolutionary phenomenon as general binding sites such as sodium and calcium transporters are inherent to every living cell (Niyogi and Wood, 2004). By considering the biotic ligand as a more general binding site, the principles underlying aquatic BLMs are likely to be valid also for terrestrial species. Attempts have been made to develop BLMs for soil invertebrates (earthworms and enchytraeids) (Steenbergen et al., 2005; Li et al., 2008; Lock et al., 2006) and plants (Lock et al., 2007; Li et al., 2009a) in solution or in solution-sand systems. These studies show that the application of the BLM to terrestrial organisms is theoretically and empirically feasible. One of the key limitations of these studies lies in the difficulty to relate the observed results to real world conditions. Increasing our understanding of the mechanisms behind metal uptake and toxicity to soil species is valuable, but the mechanistic information needs to be relevant with regard to the actual exposure of organisms in natural soils, for the terrestrial BLMs to be truly meaningful. The geochemistry of the hydroponic conditions bears little resemblance to that of real world soils, and identifying the challenges that are anticipated in soil validation of these systems would be appropriate.

A schematic diagram of the anticipated terrestrial BLM is presented in Figure 1.3, showing the interactions at the interface of the soil solid phase and the soil solution, and at the interface of soil solution and organism. Metals are exchanged between the soil matrix and the solution by sorption to reactive solid phases, such as the soil organic matter (SOM), and the mineral (hydr)oxides of Fe, Al, and Mn, and clay. Competitive sorption of cations, protons, etc., to these solid phases also affects the distribution of the metal between the soil solid phase and solution. Free metal ions (M^{2+}) can form complexes with dissolved organic and inorganic ligands. A speciation model is needed to determine the metal speciation in solution, but such a model needs to account also for the partitioning of the metal between the soil solution and the soil solid phase. In addition to these soil geochemical processes that control metal speciation, the terrestrial BLM includes a toxicity model similar to that of the aquatic BLM.

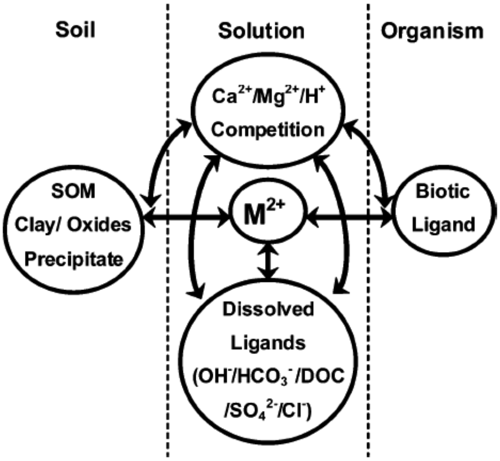

Figure 1.3 Schematic overview of the interactions considered in the terrestrial biotic ligand model (Thakali et al., 2006a).

The application of the BLM theory to soil toxicity data has rarely been done. This is because on the one hand there are not enough soil toxicity datasets suitable for such a purpose, the soil system is on the other hand much more complex than a solution system (route of exposure, environmental chemical behavior of metal) (Antunes et al., 2006; Plette et al., 1999). In soil, metal uptake by terrestrial organisms from the soil solution and the soil particles may in principle both be of substantial importance (Steenbergen et al., 2005). Like the aquatic BLM, the terrestrial BLM is based on the assumption of equilibrium partitioning. This assumption may not be valid when the surface complexation reactions between ions and biotic ligands are slow relative to the internal transport and the subsequent expression of the biological response. Besides, the environmental availability can be affected by dissolution kinetics of metal from the soil solid phase towards the soil solution phase. Equilibrium may also not be assumed when the metal transport into the organism is limited by diffusional control across the (static) boundary layer around the interfacial cells. As Thalaki et al. (2006a) argue, both kinetic control and diffusional limitations have been shown to occur, but are much more likely only under metal deficient conditions and thus not relevant to toxicity studies in general. Unlike the solution system it is difficult to univariately modify the parameters that affect metal toxicity in soil. Covariance exists among most of the physicochemical properties of soil (Lofts et al., 2004; Wang et al., 2011a). For example, the amount of protons and major cations released to the soil porewater covaries with the amount of metal salt added in soil (Wang et al., 2011a). Parameterization of the BLM, therefore, faces a variety of challenges and uncertainties. Despite these difficulties, Thakali et al. (2006a, b) developed and applied the BLMs in acidic soils for describing Cu and Ni toxicity to plants (barley, tomato), soil invertebrates (earthworm, springtail) and microbial processes (nitrification potential, respiration). Free metal ions in the soil solution were assumed to be the dominant toxic species while cation competition was supposed to modify toxicity. Interactions between the metal and the soil solid phase, and between the metal and the solution phase were taken into account when calculating metal speciation in soil solution. In applying the BLM to the toxicity data, Thakali et al. found that it is unavoidable to empirically fix some of the BLM parameters (f_{50} and even one of the binding constants of the protective cations). Therefore, the estimated binding constants should not be regarded as conventional binding constants but rather as parameters that summarize the processes underlying the observed relation between organism responses and competing cations. Since the development of terrestrial BLMs for metals like Cu, Ni, and Cd is not readily feasible, it would be highly desirable to develop alternative methods, which facilitate the application of the BLM theory in soil, to assess metal toxicity to terrestrial organisms.

1.4 Mixture toxicity

In the real world, exposure to metal mixtures is a rule rather than an exception (Kortenkamp et al., 2009). However, current regulatory approaches focus almost exclusively on single metals and rarely require assessment of mixtures, which may have little real environmental relevance (Backhaus and Faust, 2012). The development of simple and efficient approaches for modelling mixture toxicity is necessary in the sense of meeting future regulatory demands and ensuring adequate risk assessment.

The foundations for mixture toxicity modelling have been laid by pharmacologists since 1920s (Loewe and Muischnek, 1926; Bliss, 1939). Conceptual models (see below) were developed by simply adding doses and responses to predict mixture effects based on the assumption that mixture components do not impact each other’s toxicological action. However, the interactions of mixture components that may lead to decreased or increased bioavailability and toxicity were ignored. To date, our understanding of mixture toxicity is still based on those concepts, with addition as the basis for most models (Vijver et al., 2010). Only limited progress has been made to incorporate the interactions of mixture components in predicting mixture toxicity.

Conceptual models (Non-interaction)

Concentration Addition (CA) and Independent Action (IA) are the two main conceptual models that are available for calculating the toxic effects of mixtures, based on the toxicities of individual metals and their concentrations in the mixtures (Loewe and Muischnek, 1926; Bliss, 1939).

The CA concept was developed by Loewe and Muischnek (1926) to describe mixtures where the components have the same or a similar mode of toxic action (i.e., act on the same biological pathway and strictly the same molecular target):

$$\mathrm{CA} = \mathrm{TU}_{\mathrm{mix}} = \sum_{i=0}^{n} \frac{c_i}{\mathrm{EC}x_i} \qquad (1\text{-}7)$$

where n is the number of mixture components, c_i is the concentration of component i in the mixtures causing x_i% effect, ECx_i is the effective concentration of component i causing x_i% effect when applied singly. The term $\frac{c_i}{\mathrm{EC}x_i}$ is also defined as Toxic Unit (TU) which scales the relative toxicity of each component (Sprague, 1970). $\mathrm{TU}_{\mathrm{mix}}$ is therefore a dimensionless quantity that equals the sum of the TUs of the individual components in the mixture. When the EC50 of the mixture equals 1 $\mathrm{TU}_{\mathrm{mix}}$, no interactions occur and CA holds. Values of $\mathrm{TU}_{\mathrm{mix}}$ that are statistically significant below 1 indicate synergism, while values significantly exceeding 1 describe antagonism. CA deals with the issue that the relative toxicity of the metals that are present in the mixtures is the same as the relative toxicity of the metals present individually.

The concept of IA was first proposed by Bliss (1939) to describe mixtures where the components have different modes of action (i.e., act on different physiological systems):

$$\mathrm{IA} = E(c_{mix}) = 1 - \prod_{i=1}^{n}[1 - E(c_i)] \qquad (1\text{-}8)$$

where $E(c_{\mathrm{mix}})$ is the predicted effect (scaled in the range of 0-1) of mixtures, c_i is the concentration of component i, $E(c_i)$ is the effect of component i present singly at a concentration c_i. IA addresses the question whether the probability of response to one metal may be independent from the probability of response to another metal. In this model, the relative toxicity potency of metals is ignored, and the mixture effect is predicted from the joint probability of statistically independent events (Peijnenburg and Vijver, 2007).

With regard to the choice for a conceptual model, the basic idea is to use CA if the components are expected to act similarly and to use IA if the components are expected to act dissimilar (Junghans et al., 2006). However, identifying the modes of action for different chemicals is not always possible. In those cases, CA is suggested to be the more conservative choice in a risk assessment context as it estimates a higher response than IA and therefore represents the worst-case scenario for mixture exposure (Lock and Janssen, 2002; Backhaus and Faust, 2012).

Deviations from conceptual models (Interaction)

Although CA and IA are extensively used, it should be pointed out that both CA and IA approaches were put forward based on additivity, ignoring interactions between mixture components. This may explain why there are some scenarios in which the mixture effect cannot be fully described with either conceptual model. Multiple metals may interact with each other, which leads to more-than-additive (synergism) or less-than-additive (antagonism) effects. Besides, interactions between different mixture components may also depend on design of the toxicity test, such as the overall mixture concentrations and the relative proportion of component concentrations (Norwood et al., 2003; Jonker et al., 2005). In the present study, we therefore defined the following scenarios where deviations may occur:

(1) *Non-interaction*: the observed mixture effect is adequately explained by either CA or IA.

(2) *Absolute synergism or antagonism*: the observed effect of all combinations of a mixture is significantly more severe (synergism) or less severe (antagonism) than the mixture effect predicted by either CA or IA.

(3) *Dose ratio dependent deviation*: the deviation from the CA- or IA-predicted mixture effect depends on the relative proportion of mixture components. For example, in binary mixtures, antagonism can be observed when component A dominates toxicity, whereas synergism can be observed when component B dominates toxicity.

(4) *Dose level dependent deviation*: the deviation from the CA- or IA-predicted mixture effect depends on the overall mixture concentrations. For example, antagonism can be observed at low dose levels while synergism is observed at high dose levels.

In soil, mixture toxicity is complex to study because metals may interact at various levels (Calamari and Alabaster, 1980): (1) the exposure level, (2) the uptake level, (3) the target level, and (4) the internal pathway of detoxification. The first level deals with physicochemical interactions in the soil matrix, influencing sorption (partitioning of metals between soil solid phase and soil solution) and thereby the bioavailable fraction of metals. The second level involves physiological interactions during the uptake processes by the organisms, which affect toxicokinetics and subsequently the quantity available at the sites of action. The third and fourth levels describe interactions of metals at the receptors and target sites, at the intoxication processes, which affect toxicodynamics and hence the joint effect (Weltje, 1998; Conder and Lanno, 2000). Insight into these interaction levels and their relative importance is of great value for toxicity assessment of metal mixtures. This information will help to generalize study results between metal mixtures, as well as between different soil types and organisms.

Incorporation of bioavailability into mixture toxicity modelling

Another challenge in predicting mixture effects results from differences in the bioavailability and approaches used to define the bioavailable fraction among toxicological studies and subsequent ambiguity in interpreting mixture toxicity data (Peijnenburg and Vijver, 2007). For example, different conclusions (antagonistic, more antagonistic, additive) on mixture interactions were drawn when using different expressions of exposure (total soil concentration, $CaCl_2$-extractable concentration, internal concentration) (Bongers, 2007). The 0.01 M $CaCl_2$-extractable metal concentration or porewater concentration is often considered to be a suited estimate of the available metal pool for soil organisms (Peijnenburg et al., 2007). Therefore, all these expressions of exposure are considered in our research. Understanding the chemical interactions in soil could help in the interpretation of different outcomes of studies working with soils of different types.

Biotic ligand models for predicting toxicity of individual metals have been reported for more than a decade (Di Toro et al., 2001; Paquin et al., 2002). Only recently has the BLM been extended to consider mixture scenarios (Jho et al., 2011; Le et al., 2013). The basic assumption underlying the BLM (ion competition) potentially allows incorporating mixture interactions into the assessment of mixture toxicity. If two metals compete for binding to the same biotic ligand, it is possible to estimate the total amount of metal bound to that BL and, hence, to predict metal toxicity using the BLM in a CA model. Alternatively, if competitive binding does not occur, then the BLM may provide more reliable estimates of bioavailability of individual metals, which can then be incorporated into a more accurate IA model.

1.5 Species-specific responses

Metal accumulation and effects are not only driven by abiotic factors like chemical speciation and cation competition. Organisms themselves have developed effective strategies to cope with metal exposure. They have the ability to excrete or eliminate the metal to control metal accumulation and maintain homeostasis over a certain level of exposure (Chapman et al., 1996; Wood, 2001). They are also able to minimize toxic effects of reactive forms of metals in their body by sequestration, detoxification, and storage (Vijver et al, 2004). These physiological processes of organisms have not been accounted for when assessing the risks posed by metals in the environment. Studies have shown that metal accumulation and excretion rates are species dependent. Rapid zinc uptake and elimination was found in *Eisenia fetida* (Spurgeon and Hopkin, 1999), while slow uptake was found in *Lumbricus rubellus* (Mariño and Morgan, 1999). Janssen et al. (1991) studied the accumulation and elimination kinetics of cadmium in four arthropod species and observed large differences between species, which could mainly be attributed to species-specific differences in accumulation strategies. Variations in sensitivity to a given metal have been reported widely between different species. For example, Spurgeon et al. (2000) found that the earthworms *L. rubellus* and *Aporrectodea caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida*. Langdon et al. (2005) reported that the sensitivity of three earthworm species to Pb followed the decreasing order: *L. rubellus* > *A. caliginosa* > *E. fetida*. This can be a result of species-specific differences in physiological characteristics that determine detoxification and elimination strategies (Dallinger, 1993). In addition, the activity of calcium glands in earthworms may partially account for the differences in sensitivity as calcium is involved in the sequestration and elimination of many metals. It has been suggested that the more tolerant species *E. fetida* and *L. terrestris* have a higher calcium gland secretion activity than the relatively sensitive species (Morgan and Morgan, 1991; Spurgeon and Hopkin, 1996). Other species characteristics (e.g., time to maturity, habitat, food choice, immune-competent cells, etc.) may also affect metal accumulation and toxicity (Edwards and Bohlen, 1996; Plytycz et al., 2011a). More research efforts are needed on this aspect to establish solid links between species-specific responses and the relevant traits (i.e., specific characteristics of species) and processes. The exploration of such an approach, that is a traits-based approach (Baird et al, 2008; Rubach et al, 2012), might assist in explaining why one species is more sensitive to metals than another species and allows for extrapolating the results of metal accumulation and toxicity over species.

1.6 Earthworms

Earthworms constitute a large proportion of the total biomass of soil animals and play a vital part in the natural soil ecosystem. They are key species within the soil food web, which is a complex community of soil-dwelling organisms that influence the processes of soil formation (Peijnenburg and Vijver, 2009). They are also valued for their contribution to ecosystem services (i.e., provisioning, regulating, supporting and cultural services) through their action on soil processes (Blouin et al., 2013). Earthworms are commonly considered as soil ecosystem engineers because they benefit the soil ecosystem in a number of ways: mixing soil layers, recycling organic matter, increasing nutrient availability, improving soil aeration, and enhancing microbial activity, et cetera (Edwards and Arancon, 2004; Blouin et al., 2013). As earthworms play a unique role in sustaining the essential functions of soil, detrimental effects of metals on earthworms may indirectly harm soil health and subsequently the whole ecosystem.

Different species of earthworm have different habitats, behaviors, and life histories and occupy different niches within the soil ecosystem (Domınguez, 2004). Earthworm species are generally divided into three different functional groups based on their ecological strategies (Bouché, 1977): epigeic, endogeics, and anecic (Figure 1.4). Epigeic species live in the soil surface layer and feed on litter and organic rich materials. Endogeic species live in the mineral layer of the soil and feed on soil and associated organic matter. Anecic species live in deep vertical burrows but feed on litter at the soil surface (Domınguez, 2004). The Figure 1.4 illustrates where the earthworms live (their habitats), indicating that these earthworm species occupy different ecological niches.

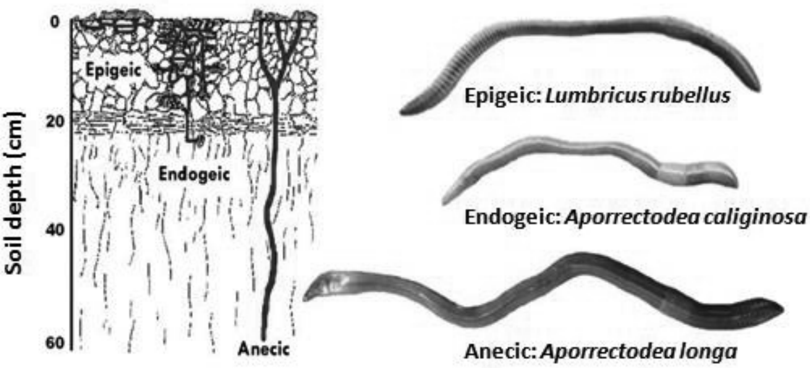

Figure 1.4 Earthworm functional groups and representative species of each group. Figure adapted from Fraser and Boag (1998) (slightly modified).

Earthworms have been considered as feasible biological indicators for metal pollution in soils due to their ecological importance, high sensitivity and great abundance (Dittbrenner et al., 2011; Maleri et al., 2007). A series of test guidelines and protocols have been developed by the Organisation for Economic Co-operation and Development, i.e., "Earthworm Acute Toxicity Tests, Guidelines for the Testing of Chemicals, No. 207" (OECD, 1984), and "Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*), Guidelines for the Testing of Chemicals, No. 222" (OECD, 2004), and by the International Organization for Standardization, i.e., "Soil quality -- Effects of Pollutants on Earthworms -- Part 1: Determination of acute toxicity to *Eisenia fetida*/*Eisenia andrei* ISO 11268-1: 2012; Part 2:

Determination of Effects on Reproduction of *Eisenia fetida*/*Eisenia andrei*, ISO 11268-2: 2012" (ISO, 2012). These guidelines are designed to estimate critical effect levels from concentration-response relationships for survival and/or reproduction of mature earthworms.

Earthworms are ideal organisms for assessing the toxicity of metals to soil-dwelling species, because they are in intimate contact with the soil porewater and the soil solid phase. Thus, they are exposed in a manner representative of many soil species, including bacteria, plants, and other soft-bodied invertebrates. Earthworms have a water-permeable surface epithelium. Although direct uptake via the gut wall (food) cannot be completely ruled out, many findings have suggested that metal uptake by earthworm takes place predominantly via the porewater or via an uptake route that is related to porewater uptake (i.e., the porewater hypothesis) (Saxe et al, 2001; Vijver et al., 2003; Jager et al., 2003; Van Gestel and Koolhaas, 2004). Vijver et al (2003) investigated a method (oral sealing using glue) to distinguish different uptake routes of metals in earthworms and found that the dermal route is the main uptake route. Janssen et al (1997) found that the same soil properties affecting metal partitioning between the soil solid phase and soil porewater were also the dominant soil properties affecting metal accumulation in earthworms. In our study, the porewater hypothesis was adopted. Metal in soil porewater is assumed to dominate toxic effects, which connects the soil solid phase and the organism.

Four earthworm species (*Eisenia fetida, Lumbricus rubellus, Aporrectodea caliginosa and Aporrectodea longa*) were used in the present study. The epigeic *E. fetida* is an artificially cultured species that inhabits only organic matter-rich locations. They are rarely found in natural soil. *L. rubellus, A. caliginosa and A. longa* are the representatives of epigeic, endogeic, and anecic earthworm species living in natural soil, respectively (Figure 1.4) (Spurgeon et al., 2000). The differences in ecological strategies and physiological characteristics between species may strongly affect effective exposure and toxicity (Morgen and Morgen, 1999). Taxonomy is not an inherently informative indicator for prospective risk assessment of metals, as two species of the same genus may show large differences in sensitivity. However, the phylogenetically related aggregations of certain species traits may provide a clue for species-specific accumulation and toxicity (Rubach et al. 2010). It has been suggested that uptake processes are physiologically driven and affected by species specific parameters (traits) such as morphology, soil habitat, feeding strategy and preferences, and life history (Peijnenburg et al., 2012). Species may possess different trait combinations to cope with a particular disturbance (De Lange et al. 2013). Incorporating traits-based approaches in metal toxicity assessment is supposed to give more explanatory power and allows for extrapolation of results of metal accumulation and toxicity between different earthworm species.

1.7 This thesis

Objective

As reviewed above, uncertainties or deviations still exist when predicting the adverse effects of metals and metal mixtures on soil organisms in different exposure scenarios. This thesis has the overall aim to improve ecological risk assessment for metals through better understanding bioavailability and developing appropriate bioavailability models for soil organisms. Therefore, the specific goals of this thesis were:

1. To develop bioavailability models to facilitate the application of BLM theory in soils;
2. To model mixture toxicity by taking into account the interactions of mixture components at different toxicological levels;
3. To extrapolate the study results to other studies reported in the literature.

To accomplish these goals, the following research questions were addressed:

[1] Which cations (H^+, Ca^{2+}, Mg^{2+}, K^+ and Na^+) exert significant effects on metal toxicity and how could these toxicity-modifying factors be incorporated into terrestrial toxicity models developed on the basis of the BLM theory? (Chapters II and III)

[2] Are the toxicity-modifying factors the same for different earthworm species (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) and for different metals (Cu, Cd, and Ni)? (Chapters II and III)

[3] Are metal (Cu, Cd, Ni, and Zn) accumulation pattern and sensitivity of earthworms species-specific? Can species-specific traits of earthworms provide a clue for predicting metal accumulation and toxicity? (Chapter IV)

[4] Where do the interactions of mixture components (Cd and Zn) possibly occur and how do they impact the observed toxicity? (Chapter V)

Biotic ligand models are increasingly being developed and applied to relate toxic effects of metals on aquatic organisms to activities of the free metal ion and competing ions. We have adopted the recent hypothesis that this modelling theory is also applicable to organisms in the soil environment. It is expected that free metal ions in soil porewater are mainly responsible for toxicity while the base cations can mitigate toxicity through competition for binding to the biotic ligand sites. Trait-based approaches are used to assist in explaining differences in metal accumulation and toxicity between earthworm species living in different habitats, with ultimately the idea to enable cross-species extrapolation of accumulation and toxicity data. Since mixture exposure represents realistic field scenarios and since mixture interactions at different toxicological levels may impact the actual risks, attempts are made to determine where mixture interactions could occur and to quantify the toxicity deviations from simple concentration addition, ultimately ensuring adequate risk assessment.

Thesis outline

This Chapter provides an overview of the basic principles of speciation, bioavailability and effect modelling of heavy metals in the terrestrial environment, particularly with regard to the development of biotic ligand models and mixture toxicity models. The main issues were highlighted and ways to tackle the associated problems were discussed.

The research questions are answered in the following chapters:

Chapter II: A Freundlich-type model, rather than the biotic ligand model, was proposed to link Cu toxicity in different earthworm species (*L. rubellus*, *A. longa* and *E. fetida*) to free Cu ion activities and activities of possibly protective cations in soil porewater. This model complies with the basic assumptions of the biotic ligand model but requires fewer parameters than the biotic ligand model, thus facilitating the application of biotic ligand model principles in soil exposure systems. The possibility of extrapolating the study results to other studies reported in literature was also explored.

Chapter III: When applying the BLM to the soil system, parameterization of the BLM faces a variety of challenges and uncertainties. Based on empirical studies and BLM theory, here we proposed an alternative method, the free ion approach, to predicting Cd and Ni toxicity to earthworms (*L. rubellus* and *A. longa*). Previously, the applicability of the free ion approach for describing Cu toxicity has been proven. However, the toxicity-modifying factors (H^+, Ca^{2+}, Mg^{2+}, Na^+, and K^+) are shown to be different for other metals. Results obtained from our study strongly suggest that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

Chapter IV: It is often stated that there is not enough ecology in ecotoxicology and that this lack can have unfortunate consequences for environmental risk assessment. Here we examined the differences in metals (Cu, Cd, Ni, and Zn) accumulation and toxicity in three earthworm species (*L. rubellus*, *A. longa* and *E. fetida*), with a special focus on the impact of earthworm traits. In this study, the ecophysiological differences between earthworms were identified and used to assist in explaining metal accumulation patterns and sensitivity. These species-specific traits of earthworms are expected to provide a clue for extrapolation across species.

Chapter V: The toxic unit method was used to quantify the mixture (Cd and Zn) toxicity in one earthworm species (*A. caliginosa*). Deviations caused by mixture interactions were assessed using the MIXTOX model. Interactions associated with different expressions of exposure (total, $CaCl_2$-extractable, and porewater concentrations) were compared. In soil, mixture toxicity is complex to study because interactions of metals can occur at various levels. By separating the interactions at the exposure level from the uptake level and the target level, it was determined where the interactions possibly occur and their influence on the toxicity pattern of binary metal mixtures was subsequently assessed.

Chapter VI: The results reported in Chapter II-V were summarized and synthesized. Based on the synthesis, this chapter further provides recommendations for the potential application of the developed models and approaches, and discusses briefly the areas of future research.