Quantitative modelling of the response of earthworms to metals

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Chapter VI

General Discussion

A comprehensive understanding of the mechanisms of toxicity is needed in order to effectively predict metal effects on soil organisms. In this PhD thesis, we investigated the responses of different earthworm species to single metals (Cu, Ni, Cd, and Zn) and a binary mixture of Cd and Zn in metal-spiked soils. These soil toxicity data allow to account explicitly for the role of bioavailability, ecophysiological factors and mixture interactions in determining metal toxicity.

Our research was based upon four research questions, which are answered in the preceding four chapters:

[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).

The biotic ligand model is often used as the state-of-the-art approach to quantify the link between chemical availability and metal toxicity in aquatic systems. It is a synthesis of decades of work on metal speciation, accumulation, toxicity and physiology (Paquin et al., 2002). In this thesis, we tried to apply the BLM theory to soil organisms, with a special focus on earthworms. The possible dependence of metal accumulation and toxicity on species-specific traits was investigated. Integration of effect models and traits-based approaches may offer a means to facilitate extrapolation of accumulation and toxicity data cross species. We also distinguished the mixture interactions at different levels and determined the impact of mixture interactions on the observed toxicity. Findings in this PhD thesis open the perspective of developing a mechanistic framework that is generally applicable to predict the effects of metals and metal mixtures on soil organisms.

This concluding chapter provides a synthesis of the preceding four separate chapters, which have been published as independent papers in peer-reviewed journals. Prior to an integrated discussion and a future outlook, answers to the research questions are provided below.

6.1 Answers to research questions

Research question 1: Only H^+ not other cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) exerted a significant role in alleviating Cu toxicity to earthworms (Chapter II). Mg^{2+} was identified as the sole factor significantly alleviating Ni toxicity, while no significant influence of cations (H^+, Ca^{2+}, Mg^{2+}, K^+ and Na^+) on Cd toxicity to earthworms was observed (Chapter III). A multicomponent Freundlich model, complying with the basic assumptions of BLM theory, was used to link Cu toxicity to free Cu^{2+} activity and possible protective cations (i.e., H^+) in soil porewater (Chapter II). The Freundlich-type model in which the protective effects of H^+ were incorporated, accounted for 84% to 96% of the variation in LC50$\{Cu^{2+}\}$ for earthworms. Based on empirical studies and BLM theory, the free ion approach was proposed to quantify Ni and Cd toxicity (Chapter III). By incorporating the competitive effects of Mg^{2+} into the free ion approach, more than 84% of the variation in LC50$\{Ni^{2+}\}$ for earthworms was explained. The free ion activity model, which is a special case of the free ion approach with no impact of cations, sufficiently described the variability in LC50$\{Cd^{2+}\}$.

Research question 2: The same toxicity-modifying factor (pH) was identified for Cu toxicity to the three tested earthworm species *L. rubellus*, *A. longa* and *E. fetida* (Chapter II). External validation also showed a pH dependence of Cu toxicity for other soil organisms with different endpoints (*E. fetida* cocoon production, *Folsomia candida* juvenile production, *Hordeum vulgare* root elongation, *Lycopersicon esculentum* shoot yield, glucose induced respiration, and potential nitrification rate). The same factor (Mg^{2+}) was found to significantly modify Ni toxicity to the two tested earthworm species *L. rubellus* and *A. longa*. No significant toxicity-modifying factor was observed for Cd toxicity to *L. rubellus* and *A. longa* (Chapter III). There was a lack of consistent effects from the presence of possible competing cations for different metals. Based on these findings it is concluded that metal toxicity to earthworms needs to be evaluated on a metal-specific basis.

Research question 3 (Chapter IV): Accumulation and toxicity of Cu, Cd, Ni, and Zn in the earthworms *L. rubellus*, *A. longa* and *E. fetida* were species-specific. At the same exposure concentration, internal concentrations followed the order: *L. rubellus* > *E. fetida* > *A. longa* for Cu and Ni, *L. rubellus* ≈ *E. fetida* ≈ *A. longa* for Cd, and *L. rubellus* > *A. longa* > *E. fetida* for Zn. The concentrations of Cu, Cd, and Zn in *E. fetida* generally levelled off at high exposure concentrations but not for the other two species. *A. longa* showed a high capability of regulating internal Ni concentrations. Based on traits theory, a set of traits (such as soil habitat, mobility, ratio of surface area to mass) can be related to the metal accumulation ranking of the earthworm species tested. However, implementing the traits theory for quantitatively explaining all the observed accumulation pattern of different earthworm species was shown to be difficult. For all the four metals tested *L. rubellus* was the most sensitive species, followed by *A. longa* and E. *fetida*. Differences in sensitivity between species might be explained using the following traits: soil habitat, calcium glands activity, ratio of surface area to mass, and immune-competent cells. While, ranking of species sensitivity cannot be quantitatively linked to one specific trait. The traits-based approaches suggested that most likely a group of earthworm traits together determined (differences in) metal accumulation and sensitivity.

Research question 4 (Chapter V): The interactions between Cd and Zn in soil (at the exposure level) were estimated by comparing partition coefficients of one metal in the presence and absence of the second metal. The partitioning of Cd and Zn between soil solid phase and porewater was affected neither by the concentrations nor by the presence of the other metal, suggesting no interactions at the exposure level. Simultaneous exposure to Cd and Zn in soil jointly affected the mortality of the earthworm *A. caliginosa*. Increasing Cd concentration decreased Zn toxicity. The addition of Zn over a certain range (100 to 1000 mg/kg) decreased Cd toxicity. Beyond a critical Zn concentration of around 1000 mg/kg, Cd toxicity dramatically increased. Interactions of Cd and Zn occurred at the organism level, limiting the predictive ability of the simple concentration addition model. MIXTOX modelling showed that mixture interactions were mainly antagonistic and the magnitude of antagonism depended on both the relative concentrations of Cd and Zn and the concentration magnitudes for the whole ranges of concentrations tested. These findings highlight the importance of identifying the relative influence of various interactions from external exposure to internal assimilation in assessing metal toxicity.

6.2 Applicability of BLM theory in soil

The results in this thesis provide evidence in favour of the applicability of BLM theory to soil organisms, especially earthworms, exposed in a range of metal-spiked soils of varying properties (Chapter II and III). Applying the BLM theory to soil toxicity data has rarely been done before. Our research therefore added new knowledge and provided alternative tools with regard to the explanation and prediction of the variability in Cu, Cd, and Ni toxicity across soils.

The BLMs for aquatic organisms have been well developed and scientifically understood (Niyogi and Wood et al., 2004; Paquin et al., 2002; Santore et al., 2002). Some of them have even been incorporated into water quality regulations, for example, the US Environmental Protection Agency's aquatic life freshwater quality criteria for copper (USEPA, 2007). And, in the EU risk assessment reports, the BLMs for chronic toxicity of Cu, Ni, and Zn have also been recommended for regulatory use (Denmark, 2008; ECI, 2008; The Netherlands, 2008). A major strength of the BLM is that it provides an operational framework for modelling bioavailability and toxicity under various environmental conditions. Because of assumed similarity in toxicity mechanisms between aquatic and terrestrial organisms (as explained in paragraph 1.3 of Chapter I), it has been postulated that the BLM can be applied to terrestrial organisms. In this thesis, we therefore aimed to expand the scope of BLM beyond the initial emphasis on metal toxicity in the aquatic environment to metal toxicity in the soil environment. However, the complexity of the soil system presents a barrier of developing biotic ligand models for soil organisms exposed in real soils.

The routes of metal uptake in soils are generally more complex than those in solution systems. It remains problematic to determine the relative importance of each exposure pathway (porewater, ingestion of food and soil particles) for different soil organisms (Steenbergen et al., 2005). The morphological (e.g., structure of the skin), physiological (e.g., mode of uptake of water and oxygen), and behavioral properties (e.g., food choice) of organisms are important variables in this respect. Uptake via the skin is the most important route of uptake of (pore)water and oxygen for earthworms and other soft-bodied organisms (e.g., enchytraeids, nematodes), while hard-bodied organisms (e.g., arthropods) rely for uptake of (pore)water and oxygen on specialized organs (Peijnenburg et al., 2012). This has consequences for the validity of the porewater hypothesis, which is a premise for applying BLM theory in soils. In our study, the Freundlich-type model and the free ion approach, complying with the basic assumptions of the BLM, well described metal toxicity to earthworms with dermal uptake of porewater as the main exposure route. However, these models may not be valid for other soil organisms with distinct exposure routes. The BLM theory in soil is more difficult to prove for soil microorganisms. When extrapolating our models to two microbial processes (glucose induced respiration and potential nitrification rate), the model performances were generally weaker than those for soil invertebrates and plants (Chapter II). Soil microorganisms are usually attached to soil particles, implying that the local composition of the microbe bathing solution may differ from soil porewater that can be sampled (Ore et al., 2010). Mertens et al. (2007) developed a model based on BLM theory to predict Zn toxicity to nitrification by *Nitrosospira* sp. in soil and found that there were up to 4 times over- and under-prediction. The main obstacle in evaluating metal bioavailability and toxicity to soil microorganism lies in the difficulty in defining the correct solution to which microorganisms are exposed (i.e., from which pool microorganisms take up metals). Therefore, before explaining and predicting metal toxicity with the BLM theory, uptake routes of the test organism need to be understood.

Besides, unlike simple solution systems it is difficult to univariately manipulate the composition of soil porewater and to separate the effects of each cation on toxicity. Challenges are met in estimating reliable and representative BLM parameters from soil toxicity data. To overcome this, previous attempts to model metal toxicity to soil organisms were often done in hydroponic solutions. But the problem is that model parameters fitted from solution cultures do not match one on one with soil cultures. In this thesis we focused on developing alternative bioavailability modes in order to facilitate the application of BLM theory in soils. The developed Freundlich-type model and the free ion approach, which share the same theoretical basis, allow us to incorporate metal speciation and interactions between metal ions and the possible competing ions in estimating metal toxicity. When applying the BLM directly to soil toxicity data, Thakali and co-workers (2006a; 2006b) found that it is necessary to empirically fix some parameters (e.g., f_{50}) before fitting the binding constants for each cation. The effectiveness, success, and regulatory relevance of BLM benefit from its mechanistic basis, which will suffer if BLM development becomes too much a data-fitting exercise based on faith that simple implementations of the approach are sufficient (Erickson, 2013). The multicomponent Freundlich model or the free ion approach avoids the need to fit separate binding constants for the metal ion and potentially competing cations as the parameters α (n_H/n_M) and β_i (n_{Xi}/n_M) express the binding affinities of H^+ and other cations relative to metal ions. This indicates that these two models require fewer parameters than the common BLM. In the BLM, biotic ligands are considered to be independent and homogeneously distributed, most often represented by a single ligand-binding constant (Slaveykova and Wilkinson, 2005), while the Freundlich-type model considers site heterogeneity. The biological surfaces usually contain multiple sites including physiologically active sites (i.e., biotic ligands or transport sites) and nonspecific, physiologically inactive sites (e.g., cell wall polysaccharides, fish mucus) (Van Leeuwen and Pinheiro, 2001). Several studies have shown the presence of multiple adsorptive sites (Plette et al., 1996) and multiple metal internalization routes (Hassler and Wilkinson, 2003; Sunda and Huntsman, 1998; Taylor et al., 2000). For instance, two different types of Cu-binding sites (high-affinity, low-capacity sites and low-affinity, high-capacity sites) have been identified on the gills of rainbow trout (Taylor et al., 2000). Therefore, site heterogeneity is expected to be important for metal toxicity. Given these considerations, the Freundlich-based models do have some conceptual and practical advantages over the BLM.

The LC50s for earthworms predicted by the Freundlich-type model or the free ion approach both were generally within a factor of two of the observed values. External validation of the model showed a similar level of precision, even though toxicity data for different soil organisms and for different endpoints were used. A factor of two is an accuracy that is commonly used in risk assessment (Van Leeuwen and Hermens, 2007). Moreover, this is the accuracy that is typically obtained due to biological variability when an ecotoxicology experiment is repeated (Jager, 2013). Our findings therefore support the applicability of BLM theory for terrestrial species exposed in soil. In spite of this accurate prediction, there were shortcomings to the models. Statistical analysis revealed a strong correlation between a fair amount of porewater parameters (Chapter III). In other words, the possible effect of each individual cation cannot be isolated and verified directly. The evident ameliorative effects of cations (e.g., Mg^{2+} on Ni^{2+}) are possibly due to the covariance of their activities with Ni^{2+} activities rather than their competitive binding at the biotic ligand sites. Here, stepwise multiple linear regression analysis and the Akaike information criterion were used to identify the variables significantly determining toxicity whilst at the same time balancing the complexity and the goodness-of-fit of the model. An important implication of our studies is that the most useful approach to model and predict effects of metals on organisms will likely involve trade-offs among the predictive ability required, porewater parameters and the cost to obtain them.

For the purpose of mechanistic studies, a solution system is often used in the toxicity test to provide direct evidence for the protective effects of cations by univariately controlling the composition of the test solutions. Previous studies with soil invertebrates and plants exposed in simple solution systems show that the application of the BLM to terrestrial organisms is theoretically and empirically feasible (Steenbergen et al., 2005; Li et al., 2008; Lock et al., 2007). This kind of research therefore lends credence to the theory behind our terrestrial models. Ideally, the results obtained in the solution system can be qualitatively or quantitatively extrapolated to natural soils. For qualitative validation, it needs to be checked whether the identified toxicity-modifying factors in solution cultures are also significantly affecting metal toxicity to organisms in soil cultures. For quantitative validation, it needs to be checked whether the same BLM parameters (binding constants and f_{50}) hold for the tested species in soil. In reality, extrapolation of the developed BLMs from solution to soil systems is generally difficult as the geochemistry of solution systems often bears little resemblance to those of real world soils (He et al., 2014). A structural underestimation of metal toxicity was found when directly applying the BLM developed from solution-sand system to soils (Koster et al., 2006). This justifies the necessity for developing models directly from soil exposure systems.

It is noteworthy to mention the work on the development of empirical bioavailability models for soils that are used in EU regulatory settings (ECB, 2009). These models were developed after it became clear that terrestrial biotic ligand models for metals like Cu, Ni, and Zn were not readily feasible. For example, Smolders et al. (2009) showed that effects of Ni (and other metals) on soil organisms were best predicted by empirical relationships based on soil CEC and pH, and not on concentrations or activities of metal in relevant soil phases, such as porewater. This does not necessarily contradict the concept that the free metal ion in (soil) solution is the directly available and toxic species. This is readily explained by the BLM theory, which states that free ions dominate toxicity while cation competition will modify toxicity. For instance, in a recent study by Ardestani and Van Gestel (2013), it was found that soil pH had surprisingly little effect on Cd uptake in a springtail species (with a porewater-related uptake route), while there was a strong increase of Cd availability in the porewater with decreasing soil pH. The absence of a pH effect on Cd uptake kinetics could be explained from processes at the level of proton competition with Cd, which occurred at the level of porewater and were not visible when looking at the level of the soil. These aspects should be borne in mind when looking at different conclusions drawn respectively from empirical relationships for linking toxicity to soil properties and mechanistically underpinned models for linking toxicity to porewater chemistry (e.g., the Freundlich-type model).

6.3 Cross-species extrapolation

In order to generalize the developed bioavailability models within a regulatory framework, it is important to assess how variable the effects of porewater chemistry parameters on metal toxicity are across different soil species. Because it is unrealistic to develop BLM-based bioavailability models for all soil species, the earthworms were used as representatives for a larger group of major soil macrofauna. In Chapter II and Chapter III, we investigated whether the bioavailability model developed for one earthworm species could be extrapolated to another earthworm species. We further evaluated whether this model could be extrapolated to other soil species with different toxicological endpoints, based on literature data. Toxicity-modifying factors (e.g., H^+ for Cu toxicity, Mg^{2+} for Ni toxicity) were the same for different earthworm species, providing qualitative evidence for the option of extrapolating the model concept across species. A pH dependence of Cu toxicity was further found for soil invertebrates, plants, and microbial processes in a range of European soils. This confirms the applicability of the developed model and suggests that earthworm species well represent other species, especially those species that are in close contact with porewater or have porewater-mediated uptake of metal. Due to the fact that the effect of pH and other cations on metal toxicity differed for different species (as reflected by the model coefficients α and β_i, see Table 2.3 and Equations 3-8 and 3-9), quantitative extrapolation of the model is difficult. Based on our findings, a generalized model framework (i.e., $\log LC50\{M^{2+}\} = \alpha\ pH - \sum \beta_i\{C_i^{z+}\} + \gamma$) with separate coefficients for each model parameter can be created to predict the effect concentrations of metals for different species and different toxicological endpoints.

The topic of Chapter IV deals with important issues of differences in metal accumulation and sensitivity between earthworm species. These topics are worth to study, because they are connected with the extrapolation of results of ecotoxicological tests conducted mainly on *Eisenia fetida* (the so-called "white mouse" among earthworm species).

Expect for the artificially cultured species *E. fetida*, two field-relevant species *L. rubellus* and *A. longa* were tested. Metal accumulation patterns and sensitivity varied largely among the different earthworm species (*E. fetida*, *L. rubellus*, and *A. longa*), although these species belong to the same class (Oligochaeta). This indicates that the taxonomy is not an inherently informative descriptor for metal effects. Some studies have suggested that phylogenetically related aggregations of certain traits could provide a useful and alternative description of species-specific responses (Barnett et al., 2007; Rubach et al, 2010). In our study, the use of traits instead of species improved our understanding of earthworm responses to metal stress, providing the possibility for intraspecies and interspecies extrapolation. Most likely more than one or two traits determined species-specific accumulation and toxicity of metals in earthworms. The use of the traits-based approach for quantitative extrapolation of accumulation and toxicity data across species still needs research efforts. The traits-based approach has been widely used in ecological and evolutionary research (Violle et al., 2007). Challenges facing the future development of traits-based approaches in ecotoxicology are met in selecting relevant traits, quantitatively integrating these traits, and linking traits with mechanistic effect models. In these respects, qualitative comparative analysis (Caren and Panofsky, 2005) could be adopted to provide indications on which traits are of most interest in explaining variations among species. Alternatively, the trait frequency analysis and multivariate analysis (De Lange et al., 2013) could be suitable methods for quantitatively describing the relation between traits and earthworm responses.

6.4 Interpretation of mixture effects

As metals are often introduced into the environment as mixtures, assessment of mixture effects is extremely relevant. The classical way of evaluating mixture toxicity is to use either the concentration addition model or the effect addition model as a reference. Interpretation of the observed interactions is therefore often limited to overall antagonism or synergism. In this thesis, more complex patterns such as dose-ratio dependent and dose-level dependent deviations from the reference model were quantified by the MIXTOX model for a binary mixture of Cd and Zn in one soil type (Chapter V). A broad range of concentration combinations (239 combinations) was employed, assuring the identification of possible deviations. Only a limited number of replicates were conducted in order to allow covering as much as possible the exposure range of both metals. Therefore, effort was devoted to the number of combinations at the expense of the number of replicates. This will not bias the interpretation of obtained results because the concentration spacing in our study is generally small and the response surface analysis for determining interactions is based on a regression model (Jonker et al., 2004, 2005). Besides, the repeated tests showed favorable reproducibility (LC50 values of duplicates were within 10% deviation).

With respect to the mixture effects, a general conclusion on interaction patterns and interaction levels cannot be drawn due to the specificity of interactions in terms of metal combination and soil being tested. In this thesis, we found that interactions of Cd and Zn were mainly antagonistic, and the magnitude of antagonism depended on both the relative concentration of each metal and the concentration magnitudes. Baas et al. (2007) investigated the lethal effect of a binary mixture of Cu and Cd on the springtail *Folsomia candida* and found that interactions of Cu and Cd were mainly synergistic. In our case, interactions of Cd and Zn occurred at the organism level but not at the exposure or soil level, while Van Gestel and Hensbergen (1997) observed physicochemical interactions of Cd and Zn when exposing *Folsomia candida* to an artificial soil. In a study by Sdepanian (2010), mixture interactions (Cu and Zn, Cd and Zn) in four soils were suggested to be related to a greater extent to the speciation effect (i.e., interactions at the exposure level) than to the interactions at the organism level. While an integrated interpretation of mixture effects is not possible, the above findings clearly show the importance of identifying interactions at relevant levels and of incorporating bioavailability effects in modelling mixture toxicity. This can be especially helpful to explain differences in interaction patterns that occur between different soil types.

Different expressions of exposure (total, porewater, and $CaCl_2$-extractable metal concentrations) yielded similar interaction patterns for Cd and Zn mixtures. This may be because of the fact that only one sandy loam soil was tested and strong correlations existed between total concentrations, porewater concentrations and $CaCl_2$-extractable concentrations (see Table 5.2). It is therefore suggested that more soil types should be tested for the purpose of comparison and extrapolation. To get the full picture of bioavailability effects on mixture toxicity in soil, metal speciation, competition and complexation, and interactions with organisms should be explicitly considered. Mixture modelling might be expanded with mechanistic bioavailability models such as the BLM and the electrostatic toxicity model (Wang et al., 2008) to optimize accurate effect prediction. In mixtures, metals may interact with each other to different extents (Vijver et al., 2010). Assuming that competition acts as a mechanism for metal mixture interactions (Niyogi and Wood, 2004), the use of BLM-based approaches for interpreting mixture effects is suitable. Jho et al. (2011) extended the BLM to predict the toxicity of Cd and Pb at different concentrations of Ca^{2+} using single metal toxicity data, and obtained good agreement between observed and predicted effects. The electrostatic toxicity model assumes that metal uptake and toxicity are determined by the ion activity at the surface of the cell membrane. Cations (e.g., Ca^{2+}, Mg^{2+}, and H^{+}) in the bulk solution can reduce the negativity of the electrical potential at the surface of the cell membrane by charge screening and ionic binding (Kinraide et al., 1998; Wang et al., 2011b), which in turn reduce toxic metal ion activities at the surface of the cell membrane. This modelling approach allows incorporating the effects of various cations simultaneously in modelling mixture toxicity and may provide mechanistic insights (in addition to competitive binding) into mixture interactions.

6.5 Implications for ecological risk assessment and soil quality criteria derivation

Ecological risk assessments for metals in the environment include two aspects: prospective and retrospective (Brock et al., 2013; Posthuma et al., 2008). Prospective risk assessment concerns the evaluation of the probability of adverse effects of metal exposure in the environment before the marketing, release, and use of the metal. It is a predictive approach which often uses toxicity data from standard laboratory toxicity tests to derive critical limits of metals in the environment (Van Gestel, 2012). Retrospective ecological risk assessment concerns the adverse impact of existing metals in the environment. It is a diagnostic approach which makes use of measured exposure concentrations or biological effects in the exposed environment and enables setting priorities for remediation. The outcome of toxicity tests in this PhD thesis can be used to establish critical limits of metals in soil, which can then be compared with the measured or predicted exposure levels to determine the potential risk for metal contaminated sites. The developed bioavailability models (Chapter II and Chapter III) provide a mechanistic framework to normalize Cu, Cd, and Ni toxicities to a range of terrestrial species among different soils. These models directly link the toxicological endpoints to soil porewater chemistry, which in turn is linked to soil properties. The ability of the parameterized bioavailability model to predict toxicity on the basis of total soil metal is useful for standard-setting and risk assessment purposes. To set critical loads of metals for soils, WHAM VI can be used to calculate the total metal concentration in equilibrium with critical free metal ion activities (e.g., LC50$\{Cu^{2+}\}$) in soil porewater. An example can be found in the BLM work of Thakali et al. (2006a). Uncertainties in calculated critical loads for a large part lie in the calculation of solution speciation. For the soil solution where natural dissolved organic matter (DOM) is present, it is often needed to specify the ratio of active humic acid to fulvic acid, and the transfer coefficient between DOM and dissolved organic carbon (DOC) (usually, DOM = 2 DOC) as the physicochemical characteristics and thermodynamic data (such as reactions with metal ions, stability constants, and the number of reactive sites) of DOM are not clearly understood (Tipping et al., 2003; Dudal and Gérard, 2004). In addition to the mechanistic speciation calculation, the developed bioavailability models could readily be coupled to an empirical partitioning model to enable the link to total soil metal concentration to be made. For example, Lofts et al (2004) proposed that free metal ion activity ($\{M^{2+}\}$, mol/L) in soil solution can be predicted based on total soil metal ($[M]_{soil}$, mg/kg), soil pH and soil organic carbon content (OC, %) with the following equation:

$$\log (\{M^{2+}\}) = a \log ([M]_{soil}) + b \text{ pH} + c \log (\text{OC}) + d \qquad (6\text{-}1)$$

where a, b, c and d are constants. With this relationship, we can easily relate, for example, the obtained critical free Cu^{2+} activity to critical total soil Cu concentration and other commonly measureable soil properties.

The current approaches for setting soil quality criteria for metals (e.g., NOECs) are strongly dependent on critical values, for example EC50s and LC50s of metals, obtained at standardized conditions. For derivation of these critical values, basic information on fate, exposure, and toxicity is investigated. However, the derivation following precautionary principles must be conservative as the critical values do not consider site-specific conditions. Exceeding the critical values does not necessarily imply a risk. This disadvantage can be overcome by using the BLM-based bioavailability models to link critical values to varying environmental conditions (e.g., pH, competing cations, and DOC). Legislators and regulators are moving forward to incorporate measures of bioavailability into regulations regarding water and soil quality. The BLM approach has been adopted by the USEPA to set aquatic life criteria for copper (USEPA, 2007). It has also been proposed for use in European Union risk assessments (Ahlf et al., 2009). In our study, the developed bioavailability models comply with the BLM theory. Their data requirements are small compared with BLMs and only routine parameters are needed as model inputs. Within regulatory frameworks, these models can help to remove the influence of test-specific abiotic conditions in the ecotoxicity database and to derive appropriate soil quality criteria.

Improving the accuracy of ecological risk assessment for metals is a primary benefit of bioavailability analysis (Ehlers and Luthy, 2003). A tiered approach which incorporates bioavailability for performing risk assessment of contaminated sites is recommended by Kördel et al. (2013). The different steps are modified and illustrated in Figure 6.1. As a start, historical examination and data gathering are needed (Step 1). If a metal contamination is suspected, soil sampling and analysis of the expected metal are performed (Step 2). A further bioavailability analysis must follow if trigger values (based primarily on total contents in current regulations) are exceeded (Step 3). The bioavailability analysis involves the determination of metal-specific exposure for specific objectives of protection and measurement of relevant bioavailability parameters (e.g., pH, competing cations). The modelling approaches developed in this thesis (Freundlich-type model and free ion approach) provide a more accurate characterization of bioavailability than traditional chemical extraction methods or empirical relationships, and these approaches thus fit well into step 3. Exceeding the trigger values corrected for bioavailability indicates a risk for a specific site. In this case, appropriate measures such as remediation or securing should be implemented.

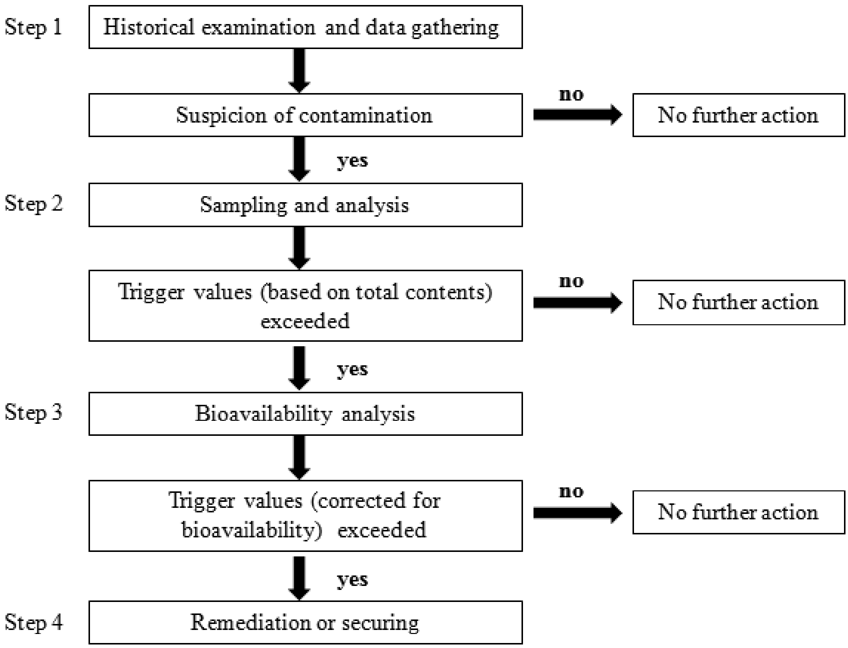

Figure 6.1 Incorporation of bioavailability in risk assessment of contaminated sites (adapted from Kördel et al., 2013, slightly modified)

Despite the fact that metals are commonly present as mixtures in the environment, mixture toxicity is not explicitly taken into account when evaluating the risks posed by metals in soil. Uncertainty factors (usually from 10 to 1000) are commonly used in risk assessment to deal with statistical uncertainties in the estimation of NOECs, biological variance, intra- and inter-species extrapolations, and extrapolation from acute to chronic effects (Posthuma et al., 2008). In the common practice of soil protection, it is sometimes argued that uncertainty factors already cover the possibility of mixture effects. But a specific mixture assessment factor is currently not employed in chemical-by-chemical risk assessment (Kortenkamp et al., 2009). It still needs to be clarified how large a mixture assessment factor would need to be to take proper account of mixture effects. In the practice of chemicals legislation, even the new European chemical legislation REACH focuses almost exclusively on the assessment of individual chemicals. Under this legislation, industry takes the responsibility for demonstrating the risks of the manufacture, use, and disposal of chemicals (Lahl and Hawxwell, 2006). Industry thus only cares about the limited number of individual chemicals they produce and thus ignore mixture effects in general. Our case study showed that interactions of Cd and Zn at the organism level significantly influenced the observed toxicity to earthworms. For effective and accurate risk assessment of metal mixtures it is required to have appropriate models or tools that enable the prediction of mixture effects, which covers both simple and complex mixtures and incorporates mixture interactions. A general tiered outline for the predictive ecotoxicological risk assessment of chemical mixtures is proposed by Backhaus and Faust (2012). Since the mode of action and interactions are not clearly understood, they suggest a sequential application of CA and IA in two tiers. The first tier uses exclusively CA as a basis for the preliminary risk assessment of the mixtures of concern, as mixture toxicities higher than predicted by CA are rare findings. Only if there are still indications for a potential risk, the second tier commences. This tier takes IA into consideration. Detailed information on this method can be found in the cited literature. As mentioned before (paragraph 1.4 of Chapter I), CA as well as IA assumes a non-interaction between the mixture components. Interactions may lead to either increased or decreased toxicity. Such cases cannot be properly handled in a generalized risk assessment scheme. The type and magnitude of interaction can vary because of its dependence on dose ratio and dose level as shown in our cases (Chapter IV). This raises questions about how these detailed aspects should be taken into account in the context of risk assessment and standard setting for a ‘cocktail’ of metals. With current scientific knowledge, it is not yet possible to predict responses to mixture effects in individual cases; at best this is possible only in terms of general response patterns (Vijver et al., 2011). Nevertheless, the assumption of additivity provides a conservative prediction of mixture toxicity, because metals act either additively or antagonistically in more than 70% of all cases as reviewed by Norwood et al. (2003) and Vijver et al. (2010; 2011). This means that mixture toxicity will be either predicted correctly or overpredicted. From a regulatory perspective, such a conservative approach is to be favored in a generic risk assessment scheme. It is only the exceptional cases (synergistic effects) that require further considerations and additional approaches.

6.6 Future outlook and recommendations

The findings of this thesis present a clear indication of the importance of bioavailability when modelling metal toxicity in soil. While the bioavailability models were developed on the basis of toxicity experiments performed in different soils covering a wide range of soil properties, the future use of these models may be limited to the range in soil properties for which they were derived. Nevertheless, we have reasons to be optimistic about regulatory application of the developed bioavailability models since they provide a mechanistic framework and can be easily modified to incorporate the effects of other toxicity-modifying factors. A series of validation studies and meta-analysis using our model concepts are encouraged to be done in future. Besides, it would be possible to apply our models to geographically oriented databases in order to do GIS-mapping of the site-specific risks of metals on a large scale, as long as the basic soil information (i.e., porewater chemistry) required by the models is available.

Various single-species screening tests are often employed to detect possible harmful effects of metals in soil. In this thesis, earthworms were selected as the test species as they are the dominant soil macrofauna and play a vital role in soil functioning. However, numerous species and a complex food web are exposed in the soil environment. Therefore, a battery of toxicity tests should be used to evaluate the effects on different trophic levels, as well as acute, chronic, and next-generation effects. Until now, very few research efforts have been made to determine the minimum battery of tests needed. To obtain a balanced battery of tests, it is recommended that a series of important criteria for selecting representative species among others need to be met, such as having different life-histories and exposure routes, and belonging to different functional and taxonomic groups. Besides, the selection of a battery of tests should be tailored to specific protection objectives.

Most of the bioavailability models are developed at a fixed time point, while the toxicodynamic part of the model is simply to relate the bioavailable faction (e.g., f value in BLM) to a toxicological endpoint (e.g., LC50 or EC50). This is also the case in our study. However, it has been recognized that bioavailability is not a static but a dynamic phenomenon (see Figure 1.1). For a better understanding of metal bioavailability, the underlying toxicokinetic and toxicodynamic processes deserve further investigation. This kind of research will enable the extrapolation of metal effects in the course of time and from soil organisms to higher organisms.

Our study distinguished the interactions of Cd and Zn at the exposure level from those at the organism level. However, the interactions of metal mixtures observed in this study yield little information about the internal processes involved. In fact, mechanistic pathways of metals inside the organism are poorly known. We therefore recommend that future studies should investigate the mechanisms of mixture interaction and identify the principles of combined toxicity for developing predictive models. Potential topics include, for example, considering the distribution and detoxification mechanisms (i.e., toxicological bioavailability) of one metal in the presence of other metals, and linking mixture effects to the expressions of specific proteins or genes (proteomics and genomics tools).

The issues of metal bioavailability and mixture toxicity have increasingly gained attention on the agenda of risk assessors and risk managers. The findings in this thesis therefore well fit into the broader picture of soil metal risk assessment. Despite the lack of mechanistic understanding of internal processes, the awareness of bioavailability and mixture toxicity has triggered the development of a series of chemical and modelling approaches to normalize the toxicity data in different environmental conditions, and thus has greatly improved risk assessment and standard setting. In case of remediation, environmental regulators are able to choose the sites for remediation where the actual risks are greater rather than blindly choosing the polluted localities where the total metal concentrations are relatively high. This also avoids unnecessarily strict requirements for cleanup and thus avoids expenditure of funds that could be better used to remediate additional areas. We recommend continuing to discuss and question the relevance of the current approaches for risk assessment and decision making, and to update them according to the available tools and scientific knowledge on bioavailability and mixture toxicity.