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Metal-specific interactions at the interface of chemistry and biology*

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Abstract: Metals have complex environmental chemistry. When metals are present at elevated levels, they cause toxicity. Some metals are essential for living organisms, and those metals occur naturally in the environment. The latter aspect has allowed biological species to adapt to long- and short-term variations in metal levels. Chemical speciation, bioavailability, bioaccumulation, toxicity, and mixture effects are key issues in assessing the hazards of metals.

In the present contribution, a global overview is given of the interactions between the chemistry and biology of metals, mostly at the interface of biological and environmental matrices. The environmental chemistry of metals and resulting methods for assessing metal availability are assumed as tokens, and the emphasis is thus on biological processes affecting the fate and effects of metals following interaction of the organism with the bioavailable metal fraction. The overview culminates in linking metal compartmentalization in organisms to bioaccumulation and toxicity.

Keywords: soil; water; metal; bioavailability; bioaccumulation; toxicity; BLM; FIAM; biotic ligand model; competition.

INTRODUCTION

Chemicals may exert adverse effects by a multitude of chemical and biological interactions. Serious deleterious effects may occur at one location, whereas the same chemical concentration can be fully harmless at other sites. A general mechanism of toxicity of hydrophobic organic compounds is the nonpolar narcotic mode of action, and it is partitioning to organic phases that is predominantly modulating effects. Organic phases of interest include in this respect soil and sediment organic matter, dissolved organic carbon, and the fat content of specific biota. As initially shown by McCarthy [1] and McCarty and Mackay [2], it actually is possible to derive critical body burdens (CBRs) for specific organisms and translate these into critical concentrations in any of the environmental compartments, assuming on the one hand that the total body concentration of a nonpolar narcotic organic contaminant is proportional to the concentration at the target or receptor of toxicity, while on the other hand assuming that (i) fat tissue is the main storage compartment for hydrophobic organic chemicals and (ii) fat tissue behaves similarly to the abiotic organic phases present in the system. McCarty and Mackay [2] showed that CBRs for polar narcotics are indeed fairly constant. The latter two assumptions imply that the CBR or a specific effect level (EC_x, with x being the extent of adverse effect) is proportional to the octanol–water partitioning coefficient of the chemical:

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$$\text{Log CBR or log EC}_x \approx a \times \log K_{ow} + b \quad (1)$$

Karickhoff et al. [3] were some of the first authors to show the equilibrium concept of partitioning of organic compounds by reporting that K_{ow} is proportional to the compound-specific organic carbon normalized partition coefficient (K_{oc}). Examples of the first sets of the K_{ow}/K_{oc} relationship reported in the literature are given in Table 1. Subsequently, it is K_{oc} that may be used to not only predict the degree of chemical partitioning between water and the sediment or soil organic carbon, but also the baseline toxicity of hydrophobic organic chemicals in a specific medium varying in organic carbon content.

$$K_{oc} = K_d/f_{oc}, \text{ with } K_d = C_w/C_{\text{solid phase}} \quad (2)$$

and

$$\text{Log CBR or log EC}_x = a \times \log K_{oc} \times f_{oc} + b \quad (3)$$

K_d is the partition coefficient of the chemical, C_w and $C_{\text{solid phase}}$ are the equilibrium concentrations of the chemical in the aquatic and solid phases, respectively, f_{oc} is the fraction organic carbon in the solid phase.

Table 1 Examples of K_{oc}/K_{ow} relationships of the form log $K_{oc} = a \times \log K_{ow} + b$.

Compounds	*a*	*b*
Alkylated and chlorinated benzenes, PCBs	0.74	0.15
PAHs	0.98	–0.32
Chlorinated phenols	0.89	–0.15
C1 and C2 halocarbons	0.57	0.66
-only chloroalkanes	0.42	0.93
-only chloroalkenes	0.96	–0.23
Brominated compounds	0.50	0.81
Phenylureas	0.49	1.05

Low-polarity organic compounds may, however, on top of equilibrium partitioning, also bind to solid phases through adsorption mechanisms, which result in greater (nonlinear) binding coefficients. Thermally or diagenetically altered forms of carbonaceous materials such as coal, kerogen from shales, soot, and charcoal have particularly high binding coefficients and nonlinear adsorption behavior, with carbon-normalized Freundlich sorption coefficients that are as much as 50 to 250 times greater than typically reported K_{oc} values [4–6]. Hydrophobic partitioning is less important for polar and ionizable compounds. These compounds are involved in more diverse binding mechanisms that contribute to compound retention, including electrostatic attraction–repulsion and bonding at specific surface sites. As the retention of these compounds is governed by a complex set of physical–chemical processes, it is difficult to generalize trends in their behavior, albeit that retention is more dependent on solution chemistry (especially pH and dissolved organic carbon, or DOC) than is the case for nonpolar compounds.

The situation is more complex for metals as a multitude of sequestration processes in the abiotic and the biotic environment affect actually occurring adverse effects. Organic contaminants are subject to various abiotic and biotic degradation processes. Metals, on the other hand, are inherently persistent as they are neither created nor destroyed by anthropogenic or biological processes, although they may undergo various speciation changes in abiotic as well as in biotic systems. Speciation is defined in this sense as the distribution of a metal across various chemical species. Metals can be present as hydrated free metal ions, hydroxo-complexes, inorganic complexes, complexes with natural organic

polymers like humic and fulvic acids, and complexes with synthetic organic chemicals like nitrilotriacetic acid (NTA) and ethylenediaminetetracetic acid (EDTA). Metals can exist in soil and sediment systems in the aqueous phase, as part of a precipitated mineral, or adsorbed on the surface of a mineral. The type of association of a metal and its binding intensity is very important in determining its mobility and availability. The availability to plants and animals, and the intensity of binding on other minerals is often the factor controlling the mobility and bioavailability of a metal. This binding intensity is strongly modulated by solution conditions, with pH and the ionic composition being the primary driving factors (e.g., competition aspects with the cations). Organic matter, clays, and oxides of Fe, Al, and Mn are the predominant sorbing phases that retain metals. Adsorption processes typically dominate the association of metals to solid phases. But, depending on the specific metal and site conditions, precipitation may play a large role in governing aqueous metal concentrations. The most pronounced example in this respect is metal precipitation in anaerobic sediments that are dominated by high concentrations of sulfide that can result in precipitation of metal sulfides [7].

In addition to the more complex environmental fate of metals, the processes underlying actually occurring adverse effects are also more complex for metals than is the case for organic compounds. It is impossible to define a single bioavailable metal pool that dictates accumulation. Therefore, metal toxicity cannot be successfully predicted by a single metal pool commonly distinguished, including the dissolved metal fraction or the total metal pool. The free metal ion often correlates best to toxicity endpoints (see, e.g., [8]), but major cations like H, Ca, K, Mg, and Na also affect uptake and toxicity. In Table 2, a summary is given of the major differences in kinetic behavior of organic compounds compared to metals and inorganic metal compounds in biota (as adapted from Goyer et al. [9]).

Table 2 Summary of major differences in kinetic behavior of organic compounds compared to metals and inorganic metal compounds in biota (adapted from [9]).

Organics	Metals
Tissue uptake is most commonly a blood flow limited process, with linear portioning into tissues.	Metals and their complexes are often ionized, with *tissue uptake* (membrane transport) having greater potential to be diffusion-limited or to use specialized transport processes.
Metabolism is generally extensive and often species-specific.	*Metabolism* is usually limited to oxidation state transitions and alkylation/de-alkylation reactions.
Persistence in body fat is common because of lipid solubility (not capacity-limited).	Often *sequestered*, bound to specific plasma or tissue proteins (intrinsically capacity-limited), or deposited in inert forms such as waste nodules, mineral concretions, and granulas.
Due to complex metabolism, organics may be *eliminated* by excretion in urine after biotransformation from lipophilic forms to hydrophilic forms.	Predominantly *eliminated* by excretion because metal compounds are generally small molecules and are hydrophilic. Thereupon, excretion of inert species.
Generally substance-specific *homeostatic mechanisms* are not available.	Essential metals have *homeostatic mechanisms* that maintain optimum tissue levels over a range of exposures.
Interactions with other structurally similar compounds may occur, especially during metabolism.	*Interactions* among metals and between metals and organics are numerous and occur commonly during the processes of absorption, excretion, and sequestration.

We recently reviewed the chemical processes underlying the fate of metals in the environment as well as extraction methods that may be used to simulate available metal fractions [10]. In the current paper, we focus on the specific interactions of metals at the abiotic and biotic interfaces present in the environment, which is more or less the follow-up on the chemical interactions. The interactions at the interface of chemistry and biology are schematically given in Fig. 1. The focus is on the processes that follow the chemical interactions between solid and aquatic (including pore water) phases, as modulated by the composition of the aquatic phase and the biotic uptake routes. A central issue in this respect is metal uptake by biota via pore water vs. ingestion of solid particles and subsequent release of metals in the gastrointestinal track of the organism. The basic assumption is that it is the free metal ion that is the predominant species actually exerting toxicological effects. It has repeatedly been shown that differences in the free metal ion activity of the test medium can explain a substantial part (but not all) of the observed toxicity variability.

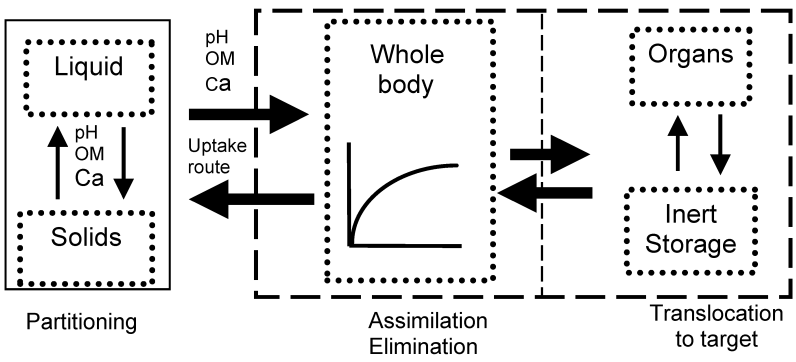

Fig. 1 Overview of the principal chemical and biological interactions at the abiotic and biotic interfaces present in the environment.

The aim of this contribution is to review the processes that are involvedin passage of metals across physiological membranes and the subsequentcirculation and sequestration within organisms. Moreover, we intend toprovide understanding of the biological significance of metalconcentrations in biota and in soils. This way, analogous to the CBR conceptfor nonpolar narcotic organics, a step forward is made toward an approachfor accurate assessment of inorganic toxicity.The general hypotheses are that:

- Toxicity is modulated in chemical terms by the composition of theexposure medium and in biological terms by the capability of organisms torespond to differences in free metal activities.
- The metal fraction interacting with the receptors of toxicity is thebest estimate of toxicity.
- It is possible to derive (pragmatic) tools that quantify the metalfraction directly associated with toxicity.

METAL-SPECIFIC ISSUES

General

In contrast to organic compounds, metals can exist in many different speciation forms. Moreover, they can transform rapidly from one speciation form to another. Biological and chemical processes jointly determine the persistence of a metal or metal compound in a specific environmental compartment. Basically, there is no difference between the principal processes by which metals are transformed in the abiotic environment and in biota: chemical binding, complexation, absorption, adsorption, and precipitation. The main factors affecting metal speciation and interaction with biota include:

- acidity (pH)
- oxidation potential—redox conditions
- salinity
- competing ions
- nature of sorbent phases and their surface areas
- surface site densities
- colloid formation

Similar to organic chemicals, high binding coefficients and nonlinear adsorption of metals are typical features that tend to increase over time due to increased chemical binding, precipitation, and inclusion of metals into solid matrices (aging). Aging is also impacted by most of the factors affecting metal speciation. Finally, organisms compete with abiotic phases for binding of metals to specific receptor sites.

The form of the metal (chemical species, compound, matrix, and particle size) influences the metal's bioavailability, bioaccessibility, fate, and effects. In the past, various empirical chemical simulations of the bioavailable and bioaccessible metal fractions have been exploited. The tests often involve chemical extractions to account for contaminant release from the solid surface to the pore water. Thus, they are most successful (predictive) when biological uptake is dominated by a pore/water-related pathway like plant uptake and metal uptake by soft-bodied organisms. However, due to the multitude of processes that sequester metals, the additional impact of factors modulating the intensity of metal-sorbent interactions, as well as additional time considerations (aging processes), no universal method is available at the moment for properly simulating the bioavailable and bioaccessible metal fractions [10]. Extractions also cannot account for other, more complicated uptake mechanisms that control an organism's overall dose, such as dietary exposure, acid extraction, removal by surfactants, and ligand complexation in solution and on membranes. No single universal extraction procedure has been shown to consistently correlate with tissue concentrations in plants or animals across complicated environmental conditions [11].

Consequences of metal origin—essentiality

Metals typically occur in the environment at concentrations ranging from geological background levels to potentially much higher levels in areas affected by natural point sources or human activities. Low metal concentrations result in deficiency effects, and high metal concentrations result in toxic effects: intermediate levels can cover a large concentration range and are the windows of essentiality [12]. Depending on the magnitude of the exposure associated with these factors, background metal concentrations can account for a significant portion of total metal exposure. Due to species-specific physiological conditions, certain essential elements can bioaccumulate to high levels in organisms (e.g., Zn in barnacles and Cu in crayfish). The fact that metals are of natural origin has important biological consequences as it has enabled all biotic species to genetically adapt to (natural) changes in bioavailable metal levels. As the Earth's flora and fauna have evolved in the presence of metals, issues like essentiality, bioaccumulation, and tolerance have become typical for metals. Many metals and metalloids are required for biological life, and therefore their presence in the ambient environment has actually shaped the natural ecosystem. Table 3 gives an overview of the known essentiality status of metals.

Table 3 Classification of metals by their status as essential element (adapted from a table presented in [13]).

Metal	Essential (known requirement for health and function)		Beneficial (but not known to be essential)		Nonessential (and not known to be beneficial)
	Plants	Animals	Plants	Animals	
Al					X
As				X	
Ba					X
Cd					X
Cr		X			
Co		X	X		
Cu	X	X			
Pb					X
Mn	X	X			
Hg					X
Mo	X	X			
Ni	X	X			
Se		X	X		
Ag					X
V				X	
Zn	X	X			

Bioaccumulation of essential metals is a natural process required by living organisms for metabolism and growth. Many organisms have developed effective means of regulating internal essential metal concentrations within quite narrow ranges (homeostasis). Homeostatic mechanisms (such as induction of stress proteins) can even play a role in the detoxification of non-essential metals such as Cd, Pb, or Hg. In terms of adverse effects, it is accepted that essentiality should be viewed as part of the overall dose–response relationship, recognizing that the shape of this relationship can vary among organisms and for given subpopulations. In this respect, the principle of optimal concentration range of essential elements (OCEE) is advocated. As graphically shown in Fig. 2, this principle states that ecosystem functioning is impaired both below and above the OCEE. Impairment of ecosystem functioning is further complicated by evolutionary processes, which may shift the OCEE. Evolution is contingent on genetic variability and environmental conditions, and as populations are not genetically homogeneous across their distribution ranges, they can locally undergo evolutionary processes that result in modified dose–response relationships [14]. Two generic mechanisms may be identified by which organisms cope with excessive metal burdens in their native (non-disturbed) habitat: adaptation and acclimation. Adaptation involves heritable, genetically determined, induced resistance resulting from directional selection during multigenerational exposure histories. Acclimation results from stress responses during the lifetime of an individual, the traits of which may be lost if the stressor is withdrawn.

To further complicate and complete the picture, it should be kept in mind that the toxicity of certain metal elements is associated with deficiency of other elements. For example, increased Zn, Cu, and Ni toxicity can be associated with Fe deficiency [15], and increased Pb and Zn toxicity can be related to P deficiency [16–18]. Also, the behavior of plant species in response to nutrient deficiencies varies, and this behavior can affect the uptake of metal elements [19]. All these factors need to be taken into

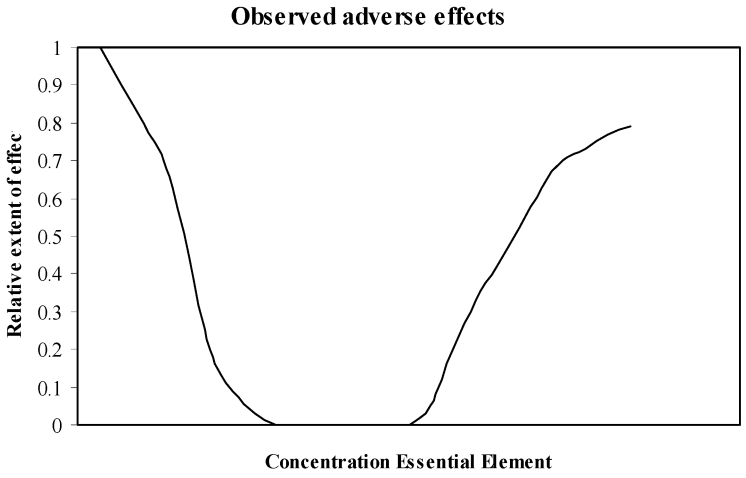

Fig. 2 Schematic representation of the OCEE principle.

consideration when assessing the impact of chemical interactions of metals at background levels with biotic species.

It is well known that metal concentrations vary widely over time and space due to differences in geology, hydrology, and anthropogenic and natural loading. The concept of metalloregions was proposed to account for differences in exposure to natural background concentrations across metal-related regions. This was done for the aquatic [20] and for the terrestrial environment [21] to provide the ability to account for the broad regional parameters affecting metal availability in soils and waters as well as for the differences in organisms' response to added metal.

Metal accumulation by biota

The chemical form of a contaminant released from the solid matrix, the geochemical environment where the release and transport take place, and the fluid properties of that environment will determine the form, delivery route, and delivery rate of the contaminant to biological receptors. A range of receptors (including plants, animals, microorganisms, and even humans) and a range of exposure routes are of interest in contaminant bioavailability. Consequently, it is difficult to generalize the process of contact and entry of chemicals in biota.

Biological uptake of most synthetic (hydrophobic) organic contaminants occurs by simple passive diffusion across a cell membrane. Membrane carriers are not involved, and, as stated above, the biological effect of organic contaminants is often characterized by narcosis, implying that the extent of adverse effect of organic contaminants is proportional to the value of the octanol–water partition coefficient. In contrast, as metals generally exist in strongly hydrated species, they are unable to traverse biological membranes by simple diffusion. Membrane transport occurs by facilitated transport, usually passive (i.e., not against a concentration gradient), and necessarily involving either membrane carriers or channels. The chemical binds to the carrier protein and is carried through the membrane by a process that requires no cellular energy. There is some specificity to the carrier protein binding, and so the process is applicable only for selected chemicals. Transport of essential metals is, for instance, facilitated by carriers or pores specific to the element, although metals are also transported on carriers designed for elements of similar physicochemical characteristics. Nies and Silver [22] showed, for instance, that Mn and Cu share a carrier in some phytoplankton species, whereas numerous examples are available for facilitated transport of Cd via Zn carriers and Ca channels (see, e.g., [23]). Facilitated diffusion may be shown experimentally by demonstrating that the influx can become saturated, thus show-

ing that a finite number of carriers exists. Uptake by passive facilitated diffusion does not preclude cellular accumulation of metals to concentrations higher than in the external media. If the chemical is rapidly sequestered, an inward flux can be sustained and the exchange between the converted form and the form crossing the membrane will ultimately determine the steady-state concentration.

Active transport uses carrier proteins to move chemicals against their concentration gradient, while requiring cellular energy. As with facilitated diffusion, there is specificity in the binding of metals to these carrier proteins. In addition to diffusion, other processes exist to bring substances across membranes. Large particles can be internalized through phagocytosis, during which the plasma membrane of a cell surrounds and engulfs a particle that is outside the cell by creating a vesicle that detaches within the cell. The quantitative importance of this specific process is difficult to demonstrate.

Metal sequestration in animals and plants

Determining the importance of levels of accumulated metals in biota as well as an organism's responses presents a number of difficulties. Intrinsic biotic (such as seasonal-linked variations, population density effects, and developmental and age-related parameters) and extrinsic abiotic factors (such as spatial heterogeneity in the habitat, climatic variations, food availability, and food variability) modulate effective exposure. Luoma and Rainbow [24] listed four interacting factors that influence metal bioaccumulation by animals:

- metal specificity
- environmental influences
- exposure route
- species-specific influences

Thus, metal accumulation is the net result of a delicate interplay between abiotic and biotic factors at interfaces like the dermis–(pore)water interface and interfaces in the intestine, internal metabolically induced redistribution, and excretion processes. Persistence in body fat is common for organic chemicals. Metals, on the other hand, typically become sequestered following chemical interaction with specific binding or sorbing phases. The general principle is that the extent of sequestration is proportional to the capacity of receptors for metal binding times the intensity of effective metal exposure (i.e., the activity of the free metal ion in the body solution).

Given evolutionary acclimation and adaptation, a variety of mechanisms exist for the detoxification of metals once they have entered an organism, precluding simple predictions of metal-induced toxicity on the basis of metal quotas or burdens, as is done with organic contaminants. Metals inside organisms can be present in the free ionic form or as soluble salts (complexed species such as, e.g., $ZnCl_2$, $ZnCl^+$, $ZnOH^+$), or bound (taken from Vijver et al. [25]) as follows:

- in the active center of functional proteins (e.g., hemoglobin, hemocyanin) and low-molecular-weight peptides (e.g., Zn finger proteins);
- in the active center of enzymes (e.g., cytochromes, carbonic anhydrase);
- to transport proteins (e.g., ferritin);
- to low-molecular-weight organic acids (e.g., citrate);
- to metallothionein (MT) or other sequestration proteins;
- to vesicles of the lysosomal system; intra- and extracellular granules; or
- to cellular constituents causing dysfunction (e.g., ion channels, DNA).

Moreover, metals may be precipitated in inert forms such as waste nodules and mineral concretions.

Given the availability of these sequestration tools in combination with the organism's capability of reducing uptake of potentially toxic metals affecting the organism's metabolism, a diversity of metal accumulation strategies is known. In response to either insufficient (essential nutrients) or excess amounts of bioavailable metal, it is the kinetics of the underlying chemical processes as well as the abil-

ity of the organism to respond to variations in the abiotic environment which determine whether an organism is capable of functioning properly. As advocated by Van Straalen et al. [26], bioaccumulation is preferably described on the basis of uptake and elimination fluxes. Conceptually, it was Rainbow [27] who distinguished different metal accumulation strategies for essential and nonessential elements. Essential metals may be subject to regulation either by limiting metal uptake at the level of the total body concentration or by involving organism-specific accumulation strategies with active excretion from the metal excess pool and/or storage in an inert form and/or excretion of stored (detoxified) metal. For nonessential metals, excretion from the metal excess pool and internal storage without elimination are the major strategies, and body concentrations generally increase with increasing external concentrations. The general concept as brought forward by Rainbow [27] is depicted in Fig. 3. As indicated in Fig. 3, toxicity starts when the elimination and detoxification fluxes are insufficient to prevent metals from interacting with important biological molecules. Formation of inclusion bodies (granules or concretions) is of importance in this respect. On the other hand, one of the best-studied metabolic changes induced by heavy metal exposure is the induction of a metal-binding protein, MT. Comparing MT across species reveals a remarkable variability of primary structure, number of metal-binding domains per MT molecule, and number of metal ions per domain. In addition, many species have more than one MT gene [28]. Exactly how excess metal is removed after being bound by MT is not completely known. One of the mechanisms suggested is that MT donates excess metals to vesicles of the lysosomal system. This may involve metal-induced transporters in the lysosomal membrane [29]. Especially in invertebrates, tissues with an intestinal and hepatic function are often full of metal-containing vesicles, which in electron microscope images become visible as electron-dense granules. These granules may be removed from the cell by exocytosis or by apoptosis of the cell. Other metal chelating substances have been found, initially in plants, hence called phytochelatins. The peptides are synthesized from three amino acids by the enzyme phytochelatin synthase.

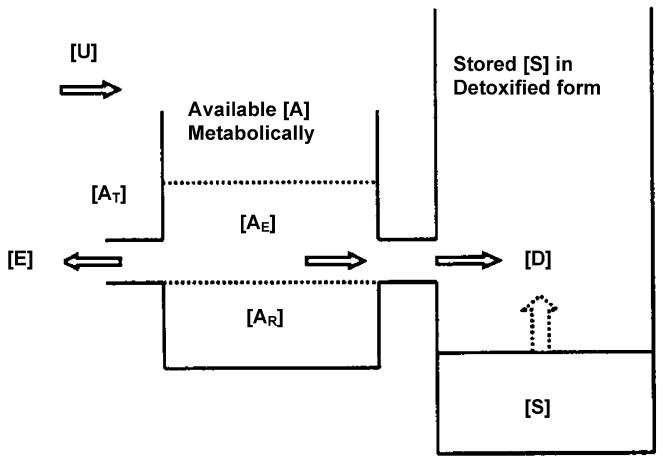

Fig. 3 Schematic representation of internal redistribution processes of metals following uptake. U = uptake flux; E = elimination flux; S = flux of storage in inert form; D = detoxified pool; A_R = metabolically required metal pool; A_E = excess metal pool above metabolic requirement, causing toxicity when the elimination and detoxification fluxes are insufficient; A_T = total metabolically available metal pool (modified from Rainbow [27]).

Vijver et al. [24] reviewed the factors that determine sequestration of metals by invertebrates and concluded that subcellular metal partitioning as the basis of internal metal sequestration over different organs and tissues is dependent on the following.

- The metal, with differences among metals linked to the affinity of the metal for specific binding groups. Metabolic pathways and internal compartmentalization of metals with closely related chemistry (such as Zn and Cd) remain distinct, whereas differences in specific ionic radius and electronegativity result in affinity to N- and S-containing groups for metals such as Cd, Cu, and Pb. Ca, Al, and Be, on the other hand, are, for example, more effective at binding with oxygen-containing ligands such as carboxylic acids and alcohols. A third group of elements, containing amongst others Ni and Zn, have no binding preference and will bind to many structurally diverse ligands. These metals are, therefore, considered borderline metals.
- Pre-exposure or tolerance induction, with external concentration and duration of exposure having significant effects on the distribution of metals inside the organism. Also, the ability of the organisms to produce the various forms of MT, and thus their ability to increase resistance following chronic exposure to increased concentrations of metals is affected by the duration of pre-exposure. Cain et al. [30] found in this respect that differences in exposure history had no influence on the bioaccumulation of Cd, but did affect the concentrations of Cd bound to MT-like proteins during Cd exposure in the insect *Hydropsyche californica*.
- The biotic species, with large differences observable across species from similar trophic levels due to differences in accumulation strategy.
- The primary uptake route, with a distinction to be made between uptake via the diet (gastrointestinal tract) and uptake via the epidermal surface or the gill. Cd and Hg, for instance, mainly accumulate in the hepatopancreas of the lobster *Nephrops norvegicus* when taken up via the food, and in the gill and the hepatopancreas when taken up via the water.

The specific internal organs and tissues in fish that accumulate Cd vary with the route of uptake and with the species. For example, in trout (*Oncorhynchus mykiss*) during water-borne exposure, the gills and the kidney are the organs with the highest Cd accumulation, whereas dietary Cd accumulates mostly in the gastrointestinal tract and kidney. Although organ-specific metal distribution has been studied for both dietary and water-borne Cd exposure, the effect of tissue-specific Cd burdens on subsequent Cd uptake and deposition is unknown. Szebedinszky et al. [31] compared the effects of chronic sublethal Cd loading via dietary vs. water-borne routes on internal organ-specific accumulation patterns and on subsequent gill Cd binding and uptake kinetics. Fish exposed to dietary Cd accumulated a much greater overall chronic Cd body burden relative to fish exposed to water-borne Cd at the same gill Cd load or control fish. The carcasses accumulated the greatest percentage of total body Cd in control and water-borne-exposed fish, whereas the intestinal tissue accumulated the greatest percentage in dietary-exposed fish. Tissue-specific Cd burdens were highest in the kidney in both dietary and water-borne treatments. The authors concluded that chronic Cd exposure alters Cd uptake dynamics, and that the route of Cd exposure results in differences of internal Cd accumulation and branchial Cd uptake characteristics.

Consequences of metal sequestration

The various ligands to which metals bind once they have been taken up by the organism, all have their own binding strength and their own binding capacity. These differences in binding strength and binding capacity have consequences for food-chain transfer to higher trophic levels and for toxicity induction in the organism itself. A limited number of studies have been carried out to relate internal sequestration to trophic transfer. Wallace et al. [32] found that only metal present in the soluble fraction of prey is available for the predator, and differences in subcellular distribution between resistant and non-resistant worms directly affected Cd availability for predatory shrimp: when offered resistant worms, shrimp accumulated about four times less Cd than when fed nonresistant worms. Similar findings are reported by Hopkin et al. [12]. These authors found that metals bound in the granules of the hepatopancreas of woodlice were not absorbed by the predatory spider *Dysdera crocata*, but metals bound to

ferritin were released and became available. Thus, metals in MT and in tissue fractions appear available for trophic transfer, while metals in metal-rich granule fractions are only partially available. One should be aware that the bioavailability of metals in prey may also depend on the digestive physiology of the predator (e.g., retention time, and especially acidity, pH, and extractive strength of the gut fluids).

Limited studies are available that link internal metal sequestration to toxicity. According to Vijver et al. [24], the amount of metal compartmentalized in cytosolic, denaturated protein, and tissue fractions seems to be most indicative of toxic pressure. The results of Conder et al. [33] confirm this general point of view as these authors found that as soon as the storage capacity of the metal-rich granulas was exceeded, toxicity started to occur for the earthworm *Eisenia fetida* exposed in artificial soil spiked with Cd salts.

Operationalization

Fractionating body burdens of metals has been suggested as a better means of identifying the bioavailable fraction. Specific metal fractions may thereupon serve as an alternative for CBRs based on total metal loadings, requiring the establishment of dose–response relationships between metal levels in an internal metal compartment and whole organism responses. Wallace and Lopez [34] developed a protocol based on homogenization of biotic samples followed by several centrifugation and/or digestion steps to hydrolyze and separate (when appropriate) metal-rich granules, tissue fragments, and intracellular and cytosolic (MT and heat-sensitive proteins) fractions. The scheme is based upon the same principles of metal speciation as applicable within the abiotic environment, and is operationalized by means of sequential extraction techniques. Metals are supposed to enter the organism in an active form, after which they are captured by labile, reversible, binding to proteins and other ligands, followed by localization to a target that has a higher affinity for metals. Vijver et al. [35] applied a modified version of the protocol to samples of the earthworm *Aporrectodea caliginosa* taken from the field, and found irregular patterns of metal uptake and elimination in this organism. The (modified) fractionation protocol of Wallace and Lopez [34] clearly is of a pragmatic nature. Further research is needed to link metal fractions thus obtained to either adverse effects or bioaccumulation. The protocols clearly show that metal accumulation is dependent on the metal and on the biotic species of interest. Table 4 shows an example of metal-specific distribution patterns of three metals across three operationally defined fractions for five soil organisms. The distribution depicted in Table 4 was obtained following the experimental details given by Vijver et al. [35]. Similar investigations of Bonneris et al. [36] on the subcellular partitioning of Cd, Cu, and Zn in the gills of a freshwater bivalve, *Pyganodon grandis*, along a polymetallic concentration gradient showed that under chronic exposure conditions, the three metals were mainly located in calcium concretions present in the gills (respectively, 58 ± 13 % of the total gill Cd, 64 ± 6 % of the total gill Cu, and 73 ± 6 % of the total gill Zn). Binding to MT could only account for a small proportion of total gill metal (about 10 % Cd; about 3 % Cu; about 1 % Zn). Metal concentrations increased proportionally in both the granules and the MT pool along the polymetallic gradient, suggesting a constant partitioning between these two compartments. Despite an increase in Cd in the "mitochondria" fraction, metal sequestration mechanisms seem to be reasonably effective in detoxifying Cd. Cd concentrations in the cytosol (i.e., the potentially metal-sensitive fraction) remained relatively low and constant, even in specimens collected from the most contaminated lakes. Although similar investigations have not been carried out with higher organisms, it is likely that mechanisms of dealing with metals are not solely yield for invertebrates but are rather uniform over biota. Therefore, similar sequestration patterns may also be observed for higher biotic species. For these animals, it is important to take the impact of the predominant uptake route via the gastrointestinal track explicitly into account.

Table 4 Typical distribution (%) of Cd, Cu, and Zn across three internal fractions in various terrestrial species sampled from the field (n.d. = not determined, remainder = fraction composed of tissue fragments, cell membranes, and intact cells).

Animal	Cd			Cu			Zn		
	Cytosol	Granules	Remainder	Cytosol	Granules	Remainder	Cytosol	Granules	Remainder
Earthworms	85.4	2.3	12.3	52.3	17.8	30.0	26.0	20.8	53.2
Beetle larvae	12.0	36.7	51.3	n.d.	n.d.	n.d.	66.3	5.6	28.2
Millipedes	65.3	6.3	28.4	76.5	3.1	20.4	54.6	7.6	37.8
Centipedes	41.7	21.0	37.3	64.7	14.1	21.2	26.8	30.9	42.3
Snails	77.7	4.4	18.0	72.9	7.0	20.1	25.7	40.9	33.4

Metal toxicity

For many metals in aquatic systems, it is the free ionic form that is most responsible for toxicity. This is despite the fact that strictly speaking, metals may be taken up via various exposure pathways and in a complexed state, bound to a number of ligands of varying binding capacity and varying binding strength. The free ion activity model (FIAM) is used to explain the relationship between speciation in the external environment and bioavailability to the organisms [8]. The FIAM produces speciation profiles of a metal in an aquatic system and provides insight into the relative bioavailabilities of the different forms of metal as well as the importance of complexation.

There is, however, an increasing body of evidence becoming available, showing that the toxicity caused by the free metal ion is modulated by a number of chemically induced competing processes. This observation was the basis for the development of biotic ligand models (BLMs). BLM theory, on the one hand, incorporates the impact of water chemistry (most notably pH and DOC) on metal speciation, whereas the model, on the other hand, quantifies the assumption of competition between the major cations like Ca^{2+}, Mg^{2+}, Na^{+} and H^{+}, and free metal ions for binding sites at the organism–water interface may result in a decreased toxicity of the free metal ion. In some cases, it is taken into account that other metal species have the potential to contribute to toxicity, such as complexes with OH^{-} and CO_3^{2-} ions and organic metabolites in the case of Cu. BLMs include all these aspects and are, therefore, gaining increased interest in the scientific as well as the regulatory community. In fact, the BLM concept, now developed for Cu, Ni, Ag, and Zn, is considered as the currently most practical technique to assess the ecotoxicity of metals on a site-specific basis. Therefore, the BLM concept is now being approved in the EU.

A basic assumption of the BLM is that metal toxicity occurs as the result of metal ions reacting with binding sites at the organism–water interface, represented as a metal–biotic ligand (metal–BL) complex. The concentration of this metal–BL complex is proportionally related to the magnitude of the toxic effect, independent of the physical–chemical characteristics of the test medium. Hence, the acute toxicity of a trace metal to an organism can be calculated when metal speciation, the activity of each cation in solution, and the stability constant for each cation to the BL(s) for the organism are known. BLMs have recently been developed for mainly algae, daphnids, and fish (e.g., De Schamphelaere et al. [37–39] and Heijerick et al. [40,41]). These species of different trophic level are considered to be representative of ecosystem structure and important for ecosystem functioning. For insects, crustaceans, and certain algae species, it is justified to extrapolate BLMs to other taxonomic groups [42], suggesting that the mechanisms of toxicity are similar across species. Once a variety of organisms is available for which toxicity can be predicted using BLM theory, the BLM-corrected toxicity data can be coupled to species sensitivity distribution (SSD) curves. From these SSD curves, the potentially affected fraction of species in a specific water body can be derived accounting for water-type specific bioavailability [43–45]. BLM models are available for individual metals, such as Cu, Cd, Zn, Ni [46]. Paquin et al. [47] provided a historical overview of the fundamentals of BLMs.

Mixture toxicity

Mixtures of metals (including metalloids and organic substances) are commonly encountered in the environment. Metal interactions, according to Calamari and Alabaster [48], occur at three levels:

- chemical interactions with other constituents in the media,
- interactions with the physiological processes of the organism, and
- interactions at the site of toxic action.

The joint action of metal mixtures may be expressed in different ways, including increasing or decreasing the toxicity relative to that predicted for individual components. Much of the difficulty in predicting the toxicity of metal mixture results from differences in the bioavailability and/or methods used to define the bioavailable fraction among toxicological studies and subsequent ambiguity in interpreting mixture toxicity data. The bioavailability of metals depends on a suite of factors that can affect their speciation, complexation with ligands, and interaction with biological systems (e.g., pH, DOC, inorganic anions, and cations). Joint action of metal mixtures is known. In a similar review the European Center for Ecotoxicology and Toxicology of Chemicals concluded that the acute and chronic toxicity to aquatic organisms of mixtures of metals could not be reliably predicted or generalized although they recommended that, in the interim, assuming response additivity is likely "a balanced approach" for acute toxicity of metal mixtures [49]. Given these difficulties in evaluating mixtures effects, risk assessors commonly use two simplifying models: concentration addition and effects (response) addition. These models are used to classify the combined effects of chemical mixtures as being antagonistic, additive, and synergistic (also referred to as "less than additive", "strictly additive", and "more than additive", respectively).

Both models use metal concentrations in media to generate concentration–response curves for individual metals, and these data are then used to generate specific critical concentrations for mixture models. In the concentration addition model, all metals in a mixture are added together to predict toxicity; differing potencies are taken into account by converting chemical concentrations to an equitoxic dose, such as toxic units (TUs) or toxicity equivalence factors (TEFs), which convert all metals to one metal concentration. Concentration addition is often used when the constituents are known or assumed to act through the same or similar mode of toxic action. In the effects addition model, differing potencies are ignored, and the effect of each metal's concentration in a mixture is combined to predict mixture toxicity. The effects addition model is used when constituents act or are assumed to act independently (i.e., different modes of action).

Apart from bioavailability differences, adverse effects have been reported to depend on other aspects of toxicity test design, including the degree of toxicity associated with the overall mixture concentration [50–53], the relative proportion of constituent concentrations [54,55], the duration of the exposure [56], and several other factors related to experimental design [54]. Considering these aspects, mixture modeling might be expanded with receptor binding models such as FIAM and BLM in the future to optimize accurate effect prediction. If two metals compete for binding to the same site of toxic action (such as competition of Zn^{2+} and Cd^{2+} for Ca^{2+} transport sites at the fish gill), it is possible to model the total metal bound to that site and, hence, to predict metal toxicity using a mechanistic receptor binding approach in a concentration additive model. Alternatively, if two metals do not compete for the same binding site (such as binding of Zn^{2+} to Ca^{2+} transport sites vs. simultaneous binding of Cu^{2+} to Na^{+} transport sites and Ni^{2+} binding to allergic reaction sites at the fish gill) then these models may provide more reliable estimates of individual metal bioavailability, which then can be combined in more accurate response additive models. At present, only a theoretical framework (including mathematical modeling) is established for simple similar (concentration addition) and independent (response addition) joint actions of toxicants. Frameworks for joint actions of toxicants having a complex similar interaction and dissimilar dependent interactions are not yet developed.

OVERALL CONCLUSION

A complex interplay of environmental and biological features determines the fate and effects of metals. Metal bioavailability is shown to be metal- and biota-specific. The hydrated free metal ion species is generally thought to be the dominant species causing toxicity. Neither the activity of the free metal ion species, nor physicochemical simulations of bioavailable metal fractions, or accumulated metal burdens in specific biota and plants serve the purpose of presenting "the" universal expression of actually occurring adverse effects. Metal chemistry has evolved in a more complex manner than can be captured in relatively simple simulation methodologies. The way organisms handle metals accumulated in their body can be determined in a pragmatic manner using biochemical fractionation procedures. The insight in internal sequestration of metals thus gradually obtained may assist in understanding the relationship between bioavailability and toxicological availability. Risk assessment tools to be developed for accurately assessing adverse effects in realistic settings need to take this relationship into account. Tools under development that quantify the most dominant chemical interactions at the site of toxic action, such as the BLM and chemical simulation of interactions at the biological target sites of toxicity, are promising features in this respect, especially when considering mixtures of toxicants and stressors. Currently, however, no validated and generally applicable pragmatic tools (analogous to the CBR concept for organics) are available to quantify our general hypothesis of toxicity being modulated by the capability of organisms to sequester metals, in order to minimize interactions with the metal-specific receptors of toxicity.

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