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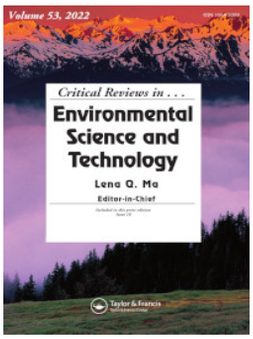

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Approaching a closer surrogate for the biologically effective dose with subcellular partitioning-based toxicokinetic models

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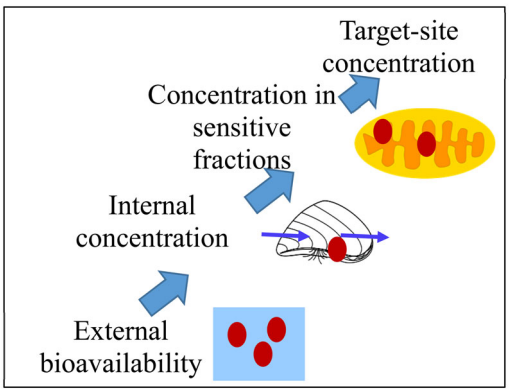
ABSTRACT

A general concept in risk assessment is that a threshold of exposure exists above which adverse effects are initiated. Toxicity is related to the target-site concentration or the biologically active dose. Although it is often a huge challenge to measure the specific dose metric of metal toxicity, significant progress has been obtained to approach closer to the target-site concentration. Such developments are reviewed in the present study. In addition, a general framework to simulate the subcellular metal partitioning, which is supposed to account for the internal metal sequestration, is developed and applied to various metals. The framework allows for delineating mechanisms of internal metal sequestration. Moreover, the specificity in these mechanisms, which varies among metals, species, and exposure conditions, might explain the complicated relationship between the internal concentration and metal toxicity. Attention should be paid to the data requirements as the high number of unknown parameters might be accompanied by potential uncertainties. In addition, future efforts should focus on linking the concentration of metals in sensitive fractions and toxicological effects.



KEYWORDS Risk assessment; toxicokinetics; toxicodynamics; inherent toxicity; sites of toxic action; metals

HANDLING EDITORS Lotfi Aleya and Robert Letcher

1. Introduction

The aquatic environment represents an important final destination for most anthropogenic contaminants, and thousands of chemical substances will continue to end up in aquatic ecosystems. Aquatic organisms are therefore exposed to a large number of contaminants, which can enter the cells via the membrane and cause noxious effects via interactions with macromolecules. Among these contaminants, metals are of particular concern because of their persistence in the

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environment (i.e. not degraded by biological or chemical processes) and their potential effects on organisms. Therefore, it is of importance to assess metal toxicity to aquatic organisms.

Chemical risk assessments are based on the concept that a threshold exists in exposures below which no unacceptable adverse effects are observed. Exposure can be expressed in metrics of the external concentration, the internal concentration (or body/tissue residue), or the target-site concentration. Only the fraction of chemicals that is taken up and reaches the target site is toxicologically active (Escher & Hermens, 2004). The closer to the sites of toxic action, the more reliable the estimate of the biologically effective dose is. Focusing on the target-site concentration and integrating toxicokinetic (TK) and toxicodynamic (TD) characteristics can provide further improvements in risk assessment for metals (Adams et al., 2011). With the potential to simulate the time-course accumulation and toxicity, TK-TD models have been increasingly applied in environmental risk assessment (Thursby et al., 2018). In this modeling approach, the concentration of chemicals at target sites is ideally the connector between toxicokinetics and toxicodynamics. Therefore, it is of importance to obtain reliable estimates of the target-site concentration. However, metal risk assessment is currently based on the external concentration or the whole-body residue because of challenges in measuring the actual concentration at the sites of toxic action. In most of the available TK-TD models, mortality and other toxicological effects are usually related to the total body burden measured or predicted from exposure concentrations (Heugens et al., 2003; Jager et al., 2011). In other words, the external concentration and the whole-body/tissue residue are commonly used as surrogates for the target-site concentration.

The relationship between the external concentration and the target-site concentration is regulated by a number of factors, leading to disadvantages of these common surrogates. For example, previous studies have demonstrated that the total internal body burden might not be a reliable indicator of metal toxicity (de Paiva Magalhaes et al., 2015; Rainbow, 2002, 2007; Vijver et al., 2004). Such a non-significant relationship is related to various mechanisms of internal sequestration as well as the requirements of essential metals for metabolic processes. A consideration of these processes, which influence the tolerance of organisms to metals, in modeling approaches helps to improve the assessment of a biologically effective dose.

In the present study, we firstly reviewed available dose metrics of metal toxicity. Among them, the concentration of metals in sensitive fractions is likely the closest to the target-site concentration. Then we introduced a subcellular metal partitioning model which allows for estimating the time-course of metal accumulation in sensitive fractions. It can be integrated into TK-TD models in which the concentration in the pool of potential toxic metal (PTM) is considered a surrogate for the target-site concentration. Furthermore, the subcellular metal partitioning model was calibrated with various data sets for the zebra mussel *Dreissena polymorpha* (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022) and the gammarid *Gammarus fossarum* (https://doi.org/10.6084/m9.figshare.20101703.v1).

2. Dose metrics of metal toxicity

Exposure can be expressed in various ways, i.e. based on the external concentration in the ambient medium, the internal concentration, or the target-site concentration (Figure 1). Since adverse effects of a toxicant are initiated by its interactions with biomolecules that are critical to biological processes (Meador et al., 2008), the concentration at these target sites is an ideal indicator of chemical-intrinsic toxicity (Wen et al., 2015). It has been suggested that the critical target-site concentration is relatively constant (Jiang et al., 2020). However it is challenging to measure the target-site concentration, and accordingly, several metrics have been proposed as its surrogates. The closer the surrogate is to the concentration at sites of toxic action, the less it is influenced by biological and/or intrinsic confounding factors (Meador et al., 2008).

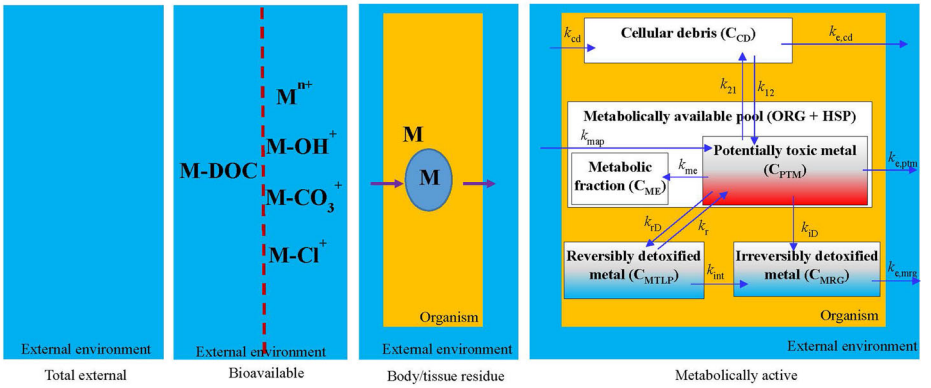

Figure 1. Various dose metrics of metal toxicity. (1) External availability: Conventional risk assessment is based on the concentration of metals in the external medium. The concentration in the bioavailable fraction has been considered a better predictor of metal toxicity than the total external concentration; (2) Internal concentration: The internal concentration has been used in the Critical Body Residue approach and the Tissue Residue Approach; (3) Concentration in sensitive fractions: Since effects are only triggered when internalized metals interact with physiologically sensitive molecules, the concentration of metals in these subcellular fractions might be a closer surrogate for the target-site concentration. This surrogate could be simulated with a subcellular partitioning model. Metals are taken up first to the cellular debris and the metabolically available pool (MAP, including metals in association with organelles ORG and heat-sensitive proteins HSP) with the uptake rate constants k_{cd} and k_{map} (L/g/d), respectively. Metals can be exchanged between these two fractions with rate constants k_{12} and k_{21} (1/d). Essential metals in the MAP can be used in metabolic processes with a rate constant k_{me} (1/d), forming the metabolic fraction (C_{ME}). Excess essential metals and non-essential metals in the MAP can be: bound to metallothionein-like proteins (MTLP) with a rate constant k_{rD} (1/d) forming the reversibly detoxified fraction (C_{MTLP}); incorporated inorganic granules with a rate constant k_{iD} (1/d) forming the metal-rich granule (MRG) or irreversibly detoxified fraction (C_{MRG}); or eliminated with a rate constant $k_{e,ptm}$ (1/d). Metals that exceed the combination of metabolic requirements and detoxification/elimination capacity form the fraction of potentially toxic metal (PTM). Reversibly detoxified metals can be released from the binding with MTLP back to the MAP with a rate constant k_r (1/d) or being incorporated into organic granules with a rate constant k_{int} (1/d) where they can be eliminated ($k_{e,mrg}$; 1/d). A more detailed description of the subcellular metal partitioning is given in Le, Nachev, et al. (2022).

2.1. External bioavailability

Conventional risk assessment is based on a comparison of the (predicted/measured) concentration of a toxicant in the medium and its noxious effects on organisms (TGD, 2003). In particular, a risk quotient is calculated based on the predicted environmental concentrations (PECs) and the predicted no effect concentrations (PNECs). In such assessment, PECs are mainly expressed as the total/dissolved concentration, and PNECs are obtained from the actual concentrations deployed in toxicity tests, which are given by a similar approach (Allen & Hansen, 1996). Classical bioassays are mostly interpreted based on the relationship between the external concentration and the biological responses of organisms (Cesnaitis et al., 2014; Le & Peijnenburg, 2013; Tarazona et al., 2014). Uncertainties are inherent in this approach as bioavailability, organotropism, and mainly the mechanism by which toxicants exert effects on organisms are not considered (Verdonck et al., 2007). The effective concentration based on the external exposure level is of limited significance since the differences in bioavailability, uptake, and metabolism result in large variations in this dose metric (Van der Heijden et al., 2015).

In the aquatic environment, metals are distributed within the solution, in suspended particles, and in sediments. In each of these phases, metals are present in various chemical forms or species. For example, metals in solution form ion pairs and complexes with organic and inorganic ligands, leading to the occurence of a number of metal species (Adams et al., 2011). The portion of the total amount of metals in the external medium that correlates with the bioaccumulation is defined as the bioavailable fraction. Usually metals as free ions or in weak complexes with inorganic ligands are bioavailable, i.e. can be taken up by organisms (Peijnenburg et al., 2002), while metals in strong complexes with organic ligands are non-bioavailable. A significant proportion of the total external concentration is non-bioavailable because of the formation of organic and

inorganic metal complexes (Di Toro et al., 2001). A large fraction of bioactive trace metals in seawater are complexed (up to > 99% for iron and copper) by natural organic ligands (Ellwood & van den Berg, 2001). Metals are usually taken up via membrane transporters (Simkiss & Taylor, 2001). These transporters are often specific for certain elements, leading to metal-specific bioavailability and uptake. Consequently, the risk assessed based on total metal concentrations might be overestimated (Jones & Bolam, 2007). These issues contribute to limitations in using the total concentration of metals in the external environment as a surrogate for assessment of metal toxicity (Adams et al., 2011).

Metal bioavailability is greatly influenced by the chemical speciation in a specific aquatic environment (Allen et al., 1980; Fairbrother et al., 2007; Pagenkopf, 1986; Pagenkopf et al., 1974), which in turn depends on water chemistry such as the concentration of dissolved organic carbon, Na^{+}, and Ca^{2+} (Allen et al., 1980; Pagenkopf et al., 1974; Playle, 1998; Sprague, 1968). Different methods have been developed to simulate metal bioavailability and to integrate it in risk assessment. For instance, metal species can be characterized and quantified by speciation models such as WHAM (Tipping, 1998), visual MINTEQ (Gustafsson, 2011), and PHREEQC (Parkhurst & Appelo, 2013). Colloidal metals (Rich & Morel, 1990) and those complexed with organic ligands (Anderson & Morel, 1982) represent the chemical species that are unable to directly react with binding sites at the cell surface. By contrast, free metal ions have been unraveled as the main reactive species determining metal toxicity (Erickson et al., 1996; Sunda et al., 1978; Sunda & Guillard, 1976; Sunda & Huntsman, 1983). Accordingly, the free ion activity model (FIAM) was developed based on the assumption that the activity of free metal ions represents the bioavailable fraction, regulating metal uptake and toxicity (Campbell, 1995; Morel, 1983; Pagenkopf, 1983). However, the activity of free metal ions is not the only regulator of metal toxicity (De Schamphelaere & Janssen, 2002). Exceptions to the FIAM have been frequently reported as ionic and organometallic complexes also contribute to the concentration of metals at the cell membrane surface, to metal uptake, and to subsequent toxicity (Allen & Hansen, 1996; Erickson et al., 1996; Ferreira et al., 2008; Markich et al., 2000; Martin & Goldblatt, 2007; McGeer et al., 2002; McGeer & Wood, 1998). More importantly, metal bioavailability is controlled not only by the geochemical speciation, but also by interactions of the reactive species with organisms. Major cations such as Ca^{2+}, Mg^{2+}, Na^{+}, and H^{+}, might compete with toxic metal ions for binding sites at the organism-water surface (De Schamphelaere et al., 2005; Pagenkopf, 1983; Plette et al., 1999; Zitko & Carson, 1976). The forms of metals, e.g. species, compound, matrix, and particle size, that regulate metal bioavailability and toxicity are influenced by both environmental conditions and biological processes (Fairbrother et al., 2007; Worms et al., 2006). The complexation of metals with ligands in solution and with the cell surface should be simultaneously considered in assessment of metal bioavailability (Parker & Pedler, 1997).

Several modeling approaches have been developed to take the role of biological factors into consideration. The fish gill surface interaction model integrates conditional metal-gill surface binding constants into a geochemical speciation model (Pagenkopf, 1983). The impacts of competition and complexation on metal binding to freshwater fish gills are incorporated in this model, providing mechanistic-based estimations of metal bioavailability. The Biotic Ligand Model (BLM) was then developed on the basis of the fish gill surface interaction model, allowing for the application of that principle to other organisms. The BLM is based on the assumption that the onset of metal toxicity originates from the binding of free metal ions or other reactive species to physiologically active sites or transport sites at biotic ligands on the organism-water interface (De Schamphelaere & Janssen, 2002; Di Toro et al., 2001; Paquin et al., 2002). This assumption is supported by a correlation between metal accumulation at the fish gill and metal toxicity reported by MacRae et al. (1999). The biological surface, such as the gill in fish, is considered a ligand that might compete with organic molecules in the external medium for binding to cations. Accordingly, metal interactions at sites of toxic action, which are influenced by competition of

protons and other major cations (e.g. Na^+, Ca^{2+}, Mg^{2+}, and K^+) (Playle, 1998; Playle et al., 1993a, 1993b), are assumed to determine metal toxicity. This chemical equilibrium-based model links metal accumulation at sites of action of toxicity at biotic ligands to toxicological responses (Di Toro et al., 2001; Paquin et al., 2002). The biotic ligands are specific proteins regulating the uptake of essential elements (Niyogi & Wood, 2003, 2004). According to the principle of the BLM, the competition for biotic ligands reduces the amount of toxic metals at the ligands, thus inhibiting metal toxicity. In other words, the influence of both chemical speciation and cation competition at biotic ligands on metal toxicity is accounted for (Deleebeeck et al., 2007; Di Toro et al., 2001; Santore et al., 2001). The original BLM development is based on the measurement of metal concentrations at gills (MacRae et al., 1999; Niyogi & Wood, 2004; Playle et al., 1993a, 1993b). However, it is difficult to determine the amount of metals at the target site of invertebrates. In recent BLMs, metal toxicity is no longer based on the actual measurements of a tissue residue. Instead, affinity constants for metal binding to sites at biotic ligands are derived from toxicity data, and the effective concentration is still based on the external concentration (De Schamphelaere et al., 2005; De Schamphelaere & Janssen, 2002, 2010; Le et al., 2012; Le & Peijnenburg, 2017; Santore et al., 2021). In the BLM, the hypothetical biotic ligand is usually the surface membrane while only metals internalized into the cells can cause effects (Croteau & Luoma, 2009).

2.2. Internal concentration

Bioaccumulation, which is influenced by both chemical characteristics of metals and biological factors (Rainbow et al., 2011), has been widely used as an integral part of the exposure-effect causality chain (Hansen et al., 2002; Jager et al., 2011; Ng et al., 2012; Schmidt et al., 2011). Biological compartments have become major objects in legislations (e.g. EU Water Framework Directive; Marine Strategy Framework Directive; US Clear Water Act and Oceans Policy; Oceans Act in Canada). A number of aquatic organisms, especially filter-feeder bivalves (mussels and oysters) and fish, have been deployed as sentinels in biomonitoring (Albuquerque et al., 2021; Evariste et al., 2018; Goldberg, 1975; Hinck et al., 2008). With the ability to accumulate various toxicants at high levels, several groups of parasites such as acanthocephalans have been considered potential sentinels (Sures et al., 2017). Consequently, parasites were further included in toxicokinetic models for predicting metal accumulation in fish-parasite systems (Le et al., 2014, 2018; Le, Kiwitt, et al., 2022). By using sentinel organisms in biomonitoring, the bioavailable fraction that is the most able to exert effects on organisms is directly measured. In other words, a comparison of the internal concentration versus a threshold value (critical internal concentration) is an alternative to the conventional assessment based on the external concentration. The Critical Body Residue (CBR) approach has been developed to link toxic effects to the internal concentration (Adams et al., 2011; McCarty & Mackay, 1993; Mendez-Fernandez et al., 2013; Nadella et al., 2013; Rosen et al., 2008; Sonne et al., 2016). It allows for determining the actual body burden that triggers toxic effects (McCarty & Mackay, 1993). However, toxicants must interact with a receptor on or in cells or tissues to trigger adverse effects. Consequently, the concentration of metals at specific tissues has been used as a predictor of adverse effects. In the Tissue Residue Approach (TRA), the concentration at the critical tissue or sites of toxic action is considered the dose metric in dose-response relationships in risk assessment (Adams et al., 2011; Gimbert et al., 2016; McCarty et al., 2011; Meador et al., 2011; Ng et al., 2012; Sappington et al., 2011). The TRA assumes that toxic effects are initiated when toxicants reach sites of toxic action (Gimbert et al., 2016). So, in principle, modes or mechanisms of toxic action are considered in this approach.

Both the CBR and TRA approaches are based on the idea that a body/tissue residue concentration is indicative of the amount of metals taken up by organisms in which the variability in the

bioavailability induced by external conditions is accounted for Adams et al. (2011). Risks assessed based on the body/tissue residue are less influenced by a myriad of factors (bioavailability and toxicokinetics), leading to decreased variability in the expression of toxicity among species (Fay et al., 2000; Meador et al., 2008). In addition, the toxicity parameters expressed by the internal concentration are less influenced by exposure time than the parameter based on the external concentration (Mackay et al., 1992). The assessment based on the internal concentration allows for linking various compartments, which is often not feasible in evaluations based on the external concentration (Escher & Hermens, 2004). The internal concentration is a better surrogate for the concentration at sites of toxic action, and a better predictor of metal toxicity than the external concentration (Escher & Hermens, 2004; Rosen et al., 2008) as bioavailability, kinetics, and the contribution of various exposure pathways are accounted for (Steevens et al., 2005). With these advantages, the CBR and TRA approaches might improve toxicity assessment (Pery et al., 2005), providing more reliable indicators of the bioavailable and effective target dose. However, the application of the TRA is currently restricted due to limitations in the quantity and quality of critical residue data (Beckvar et al., 2005; Hendriks et al., 2005; Steevens et al., 2005).

The development of multi-compartment models like physiologically-based toxicokinetic (PBTK) models (Gestin et al., 2021; Le et al., 2018; Le, Kiwitt, et al., 2022) allows for estimating tissue-specific metal concentrations. The concentration of pollutants in some tissues might be of limited toxicologically significance. It is preferable to choose tissues that are closest to the sites of toxic action. However, it is practically constrained by challenges in the performance of chemical analyses on individual tissues while the tissues involved in the sites of toxic action may be unknown (Sappington et al., 2011). Most of the studies using the TRA are based on the whole-body tissue residue, which does not reflect the target-site concentration or the biologically effective dose (Beckvar et al., 2005; Sappington et al., 2011; Steevens et al., 2005). For metals, the relationship between the tissue residue and toxic effects might be influenced by the exposure and uptake routes (Sappington et al., 2011), which determine the degree that metals in a tissue are inactivated via various mechanisms. As such, the effective tissue residue depends on the exposure duration (Landrum et al., 2004; Lee et al., 2002). More importantly, metals in the whole body or in a tissue do not necessarily reflect the metabolic or metabolically available (or active) fraction that is available to modulate physiologically sensitive molecules (Adams et al., 2011). In other words, the whole body/tissue concentration is not always a reliable indicator of toxicologically relevant accumulation since the detoxified forms and the biologically active forms are not distinguished (Rosen et al., 2008). Therefore, their application to metals should be done with caution (Adams et al., 2011).

2.3. Metabolically available pool

In order to obtain a reliable relationship between the tissue concentration and biological responses, it is of importance to consider the biologically effective dose, that is, the internally bioavailable proportion of the total body burden that interacts with biomolecules at sites of toxic action (Paustenbach, 2000). For specific-acting compounds like metals, responses of organisms are influenced by the mode of toxic action, the concentration of subcellular ligands, as well as the metabolism capacity (Escher & Hermens, 2004). Because of the importance of these factors, the target-site concentration might be a more reliable predictor of metal toxicity than the whole-body concentration. The concentration of metals at target sites that determines adverse effects (toxicodynamics) is regulated by toxicokinetic processes including uptake, metabolism, interactions with target and non-target sites, and excretion. The ultimate objective of exposure assessment is to estimate the target-site or biologically effective concentration (Paustenbach, 2000).

Different fractionation approaches have been developed and applied to experimentally quantify metal concentrations in various subcellular fractions (Blackmore & Wang, 2002; Le, Grabner,

et al., 2021; Wallace et al., 2003), providing estimates of the concentration of metals that interact with physiologically active molecules. However, the partitioning of internalized metals into subcellular fractions is a dynamic process (Campana et al., 2015; Wang & Rainbow, 2006). Kinetic models allow for simulating such dynamics (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022). Considering the significance of the sequestration of subcellular metals to metal toxicity, in our previous TK-TD models, the chronic toxicity of copper (Cu) to the zebra mussel has been related to either the active species of subcellular metals (Le, Nachev, et al., 2021) or to the excess metal in the metabolically available pool (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022). Such approaches are developed based on a significant correlation between sublethal effects of metals and their accumulation in non-detoxified forms (Rainbow & Smith, 2013).

3. Toxicokinetic-toxicodynamic modeling and the relationship between the external concentration and the target-site concentration

In ecotoxicology, it is a common practice to reduce all of the information from toxicity tests to a single value, e.g. the median lethal/effective concentration (LC50 or EC50), which is derived by fitting the data on biological responses versus concentration to a standard curve. The median lethal/effective concentration based on the external level decreases with increasing exposure time, presumably attributed to a relatively slow distribution to target sites (Jiang et al., 2020). Such declines occur until the median lethal/effective concentration asymptotically approaches a stable value, i.e. the incipient LC50 or EC50 (Sprague, 1969). However, the time required to reach this incipient value depends on chemical properties of the toxicant and characteristics of the organism (Jager & Ashauer, 2018). Such influence of exposure time on metal toxicity contributes to further complications in risk assessment for metals (Chapman et al., 2003). A more mechanistic approach that explicitly includes the factor of "time" is required to address this issue. A model that covers both toxicokinetics and toxicodynamics is required as toxicants need to be taken up into the body first before they can affect the organism. With the potential to simulate the time-course processes that regulate adverse effects on organisms (Ashauer & Escher, 2010), TK-TD models have been increasingly applied to estimate acute toxicity (Feng et al., 2018; Gao et al., 2017, 2019), and recently also chronic toxicity (Le, Grabner, et al., 2021; Le, Nachev, et al., 2021). Acknowledging the disadvantages in the use of whole body/tissue residues, Adams et al. (2011) suggested that further improvement could be obtained by focusing on the target-site concentration and by integrating toxicokinetic and toxicodynamic characteristics.

Toxicokinetics and toxicodynamics are complementary components of toxicology. Toxicokinetics describe the effects that an organism exerts on a toxicant, while toxicodynamics show the effects that the toxicant exerts on the organism. The former addresses absorption, distribution, metabolism (biotransformation), and excretion of the toxicant, whilst the latter refers to the interactions between the toxicant and its target sites, which trigger toxicity (Escher & Fenner, 2011). Ideally, the toxicokinetic phase in TK-TD models provides estimates of the concentration of the toxicant at sites of toxic action. However, in most of the available models, toxic effects are related to the total internal concentration, excluding the significance of subcellular metal sequestration (Ashauer et al., 2013; Feng et al., 2018; Gao et al., 2015).

4. Development of a subcellular metal partitioning model for predicting the concentration at target sites

The review above demonstrates that we are approaching a closer surrogate for the concentration of metals at target sites. With the progress until now, the concentration of metals in sensitive fractions is likely the closest one. Hereby we provide a general framework that can provide the

time-course of metal accumulation in these fractions, which is related to the dynamics of subcellular fractionation and could be integrated into TK-TD modeling.

4.1. Methods

4.1.1. Model characterization

A general framework of toxicokinetic modeling based on subcellular metal partitioning was developed by modifying our previous modeling approaches (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022). In the general framework of the present study, the metal fraction in the cellular debris is integrated together with the metal content of other subcellular fractions (Figure 1). A distinction of the metal in the metabolic fraction facilitates the application of the models to various metals, both essential and non-essential.

Our conceptual model (Figure 1) is based on the mechanism described by Rainbow and Luoma (2011a, 2011b). Specifically, metals are initially taken up into the metabolically available pool (MAP; Rainbow, 2002; Rainbow & Luoma, 2011a, 2011b) with a rate constant k_{map} (L/g/d) and into the cellular debris with a rate constant k_{cd} (L/g/d). Metals can be transported between these two fractions with rate constants k_{12} and k_{21} (1/d). Essential metals can be used in metabolic processes with a rate constant k_{me} (1/d), forming the metabolic fraction (C_{ME}). The potentially toxic fraction of essential metals is the excess in the MAP above the metabolic requirements, while non-essential metals in the MAP are all potentially toxic (i.e. without metabolic fraction: k_{me}=0). The concentration of metals in the fraction of potentially toxic metal (PTM) depends on their removal from the MAP via incorporation into inorganic granules or binding to metallothionein-like proteins (MTLP). These mechanisms form the detoxified pool, as metals in these fractions are unable to trigger adverse effects. In particular, metals in the MAP can be reversibly detoxified by binding to MTLP at a rate constant k_{rD} (1/d) (forming the reversibly detoxified or MTLP fraction C_{MTLP}); irreversibly inactivated by incorporation into inorganic granules at a rate constant k_{iD} (1/d) (forming the irreversibly detoxified or MRG fraction C_{MRG}); or eliminated at a rate constant $k_{e,ptm}$ (1/d). Reversibly detoxified metal can be released back to the MAP at a rate constant k_r (1/d) or be incorporated into the MRG due to the breakdown of lysosomes (Luoma & Rainbow, 2008) at a rate constant of k_{int} (1/d).

Considering the above processes, the accumulation of metals in the cellular debris could be described as a function the uptake from water (1st factor), the exchange with the PTM (2nd factor), and elimination (last factor) by the following equation:

$$\frac{dC_{CD}}{dt} = k_{cd} \times C_w + (k_{21} \times C_{PTM} - k_{12} \times C_{CD}) - k_{e,cd} \times C_{CD} \quad (1)$$

with C_{CD} and C_{PTM} (μg/g) being the metal concentrations in the cellular debris and the PTM fraction, respectively; C_W (μg/L) being the dissolved metal concentration in the external medium; k_{cd} (L/g/d) being the rate constant for metal uptake into the cellular debris; k_{12} and k_{21} (1/d) being the rate constants for the exchange between the cellular debris and the PTM fraction; and $k_{e,cd}$ (1/d) being the rate constant for elimination of metals from the cellular debris.

The concentration of essential metals in the PTM fraction could be written as a function of the uptake of dissolved metal (1st factor), exchange with the cellular debris (2nd factor), metabolism (3rd factor), back-flow of the reversibly detoxified metal (4th factor) as well as detoxification and elimination (last factor):

$$\begin{aligned}\frac{dC_{PTM}}{dt} = {} & k_{map} \times C_w + (k_{12} \times C_{CD} - k_{21} \times C_{PTM}) - \frac{dC_{ME}}{dt} + k_r \times C_{MTLP} \\ & - (k_{rD} + k_{iD} + k_{e,ptm}) \times C_{PTM}\end{aligned} \quad (2)$$

where C_{ME} and C_{MTLP} (μg/g) are the metal concentrations in the metabolic and reversibly

detoxified fractions, respectively; k_{map} (L/g/d) is the rate constant for metal uptake into the MAP; k_r (1/d) is the rate constant at which reversibly detoxified metal is released back to the MAP; k_{rD} and k_{iD} (1/d) are the reversible and irreversible detoxification rate constants; and $k_{e,ptm}$ (1/d) is the rate constant for elimination of metals from the PTM fraction. Among these factors, the metabolic fraction could be expressed as a function of the maximum metabolic requirements as applied for simulating Na^+ influx by Veltman et al. (2014) or Na^+ binding to Na^+/K^+-ATPase enzymes by Le, Nachev, et al. (2021):

$$\frac{dC_{ME}}{dt} = k_{me} \times C_{PTM} \times \left(1 - \frac{C_{ME}}{Me_{max}}\right) \tag{3}$$

where k_{me} (1/d) is the rate constant that essential metals are used in metabolic processes; and Me_{max} (μg/g) is the maximum requirement of the essential metals for metabolic processes.

For essential metals, the MAP consists of the metabolic fraction and the excess in the PTM. In other words:

$$C_{MAP} = C_{ME} + C_{PTM} \tag{4}$$

For non-essential metals, all metals in the MAP are potentially toxic ($k_{me} = 0$; $C_{MAP} = C_{PTM}$). Similarly, metal accumulation in detoxified fractions could be written by mass-balance equations:

$$\frac{dC_{MTLP}}{dt} = k_{rD} \times C_{PTM} - k_{int} \times C_{MTLP} - k_r \times C_{MTLP} \tag{5}$$

$$\frac{dC_{MRG}}{dt} = k_{iD} \times C_{PTM} + k_{int} \times C_{MTLP} - k_{e,mrg} \times C_{MRG} \tag{6}$$

with C_{MRG} (μg/g) being the concentration of irreversibly detoxified metal in the MRG fraction; k_{rD} and k_{iD} (1/d) being the rate constants of reversible and irreversible detoxification, respectively; k_{int} (1/d) being the rate constant for the incorporation of reversibly detoxified metal into the granules; and $k_{e,mrg}$ (1/d) being the rate constant for elimination of irreversibly detoxified metals.

The bioaccumulation of toxicants is usually expressed by the bioconcentration factor, defined as the ratio of the uptake and elimination rate constants. To unravel metal accumulation in the MAP, we introduce a similar factor to the model described above or in our previous studies (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022). This factor allows for comparing the uptake from the dissolved phase with processes that reduce the accumulation in this compartment (i.e. metabolism, detoxification, and elimination). In particular, the bioconcentration factor of metals in the MAP (BCF_{map}; L/kg) is calculated as a function of the rate constants of influx (uptake from the external medium) and efflux (metabolism, detoxification, and elimination):

$$BCF_{map} = \frac{k_{map}}{k_{me} + k_{rD} + k_{iD} + k_{e,ptm}} \times 10^3 \tag{7}$$

4.1.2. Model calibration

In our previous studies (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022), the TK phase based on the subcellular metal partitioning model was calibrated simultaneously with the TD phase for essential metals. In the present study, the subcellular metal partitioning model was calibrated for various non-essential metals as well. Model calibration was conducted with various data sets on subcellular concentrations of: (1) Cu in the zebra mussel *Dreissena polymorpha* (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022); (2) cadmium (Cd), lead (Pb), and nickel (Ni) in the gammarid *Gammarus fossarum* (https://doi.org/10.6084/m9.figshare.20101703.v1). This step was implemented using the AMIGO2 Toolbox running on MATLAB (Balsa-Canto et al., 2016). Code samples for both essential and non-essential metals that allow for simulating subcellular

distribution are given in the Supporting Information. In addition, statistical parameters are given in Table S1, Supporting Information. The 95% confidence intervals are computed using the Fisher Information Matrix and the Crammer-Rao inequality (Balsa-Canto et al., 2010, 2021).

Subcellular partitioning of copper in the zebra mussel. A detailed description of Cu fractionation in the zebra mussel is given in Le, Grabner, et al. (2021). In brief, zebra mussels were exposed to waterborne Cu at nominal concentrations of 25 and 50 μg/L. The exposure was conducted at varying Na^+ concentrations up to 4 mmol/L. The maximum metabolic requirement was parameterized based on the estimates of enzyme requirements by White and Rainbow (1985) as described in Le, Nachev, et al. (2022). Copper requirement for respiratory pigment proteins, such as hemocyanins, is excluded as these proteins are restricted to the Protobranchia (Markl, 2013). At the investigated concentrations of Na^+ in water, Na^+ had negligible effects on Cu accumulation in the zebra mussel (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022). Therefore, model calibration was performed with data obtained at various Na^+ concentrations.

Subcellular partitioning of cadmium, lead, and nickel in Gammarus fossarum. Specimens of *Gammarus fossarum* were collected using a hand net from an unpolluted stream, La Bourbre (near Lyon, France). The organisms were quickly transported in plastic vessels to the laboratory, where they were kept at 12 °C in well-water for 10 days before being used in experiments. Male gammarids were exposed to waterborne Cd (0.3, 1, and 3 µg/L), Pb (2, 10, and 50 µg/L), and Ni (0.2, and 1 µg/L) for three weeks. All the experiments were carried out in 12 ± 0.5 °C water baths. Male gammarids ($n = 160$ per condition, 8 replicates of 20 size-calibrated males) were randomly placed in 500 mL-beakers in polypropylene. Continuous exposure was provided (i.e. exposure medium was renewed four times a day), based on the methodology described by Geffard, Xuereb, et al. (2010). Briefly, a stock solution was prepared daily and used to obtain all tested exposure media. Peristaltic pumps were used to dilute the stock solution and obtain the highest tested concentration, which was in turn diluted to obtain the lowest concentrations. The dilution factors were established by controlling the flow of water and stock solution or intermediate dilution. In addition, peristaltic pumps were used to dispense each solution in beakers with a constant flow of 1.34 ml/min. During the acclimation and exposure, animals were fed on alder leaves (*Alnus glutinosa*). On each sampling day (days 0, 4, 8, 16 and 21), amphipods (3 replicates of 10 individuals) were randomly sampled from all the beakers. They were gently dried with a paper towel, weighed (± 0.1 mg), and stored at −80 °C until compartmentalization analyses. The procedure for subcellular fractionation and metal analyses in all subcellular fractions was described previously in Geffard, Sartelet, et al. (2010).

4.2. Results and Discussion

4.2.1. Specificity of subcellular metal sequestration and the relationship between potentially toxic metal and total body burden

Although the results are encouraging, further work is needed to generalize this framework for various metals, organisms, and conditions. In general, it is able to cover the variations in the concentration of Cu in various subcellular fractions of the zebra mussels (Figures 2 and S1) and of Cd (Figures 3 and S2), Pb (Figures 4 and S3), and Ni (Figures 5 and S4) in the gammarids. A kinetic model based on subcellular partitioning allows for delineating the specific pathways of internal sequestration, which vary among metals, organisms, and exposure conditions (Table 1). McGeer et al. (2003) demonstrated that the BCF values calculated based on the whole body burden depend on the exposure level. Such a relationship is attributed to the involvement of various physiological mechanisms, including the use of essential metals for essential functions as well as detoxification mechanisms for both essential and non-essential metals. These processes are accounted for in the parameter of BCF_{map}.

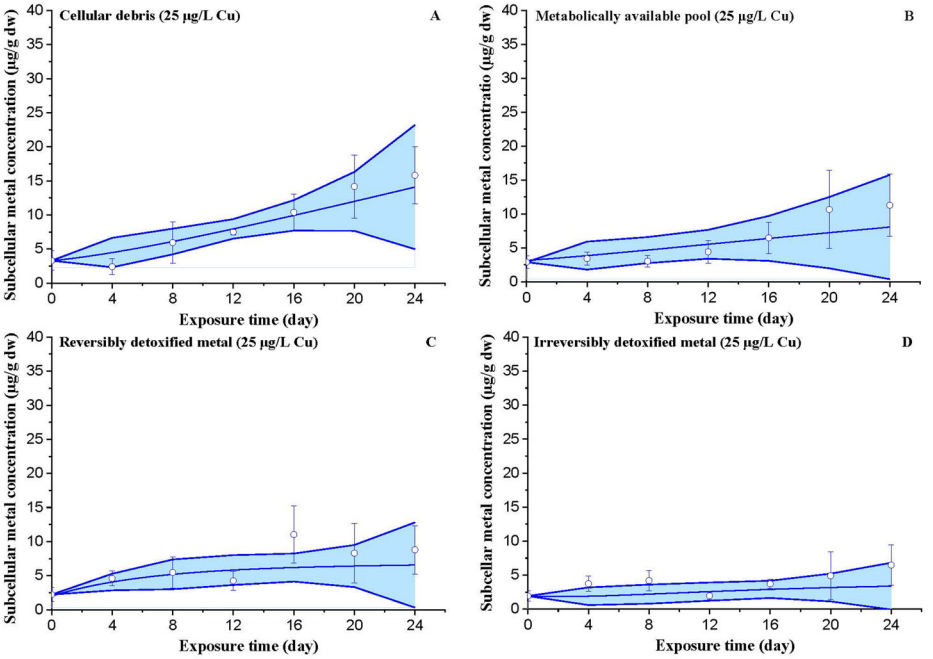

Figure 2. The relationship between the measurements and the estimates of Cu concentrations in subcellular fractions when the zebra mussels were exposed to Cu at a nominal concentration of 25 μg/L and a Na^+ concentration of 0.5 mmol/L. The areas covered by the solid lines represent the 95% confidence interval with the line in the middle representing the mean estimate. Dots and bars represent the measurements (average ± standard deviation). The calibration was implemented with data on the concentration of metals in subcellular fractions, not with data on biomarker responses as in TK-TD modeling in Le, Nachev, et al., (2022).

In the zebra mussel, the higher BCF_{map} at 50 μg/L compared to the corresponding value at 25 μg/L might be related to the decreased copper-binding capacity of MTLP (Table 1). This might reflect some saturation at binding sites of these proteins and/or dose-dependent induction of MTLP. These factors lead to increasing accumulation of excess Cu in sensitive fractions, as also observed in Voets et al. (2009). With a 24-d exposure duration (Le, Grabner, et al., 2021; Le, Nachev, et al., 2022), the binding by MTLP seems to play an important role among detoxification mechanisms, compared to the incorporation of Cu into MRG and elimination (Table 1; Figures 2 and S1). Cadmium was negligibly eliminated from the gammarid, while both the binding to MTLP and incorporation into MRG played a more important role in limiting the Cd concentration in sensitive fractions (Table 1). Khan et al. (2010) unraveled that the MRG and MTLP fractions accounted for the majority of the Cd internal concentration (43 and 24.8%, respectively). The capacity of the gammarid to detoxify Ni was likely lower than the uptake, potentially leading to the high value of BCF_{map} (Table 1). By contrast, internalized Pb could be effectively detoxified by this organism, leading to its lowest BCF_{map} (Table 1). The specific sequestration pathways as mentioned above contribute to a complicated relationship between the total internal concentration and the concentration of metals in the sensitive fractions. This might explain the finding that indicators based on the total internal concentration might not be a good predictor of metal toxicity (Voets et al., 2009; Zhang et al., 2019). The above differences among internal sequestration pathways are not statistically significant when estimates of the model parameters are not significantly deviating from zero (Table 1). Therefore, such discussion on the subcellular partitioning of various metals should be supported by further information on the underlying mechanisms or examined with further data.

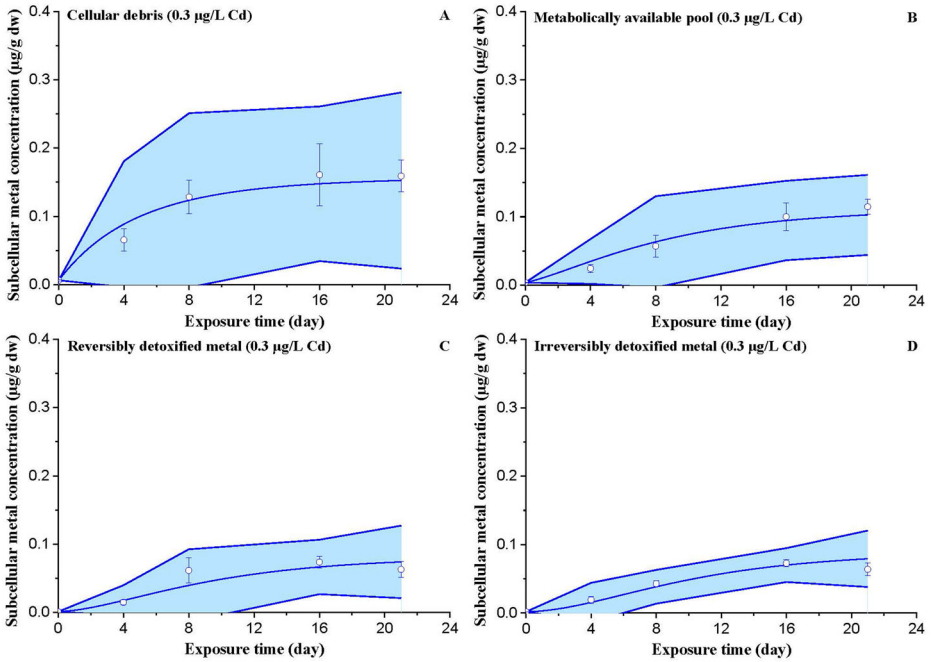

Figure 3. The relationship between the measurements and the estimates of Cd concentrations in subcellular fractions when gammarids were exposed to Cd at a nominal concentration of 0.3 μg/L. The areas covered by the solid lines represent the 95% confidence interval with the line in the middle representing the mean estimate. Dots and bars represent the measurements (average ± standard deviation).

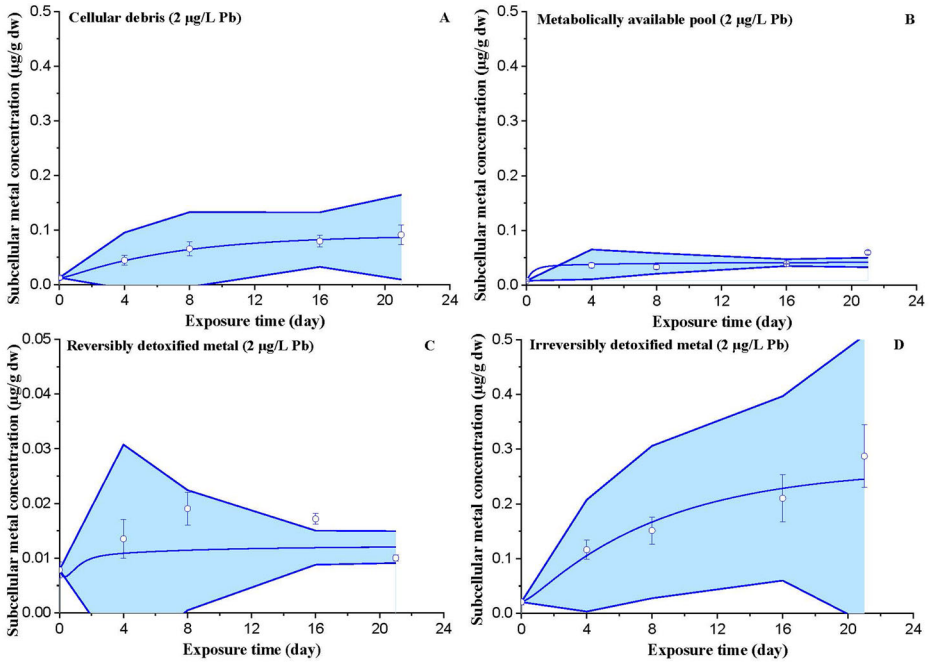

Figure 4. The relationship between the measurements and the estimates of Pb concentrations in subcellular fractions when gammarids were exposed to Pb at a nominal concentration of 2.0 μg/L. The areas covered by the solid lines represent the 95% confidence interval with the line in the middle representing the mean estimate. Dots and bars represent the measurements (average ± standard deviation).

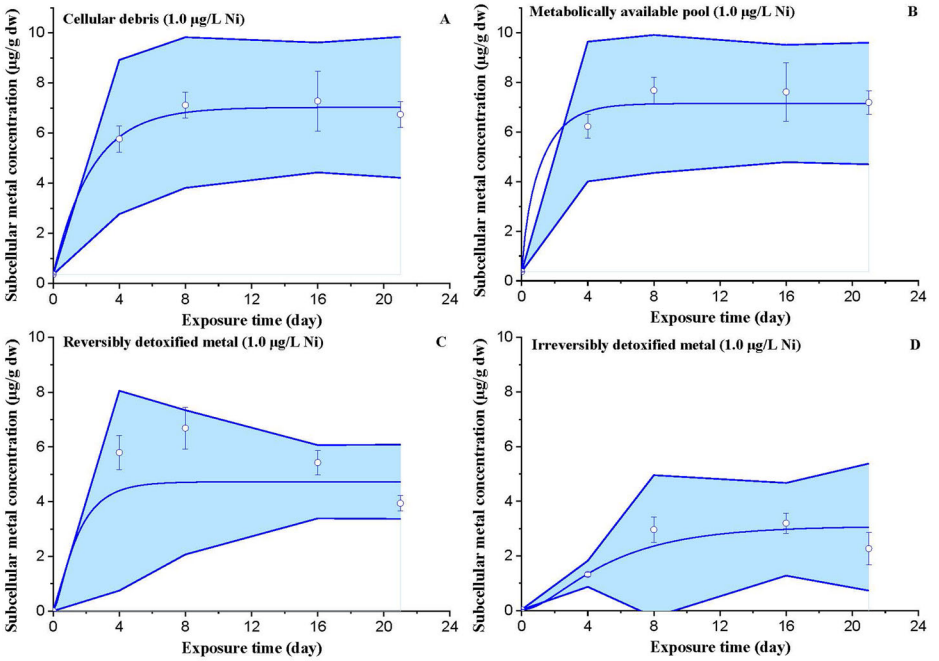

Figure 5. The relationship between the measurements and the estimates of Ni concentrations in subcellular fractions when gammarids were exposed to Ni at a nominal concentration of 1.0 μg/L. The areas covered by the solid lines represent the 95% confidence interval with the line in the middle representing the mean estimate. Dots and bars represent the measurements (average ± standard deviation).

4.2.2. Model uncertainties and future outlook for toxicokinetic-toxicodynamic modeling

Until now, responses of organisms to toxicant exposure are mostly related to the total internal concentration, measured or predicted from the actual exposure concentration. The lack of data on subcellular metal distribution in toxicity experiments prevents a thorough evaluation on the effectiveness subcellular metal partitioning-based TK-TD models compared to conventional total body burden-based TK-TD model. However, our brief assessment with data generated from our study (Le, Nachev, et al., 2022) shows that the concentration of metals in the MAP pool could explain the variation in the glutathione-S-transferase (GST) activity in Cu-exposed zebra mussels significantly better than the total internal concentration according to a sigmoid curve (Figure S5, Supporting Information). While 35% of the variations in the GST activity could be captured in relation to the concentration in the MAP pool, a similar analysis based on the total internal concentration was interrupted. However, with a large number of parameters of the general model based on subcellular partitioning, model calibration with limited data will lead to high uncertainties in the estimates of unknown parameters. This is indicated by large confidence intervals compared to the mean estimate (Tables 1 and S2). The results of model calibration for subcellular Ni partitioning in the gammarid is one evident example (Table 1). Specific simplification by excluding insignificant processes might apply to each metal to reduce such uncertainties. For example, based on the low sensitivity of the model to the transport between the cellular debris and the MAP for Ni, the exclusion of this process considerably reduced the confidence interval (Table S2). Uncertainties in model estimation could be reduced by calibration with long time-series datasets or by parameterizing some parameters prior to model calibration.

With a subcellular partitioning model as presented here, it is possible to predict the time-course of metal accumulation in sensitive fractions. With this model, the influence of confounding factors on metal availability and uptake has been taken into consideration. In addition, the

Table 1. Estimates (mean and 95% confidence interval) of parameters in the toxicokinetic model based on subcellular metal partitioning.

		Dreissena polymorpha		*Gammarus fossarum*							
		Cu		Cd			Pb			Ni	
Parameter	Unit	25 μg/L	50 μg/L	0.3 μg/L	1 μg/L	3 μg/L	2 μg/L	10 μg/L	50 μg/L	0.2 μg/L	1 μg/L
BCF_{map}	L/kg	8.32 (± 1360)	34.32 (± 200)	23.85 (± 503)	403 (± 8644)	317 (± 11945)	18.27 (± 1152)	0.39 (± 18635)	4.22 (± 155)	11576 (± 230499)	3709 (± 25180)
k_{map}	L/g/d	0.09 (± 1.41)	0.14 (± 0.52)	0.02 (±0.18)	0.14 (± 1.66)	0.18 (± 3.75)	0.04 (± 1.22)	0.001 (± 55.85)	0.003 (± 0.03)	11 (± 448)	9.95 (± 63.66)
k_{cd}	L/g/d	$2.13 \cdot 10^{-4}$ (± 1.40)	0.03 (± 0.14)	0.11 (± 0.20)	$2.16 \cdot 10^{-13}$ (± 1.65)	$3.33 \cdot 10^{-13}$ (± 3.51)	$4.28 \cdot 10^{-8}$ (± 0.40)	0.05 (± 9.98)	0.02 (± 0.05)	0.89 (± 9.82)	3.01 (± 15.84)
$k_{e,cd}$	1/d	$1.28 \cdot 10^{-5}$ (± 0.23)	$1.63 \cdot 10^{-6}$ (± 0.42)	$5.44 \cdot 10^{-14}$ (± 2.99)	$5.13 \cdot 10^{-14}$ (± 3.59)	$1.42 \cdot 10^{-13}$ (± 2.14)	0.003 (± 5.39)	0.04 (± 3042)	$1.94 \cdot 10^{-13}$ (± 5.65)	0.001 (± 0.46)	0.43 (± 7.26)
k_r	1/d	0.06 (± 201)	0.22 (± 1.56)	0.71 (± 11.45)	0.26 (± 5.59)	0.23 (± 3.79)	0.99 (± 420)	1.16 (± 3856)	$6.34 \cdot 10^{-11}$ (± 121)	$1.07 \cdot 10^{-11}$ (± 18.52)	1.96 (± 22.74)
k_{rD}	1/d	9.93 (± 1553)	1.51 (± 6.17)	0.52 (± 1.10)	0.16 (± 0.35)	0.17 (± 0.43)	0.56 (± 31.93)	0.31 (± 23.55)	$7.01 \cdot 10^{-18}$ (± 0.0002)	0.95 (± 11.12)	1.36 (± 14.86)
k_{iD}	1/d	$2.56 \cdot 10^{-4}$ (± 7.27)	$1.22 \cdot 10^{-4}$ (± 2.38)	0.35 (± 2.24)	0.19 (± 0.97)	0.39 (± 2.50)	0.63 (± 99)	2.82 (± 890)	0.67 (± 1.73)	$2.21 \cdot 10^{-14}$ (± 6.56)	0.03 (± 1.28)
k_{int}	1/d	0.07 (± 0.62)	0.10 (± 0.29)	$5.55 \cdot 10^{-16}$ (± 10.51)	$4.59 \cdot 10^{-14}$ (± 5.12)	$2.46 \cdot 10^{-13}$ (± 4.06)	0.95 (± 352)	0.002 (± 3769)	0.03 (± 121)	0.90 (± 28.36)	0.10 (± 2.22)
$k_{e,ptm}$	1/d	$2.79 \cdot 10^{-5}$ (± 6.27)	1.47 (± 21.89)	$3.77 \cdot 10^{-14}$ (± 9.41)	$9.86 \cdot 10^{-14}$ (± 7.84)	$5.50 \cdot 10^{-13}$ (± 22.99)	0.97 (± 51.92)	0.002 (± 6022)	$1.33 \cdot 10^{-12}$ (± 23)	$3.74 \cdot 10^{-13}$ (± 61.44)	1.29 (± 15.88)
k_{me}	1/d	0.46 (± 27.91)	1.07 (± 9.46)								
$k_{e,mrg}$	1/d	0.12 (± 0.65)	0.41 (± 1.55)	0.44 (± 7.02)	0.14 (± 1.82)	0.54 (± 7.14)	0.14 (± 0.50)	0.47 (± 21.96)	0.03 (± 0.27)	2.77 (± 68.28)	0.23 (± 0.55)
k_{12}	1/d	0.001 (± 1.40)	$7.52 \cdot 10^{-6}$ (± 0.37)	0.25 (± 3.30)	0.21 (± 5.15)	0.18 (± 4.39)	0.17 (± 7.27)	0.98 (± 3447)	0.14 (± 4.44)	$4.73 \cdot 10^{-12}$ (± 0.43)	0.002 (± 6.92)
k_{21}	1/d	0.88 (± 83.19)	$4.55 \cdot 10^{-4}$ (± 4.09)	0.05 (± 1.66)	0.48 (± 12.18)	1.46 (± 43.38)	0.37 (± 23.55)	0.003 (± 774)	0 (± 23)	$1.91 \cdot 10^{-12}$ (± 1.03)	0.007 (± 4.43)

Abbreviations: BCF_{map}: bioaccumulation factor for the MAP representing the ratio between the rate of the influx and the outflux for this compartment; k_{map}: the rate constant for metal uptake into the MAP; k_{cd}: the rate constant for metal uptake to the cellular debris; $k_{e,cd}$: the rate constant for metal elimination from the cellular debris; k_r: the rate constant that metals are released from binding with MTLP to the MAP; k_{rD}: the reversible detoxification rate constant; k_{iD}: the irreversible detoxification rate constant; k_{int}: the rate constant for metals from binding to MTLP to be incorporated into granules; $k_{e,ptm}$: the rate constant for metal elimination from the PTM; k_{me}: the rate constant for metal use in metabolic processes; $k_{e,mrg}$: the rate constant for metal elimination from the irreversible detoxified fraction (MRG); k_{12} and k_{21}: the rate constant for metal transport between the cellular debris and the MAP.

detoxification capacity that affects the effect of internalized metals is revealed as both the association of metals with insoluble metal-rich granules and with heat-stable proteins in the cytosol are quantified. The disturbance of physiological processes is related to the interactions of metals with sensitive molecules such as small peptides, nuclei, and membranes (Blanchard et al., 2009; Kamunde, 2009; Pan & Wang, 2008; Serafim & Bebianno, 2007). Therefore, the delineation of the subcellular metal distribution facilitates more accurate estimates of metal toxicity. However, further steps should be taken in both toxicokinetics and toxicodynamics to achieve more progress in risk assessment for metals.

Regarding toxicokinetics, further attempts should be paid to get closer to the biologically effective dose. Semi- and full-PBTK models (Gestin et al., 2021; Le et al., 2018; Le, Kiwitt, et al., 2022) enable simulating metal organotropism, providing estimates of metal concentrations at specific organs/tissues. Integrating the subcellular metal simulation into these models might facilitate predicting the concentrations of metals at sites of toxic action in the sensitive tissues. With such an approach, we might take another step toward the target-site concentration.

Further delineation of toxicodynamics will contribute to significant progress in TK-TD modeling and risk assessment. The potential of the metal concentration in sensitive fractions to indicate metal toxicity should be evaluated in comparison with the whole body/tissue residue. The relationship between the concentration of metals in sensitive fractions and toxicological effects might not be direct. In the TK-TD model for survival, the survival probability is related to the internal concentration via "abstract" damage. With the increasing application of biomarkers to present signals of early warning, they can be considered as potential damage, which are induced by metal accumulation in sensitive fractions and might lead to toxicological effects like mortality. The interactions of metals with subcellular fractions, as delineated by the subcellular partitioning model, might firstly lead to cellular and molecular changes via various mechanisms including oxidative stress, osmoregulation, and energy metabolism. Oxidative stress is related to stimulated peroxidation of poly-unsaturated fatty acids by metal-enhanced formation of reactive oxygen species (Frias-Espericueta et al., 2022; Schaich, 1992; Valavanidis et al., 2006). Covalent bonds to thiol groups (Kone et al., 1990) or interactions with magnesium binding sites (Li et al., 1996) inhibit the activity of osmoregulatory enzymes (Griffith, 2017), causing ionoregulatory and osmotic disruption (Grosell et al., 2002). Metals might replace Zn^{2+} at active sites or bind to histidine and cysteine residues (Caricato et al., 2018), thus inhibiting the activity of carbonic anhydrase (Lionetto et al., 2006; Santini et al., 2011) and consequent interference of calcium metabolism. In addition, energy is diverted into detoxification and damage repair, resulting in effects on energy metabolism (Sokolova & Lannig, 2008). The production of metallothioneins to detoxify metals might inhibit mitochondrial oxygen consumption (Simpkins et al., 1998). These findings indicate potential causality links between subcellular metal distribution and responses of organisms at cellular and molecular levels, as addressed in our previous TK-TD models (Le, Nachev, et al., 2021). Furthermore, damage at this level might trigger toxicological consequences such as mortality. For instance, the increase in the energetic costs of osmoregulation is lethal as organisms are unable to regulate hemolymph osmolarity (Thwala et al., 2011). As such, the delineation of subcellular metal distribution facilitates predicting the concentration of metals in sensitive fractions, thus providing more accurate estimates of adverse effects and substantial improvement in TK-TD modeling.

5. Conclusions

As toxicity is determined by the target-site concentration, this dose metric is the ideal connector between toxicokinetics and toxicodynamics. It is usually difficult to measure the concentration of metals at sites of toxic action. However, with significant progress in risk assessment for metals, we are approaching to a closer surrogate for the biologically effective dose. In the present study,

various surrogates were reviewed. Among them, the concentration of metabolically active metals, i.e. potentially available for modulating sensitive molecules, is likely the closest one. A general modeling framework that allows for simulating the time-course of metal accumulation in this fraction was calibrated for various metals. With a number of parameters, model calibration with limited data availability might lead to significant uncertainties. This factor should be considered in applying the model.

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