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Predicting the Bioavailability of Metals and Metal Complexes: Critical Review of the Biotic Ligand Model

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Environmental Context. *The chemical speciation of trace metals greatly influences their biological effects. Nonetheless, no clear consensus currently exists as to when metal complexes are bioavailable, especially for field conditions. Recently, the USA EPA has incorporated the biotic ligand model (BLM) into their regulatory framework and many other countries are now examining the implications of following suit. This review examines the fundamental basis of the BLM in order to provide the reader with an understanding of its potential uses and limitations.*

Abstract. The biotic ligand model is a useful construct both for predicting the effects of metals to aquatic biota and for increasing our mechanistic understanding of their interactions with biological surfaces. Since biological effects due to metals are always initiated by metal bioaccumulation, the fundamental processes underlying bio-uptake are examined in this review. The model assumes that the metal of interest, its complexes, and metal bound to sensitive sites on the biological surface are in chemical equilibrium. Therefore, many of the equilibrium constants required for the model have been compiled and their methods of determination evaluated. The underlying equilibrium assumption of the BLM is also examined critically. In an attempt to identify which conditions are appropriate for its application, several documented examples of failures of the BLM are discussed. Finally, the review is concluded by identifying some important future research directions.

Keywords. bioavailability measurement — contaminant uptake — metals — thermodynamics

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Introduction

Trace metals are found in many different forms in the environment, including free hydrated ions, complexes with poorly defined natural ligands, or adsorbed species on the surfaces of particles and colloids.[1,2] Although, it is now well accepted that the chemical speciation of trace metals will greatly influence their biological effects, no clear consensus currently exists as to when metal complexes are bioavailable, especially for field conditions. For this reason, a majority of regulatory agencies still routinely employ total or 'dissolved' metal

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Kevin Wilkinson is interested in 'molecular' environmental chemistry. Current projects include the examination of the main physicochemical processes influencing trace metal bioaccumulation by microorganisms. The influence of the natural biopolymers on environmental processes, including flocculation and trace metal speciation are also of great interest. Various single molecule detection techniques are currently being used to characterize biopolymers, colloids and biological effects. He is a member of the Editorial Board of Environmental Chemistry.

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concentrations to set maximum acceptable levels for effluents and pollutant point sources. Recently, the USA Environmental Protection Agency has incorporated the BLM into their regulatory framework and other countries are now examining the implications of following suit. The goal of this review is to (re-)examine the fundamental basis and assumptions of the BLM in order to provide the reader with an understanding of its potential uses and limitations. While the review focusses on aquatic systems, for which the majority of data are available, the theoretical principles are also applicable for soils and sediments.

Environmental systems are always dynamic and often far from equilibrium. In spite of this, the BLM assumes that the metal of interest and its complexes are in chemical equilibrium with each other and with sensitive sites on the biological surface. Since biological effects due to metals are always initiated by metal bioaccumulation, the first step in any evaluation of the BLM is to attain a thorough understanding of the fundamental processes underlying bio-uptake. This review will focus on the uptake processes, especially as they are related to the underlying equilibrium assumption of the BLM. Since the BLM is designed to predict metal–organism interactions, many of the equilibrium constants required for the model have been compiled and the methods of their determination examined critically. In an attempt to identify which conditions are appropriate for its application, several documented examples of the failure of the BLM are also given. The review concludes by identifying some necessary future research directions with respect to the biotic ligand model.

Theory Behind the BLM, Including some Critical Assumptions and Implications

A general consensus exists in the literature with respect to the key processes that control trace metal uptake and bioavailability (Fig. 1).[3–8] To interact with the organism, the metal must first be transferred from the external medium to the vicinity of the organism (mass transport). Metal complexes (Eqn 1) are not necessarily inert during transport; dynamic complexes can dissociate/associate in the time that it takes to diffuse to the surface of the organism. To invoke a biological effect, the trace metal must first react with sensitive sites on the biological membrane, $\{R_S\}$, often (but not necessarily) followed by transport (internalization) (Eqn 2)

$$M + L \rightleftharpoons ML \qquad [ML] = K_{ML} \cdot [M] \cdot [L] \quad (1)$$

$$M + R_S \underset{k_d}{\overset{k_f}{\rightleftharpoons}} M\text{-}R_S \xrightarrow{k_{int}} M_{int} + R_S \qquad \{M\text{-}R_S\} = K_S \cdot \{R_S\} \cdot [M] \quad (2)$$

where [] and {} are the bulk and surface concentrations, respectively; M and L are the metal and ligand in solution (charges omitted for simplicity); R_S refers to the free concentration of a sensitive (physiologically active) site on the surface of the organism (e.g. transporter, carrier, ion channel); k_f, k_d, and k_{int} are the formation, dissociation, and internalization rate constants; K_{ML} and K_S are the stability constants defining the equilibrium reactions; and M_{int} represents the metal that has been internalized with a concurrent recycling

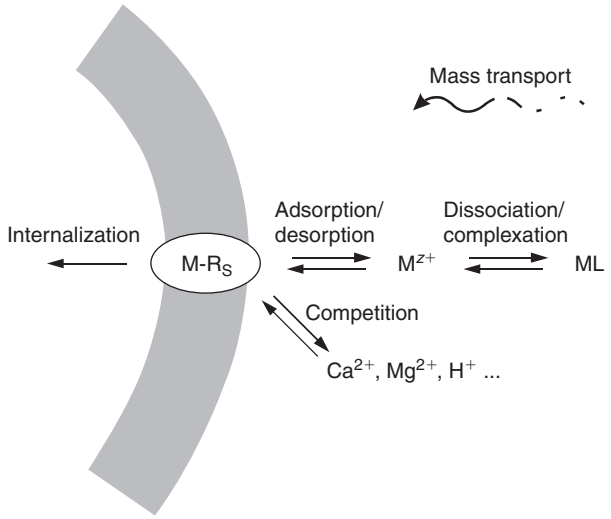

Fig. 1. Conceptual model of the important physicochemical processes leading to the uptake of a trace element by an aquatic microorganism.

of membrane carrier ligands. In the BLM, R_S can be considered as the site of toxic action. The transfer of metal across the biological membrane is generally assumed to be a first-order process such that the internalization flux, J_{int}, can be directly related to any metal species in equilibrium, including metal bound to the sensitive sites on the organisms, $\{M\text{-}R_S\}$ (Eqn 3, basis of the BLM),[9–11] or free metal ion in the solution, [M] (Eqn 4, basis of the Free Ion Activity Model, FIAM).[4,12,13]

$$J_{int} = k_{int} \cdot \{M\text{-}R_S\} \quad (3)$$

$$J_{int} = k_{int} \cdot K_S \cdot \{R_S\} \cdot [M] \quad (4)$$

Although the BLM and FIAM are mathematically equivalent, the major difference between the two models is their ability to take competition into account, since determinations of $\{M\text{-}R_S\}$ in the BLM will take competing ions into account implicitly whereas in the FIAM stability constants for the competing ions must be taken into account explicitly. In their simplest forms, both models predict that the formation of complexes in solution will reduce trace metal uptake and thus reduce metal bioavailability. While a decrease in bioavailability has usually been observed experimentally, several examples exist where either no effect is observed or, indeed, that bioavailability increases in the presence of trace metal complexes (see e.g. refs [14–16]).

An accurate assessment of trace metal bioavailability will depend upon the nature of the rate-limiting flux. The magnitude of the fluxes (diffusive, internalization, dissociation, etc.) will vary according to the chemical nature of the compounds being accumulated, the size and type of the organism, and the physicochemical nature of the surrounding medium, among other factors.[8,17,18] In spite of the fact that the FIAM and BLM models have been used fairly ubiquitously across a wide range of conditions for a wide range of aquatic organisms, they are only strictly applicable for cases where:[13,19]

(a) the plasma membrane is the primary site of interaction of the trace metal with living organisms. It is further

assumed to be chemically homogeneous (i.e. it only contains one type of site).
(b) a single 1 : 1 binding site with a homogenous distribution of charges is involved, and a single compound is transported.
(c) the carrier molecule does not possess regulatory sites and no significant modification (degradation, synthesis) of carrier concentrations occurs.
(d) mass transport towards the biological interface is not rate-limiting (i.e. diffusion layers can be neglected).
(e) surface complexation kinetics at the biological interface are not rate-limiting.
(f) the dissociation constant of $\{M\text{-}R_S\}$ has the same value on both sides of membrane.
(g) chemical gradients at the bulk solution–biological interphase (e.g. concentration or pH gradients, etc.) do not affect transport to or reaction with the transport sites.
(h) the induced biological response is directly proportional to metal internalization fluxes, J_{int}, or concentrations of the surface complex, $\{M\text{-}R_S\}$ (Eqn 3).

Clearly, there are many, perhaps unrealistic, assumptions that must be fulfilled for the BLM to be valid. Unfortunately, few studies have rigorously examined the uptake of trace metals in a context other than the simple thermodynamic models.[20] For the most part, BLM constants have been determined for the uptake or toxicity of a specific trace metal by a single organism, most often fish. Although most data have been gathered for aqueous solutions of metals, a recent thrust of the model has been towards predicting metal toxicity in soils. To date, the most studied metals have been silver and copper with some data available for nickel, cobalt, cadmium, lead, and zinc. More recently, the model has been extended to metal mixtures (e.g. lead, cadmium, zinc, cobalt).[21] Nonetheless, due to the lack of experimental verification, it is currently unclear to what extent (a) chemical reactions, mass transport, and surface charges may influence solute fluxes, (b) BLM model constants vary across species, and (c) laboratory-determined BLM parameters can be employed under field conditions.

Nature and Characteristics of the Biotic Ligand

The current BLM framework considers the biotic ligands to be independent and homogeneously distributed, most often represented by a single binding constant. Similar to simple ligands in solution, the binding of metal to the biotic ligand present can be characterized by an affinity constant, K_S (Eqn 5) and a maximal binding site concentration, $\{R_S\}_{max}$, that is equal to the sum of concentration of free biotic ligand, $\{R_S\}$, metal bound to biotic ligand, $\{M\text{-}R_S\}$, and competitors bound to the biotic ligand, $\sum_i\{C_i - R_S\}$ (Eqn 6).

$$K_S = \frac{\{M\text{-}R_S\}}{\{R_S\}[M]} \tag{5}$$

$$\{R_S\}_{max} = \{R_S\} + \{M\text{-}R_S\} + \sum\nolimits_i \{C_i - R_S\} \tag{6}$$

In this paper, the terms binding constant and stability constant are used synonymously.

Nevertheless, in contrast to the above assumption of a single type of binding site, all biological surfaces contain multiple sites including physiologically sensitive sites (i.e. biotic ligands, transport sites, or specific sites, R_S) and non-specific, non-physiologically active sites that are unlikely to participate in the internalization process, including cell wall polysaccharides, peptidoglycans, fish mucus, etc. (R_{NS}).[22] Indeed, several studies have demonstrated the presence of both multiple adsorptive sites[23–25] and multiple trace metal internalization routes.[8,26–28] Fish gills are known to possess a complex mixture of metal-binding functional groups, each with characteristic metal- and proton-binding constants and capacities.[29–31] For instance, for 3 to 72 h exposures, two different types of copper-binding sites have been identified on the gills of rainbow trout.[32] In that case, at very low (environmentally relevant) metal concentrations, low-capacity, high-affinity uptake sites were filled until saturation while at high concentrations, a mixture of low-affinity, high-capacity binding was observed that did not lead to bio-uptake. In spite of the chemical heterogeneity of the biological surface, bioaccumulation is often predicted reasonably well by simple 1 : 1 carrier models, suggesting that the conditional stability constants for the surface complexes are sufficiently different (i.e. at least one order of magnitude) so as not to interfere with each other.

Furthermore, it should be noted that biological ligands are generally polyfunctional and polyelectrolytic, with an average pK_a value between 4.0 and 6.0.[8] This implies that at circumneutral pH, a high percentage of the binding sites will be deprotonated and anionic. Ionic strength and metal-loading effects will thus play important roles in metal binding. The strength of metal complexation will be decreased with increasing ionic strength due to a screening of the surface potential (Eqn 7) and metals will be more strongly bound at lower metal concentrations (i.e. K_S decreases with an increasing ratio of metal to the concentration of binding sites).[33]

$$K_S = K_{S,int} \exp(-nF\psi_0/RT) \tag{7}$$

where $K_{S,int}$ is the intrinsic stability constant, ψ_0 is the surface potential of the organism surface (e.g. algae or bacteria), R is the gas constant, T is the temperature, and F is the Faraday constant. This implies that constants determined in a given concentration range will not necessarily be the same as constants determined in another concentration range (i.e. each determination will have its own detection window).[34]

Finally, biotic ligands are part of living organisms that are often under tight regulatory control and able to change in response to environmental perturbations.[35] For example, metal-transport sites are continually being recycled, degraded, and synthesized at rates that can be modified by the cell.[36,37] Indeed, once adsorbed to a carrier, a compound can activate numerous intracellular enzymes and entire cascades of intracellular reactions triggering both short (milliseconds to minutes) and long-term (e.g. protein synthesis) modifications of the uptake process.[38] The dynamic nature of biotic ligand[39] is not taken into account in the current model framework.

Existing Methodologies for Determining Constants for the Biotic Ligand Model

Biotic ligand stability constants have been estimated for fish gills (see e.g. refs [10,40]), uptake sites on the cell membrane including carrier proteins (see e.g. refs [41–43]), and hypothetical ligands provoking metal toxicity.[44] Concentrations (numbers) of sites and conditional binding constants for the interaction of metals with a biotic ligand have been incorporated into thermodynamic speciation programs such as *MINEQL*, *CHESS*, or *WHAM*.[45] In such cases, the BLM takes into account the *complexation* of the metal ion with environmental ligands, including small inorganic and organic ligands, humic substances (HS), the *competition* with ions such as Ca^{2+} and H^+ and ionic strength (ion activity) effects. Constants for the interaction of the metal with the biological surface have been estimated by measuring metal internalization fluxes (bio-uptake), metal loading, and metal toxicity. Following a mathematical manipulation, each determination provides a different measurement of the stability constants and each technique has its own advantages and disadvantages. An evaluation of the available experimental methodologies and data treatment employed to derive the stability constants are detailed below.

Metal Internalization Fluxes

The measurement and analysis of metal uptake fluxes is surely the technique that most closely retains the spirit of the theory of the conceptual model presented above. Organisms are placed in contact with metal from the exposure medium and the quantity of accumulated metal is monitored as a function of time. Metal uptake rates (normalized for cell number or biomass) or fluxes (normalized for cell surface area) are determined from the slope of the plot of accumulated metal as a function of contact time. Internalized or cellular metal that has crossed a biological membrane may be distinguished from total metal burdens, often using chemical extraction techniques (see e.g. ref. [46]). Metal internalization fluxes are determined and plotted against concentrations in the bulk solution over several orders of magnitude (Fig. 2).

The Michaelis–Menten equation (Eqn 8) is used to quantify the saturation flux and the half saturation constant, K_M.

$$J_{int} = J_{max}\frac{[M]}{K_M + [M]} \tag{8}$$

If metal internalization across the biological membrane is rate-limiting[4,8] then the conditional affinity constants of metal binding to uptake sites can be determined from the reciprocal values of K_M (Eqns 9 and 10):

$$K_M = \frac{k_{int} + k_d}{k_f} \tag{9}$$

$$\text{For } k_{int} << k_d,\ K_M = \frac{k_d}{k_f} = \frac{1}{K_S} \tag{10}$$

Competitive effects due to pH and other ions including water hardness, can be taken into account by measuring internalization fluxes in the presence of the competitor and by analyzing the data using Eqn 11 (e.g. effect of Cd and Zn on Mn uptake;[47] effect of H^+, Ca^{2+}, and Mg^{2+} on Ag uptake;[48] effect of pH or Ca on Pb uptake;[43,49] effect of Cd or Ca on Zn uptake[46]). From Eqn 11, one can conclude that for the low concentrations of trace metals that are generally found in natural waters, i.e. $[M] < 1/K_S$, J_{int} will be reduced by a factor $1/(1 + \sum_i K_{C_i}[C_i])$ in the presence of a competing ions C_i with binding constants of K_{C_i}. Using this approach, affinity constants for the interaction of trace metals with various organisms have been determined by several authors (Table 1).

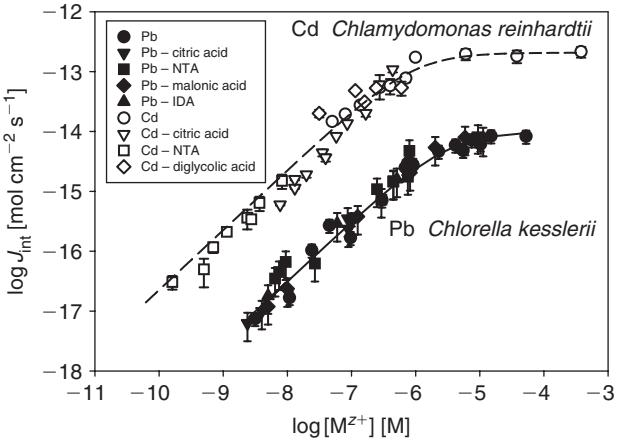

Fig. 2. Logarithmic representation of the internalization fluxes for lead by *Chlorella kesslerii* and for cadmium by *Chlamydomonas reinhardtii* as a function of free metal ion concentrations in the absence or presence of different ligands. The Michaelis–Menten fit is given as a solid line for Pb^{2+} (K_M 3×10^{-6} M, J_{max} 1×10^{-14} mol cm^{-2} s^{-1}) and as a dashed line for Cd^{2+} (K_M 1×10^{-6} M, J_{max} 6.7×10^{-2} mol cm^{-2} s^{-1}). Standard deviations are given when larger than the symbol size. Reproduced with permission from (Pb) *Environ. Sci. Technol.* **2002**, *36*, 969[43] and (Cd) *Environ. Sci. Technol.* **2005**, in press,[121] copyright the American Chemical Society.

The major disadvantage of this approach is that it assumes that the biological effects are directly related to the rate of metal crossing the biological membrane (internalization flux), as opposed to the concentration of cellular metal (flux integrated over time) or some other internalized metal fraction. While this assumption is reasonable when predicting acute toxicity, it is less likely to be valid when evaluating chronic effects.[50,51] Furthermore, the method is technically difficult in that it requires determinations of metal uptake fluxes over several orders of magnitude of dissolved metal, including saturating concentrations that would not normally be environmentally relevant.[52,53] Other disadvantages of the Michaelis–Menten approach include the large number of assumptions, similar to those given above,[46] including the assumption of overall steady-state conditions (cf. refs [54,55]).

$$J_{int} = J_{max}\frac{K_S[M]}{1 + K_S[M] + \sum_i K_{C_i}[C_i]} \tag{11}$$

Metal Loading Experiments

Total metal contents (both intracellular and extracellular, $\{M\text{-}R_{TOT}\}$) of unicellular organisms including algae and bacteria are often determined by bioaccumulation experiments

Table 1. Representative affinity constants (log K_S) for the binding of metal to various organisms based on a Michaelis–Menten interpretation of trace metal uptake fluxes

Due to the nature of the determination, these constants are virtually all conditional stability constants that are valid under the given conditions of pH, ionic strength, [Ca], etc. used in the experiment. In most cases, proton effects were not observed above pH 6.0, so that constants determined at pH > 6.0 may provide a good estimate of the intrinsic affinity constant. Where constants for competitive interactions are given, these cannot be considered to be affinity constants for the competing ion but rather constants for the interaction of the competing ion with metal uptake sites of the ion of interest. Unless mentioned otherwise, values of maximal uptake fluxes (J_{max}) and rates (V_{max}) are given in mol cm^{-2} s^{-1} and mol g^{-1} s^{-1}, respectively. L = low concentration; H = high concentration.

Organism		Metal	log K_S [M^{-1}]	Comments	References
Bacteria	*Rhodospirillum rubrum*[A]	Ni	4.7	pH 7.5; V_{max} 5.2×10^{-9}	[134]
Fungus	*Penicillium ochrochloron*	Cu	3.4	pH 3.0; no saturation at pH 6	[135]
	Saccharomyces cerevisiae[B–D]	Mn	6.5 (L), 4.2 (H)	pH 6.5; V_{max} 5.7×10^{-14}; C = Co, Zn	[136]
Phytoplankton	*Chlorella pyrenoidosa*[B,D]	Cd	5.5	pH 7.0; V_{max} $\sim 1.8 \times 10^{-13}$; C = Mn	[137]
	Chlamydomonas sp.[C]	Mn	7.0	pH 8.2; V_{max} $\sim 1.2 \times 10^{-7}$	[42]
		Zn	7.7		
	Chlamydomonas reinhardtii	Cd	6.0	pH 7.0; J_{max} 6.7×10^{-12}	[121]
		U	6.3	pH 5.0; J_{max} 1.7×10^{-9}	[138]
	Chlorella kesslerii	Pb	5.5	pH 6.0; J_{max} 1×10^{-14}	[43]
	Chlorella salina[E]	Co	4.7 (L), 3.6 (H)	pH 8.0; V_{max} 7.8×10^{-17} (L), 8.3×10^{-16} (H)	[139,140]
		Mn	5.7 (L), 3.1 (H)	V_{max} 1.7×10^{-18} (L), 2.8×10^{-16} (H)	
		Zn	5.4 (L), 3.2 (H)	V_{max} 3.8×10^{-17} (L), 2.6×10^{-15} (H)	
	Chlorella vulgaris	Cd	4.4	pH 6.8	[141]
		Zn	4.1		
	Emiliania huxleiyi[H]	Zn	8.4[F]	pH 8.2; V_{max} 1.3×10^{-9}	[117]
			9.6[G]	V_{max} 3.0×10^{-9}	
	Selenastrum capricornutum	Zn	8.2	^{65}Zn; pH 6.2; V_{max} 3.3×10^{-10}	[142]
	Thalassiosira pseudonana[G,H]	Mn	7.1	pH 8.2; V_{max} 8.6×10^{-9}	[47,143]
		Zn	7.5	V_{max} 1.7×10^{-9}	
		Cd	8.1	V_{max} 8.6×10^{-9}	
	Thalassiosira weissflogii	Fe	8.5	pH 8.2; V_{max} 5.0×10^{-20}	[95]
	Pleurochrysis carterae (coccolothophorid)	Fe	9.2	pH 8.2; V_{max} 8.6×10^{-22}	[95]
Fish	*Caprinus caprio*	Cd	6.5	pH 7.0; V_{max} 1.3×10^{-12}	[144]
		Cd	6.5	^{109}Cd; pH 8.0; V_{max} 1.0×10^{-13}	[112]
		Zn	5.47	^{65}Zn; pH 8.0; V_{max} 6.3×10^{-13}	
Bivalve	*Mytilus edulis*	Zn	7.15	pH 7.0; V_{max} 1.0×10^{-13}	[144]

[A] V_{max} in mol s^{-1} g^{-1} total protein. [B] V_{max} in mol s^{-1} per 5×10^6 cells. [C] V_{max} in mol s^{-1} L^{-1}. [D] C is a competing ion. [E] Double Michaelis–Menten. [F] Acclimation at 1.6×10^{-9} M [Zn^{2+}]. [G] Acclimation at 10^{-11} M [Zn^{2+}]. [H] V_{max} in mol s^{-1} (mol C)$^{-1}$.

or by whole cell titrations in which increasing concentrations of metal are added to cell suspensions (see e.g. refs [23,56]). Data can be interpreted using relatively simple treatments such as a Langmuir adsorption isotherm (one site, Eqn 12), a Scatchard plot (multiple sites, Eqn 14),[2] or by more complex treatments such as the Non-Ideal Competitive Adsorption (NICA)–Donnan approach (see e.g. ref. [24]) that take into account both the polyelectrolytic and polyfunctional nature of the cell surface. In the Langmuir approach, competing ions and pH effects can be taken into account (Eqn 13) in a similar manner to the Michaelis–Menten approach above (Eqn 9).

$$\{\text{M-R}_{\text{TOT}}\} = \{\text{M-R}_{\text{TOT}}\}_{\max} \frac{K_{\text{NS}}[\text{M}]}{1 + K_{\text{NS}}[\text{M}]} \quad (12)$$

$$\{\text{M-R}_{\text{TOT}}\} = \{\text{M-R}_{\text{TOT}}\}_{\max} \frac{K_{\text{NS}}[\text{M}]}{1 + K_{\text{NS}}[\text{M}] + \sum_i K_{\text{C}_i}[\text{C}_i]} \quad (13)$$

$$\frac{\{\text{M-R}_{\text{TOT}}\}}{[\text{M}]} = K_{\text{NS}}\{\text{M-R}_{\text{TOT}}\}_{\max} - K_{\text{NS}}\{\text{M-R}_{\text{TOT}}\} \quad (14)$$

Based upon the above Equations, both graphical and numerical methods can be used to determine the concentration of total binding sites and the equilibrium constant, K_{NS}, corresponding to the formation of a cellular surface complex. In the Langmuir treatment, the reciprocal adsorption density ($1/\{\text{M-R}_{\text{TOT}}\}$) is generally plotted as a function of the reciprocal (equilibrium) concentration of the trace metal in solution to obtain the affinity constant and the total adsorption capacity. It should be noted that a satisfactory fit of Eqn 12 does not imply that the model is mechanistically correct, namely that only one site is available for a reversible adsorption of the metal up to monolayer coverage. Similarly, a (Scatchard) plot of $\{\text{M-R}_{\text{TOT}}\}/[\text{M}]$ versus $\{\text{M-R}_{\text{TOT}}\}$ should yield a straight line with a slope equal to the conditional stability constant and an intercept equal to the maximum binding capacity (see e.g. refs [2,57]). If only a single type of binding site is present, then the Scatchard analysis converts the non-linear surface complexation data to a linear form. Non-linearity indicates that either multiple binding sites exist or that there is cooperativity among the various sites. The interpretation of the Scatchard plots is especially ambiguous in the presence of

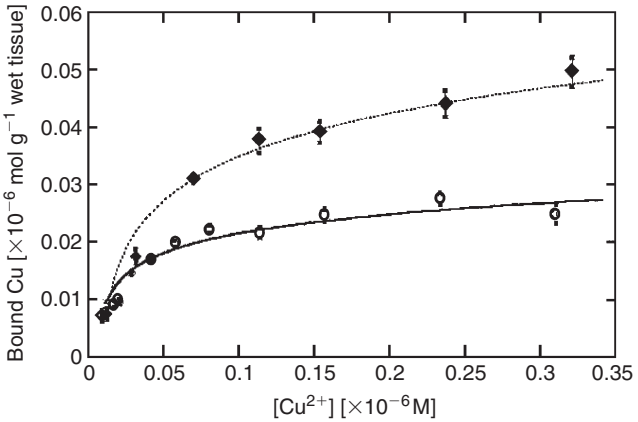

Fig. 3. Langmuir fit of copper bound to the gills of rainbow (○) and brook trout (♦) after a 24 h exposure to different free Cu^{2+} in an experimental medium. Reproduced with permission from *Environ. Toxicol. Chem.* **1999**, *18*, 1180,[57] copyright Alliance Communications Group.

several independent sites. A plot of log $f_{\text{M-R}_{\text{TOT}}}/(1-f_{\text{M-R}_{\text{TOT}}})$ against the log of the free metal concentration (Hill analysis), where f is the fraction of bound sites, can be used to establish the type of interaction. In this case, the Hill coefficient (slope) of less than one indicates a negative cooperativity and/or multiple binding sites, whereas a coefficient greater than one suggests positive cooperativity.[57] More recently, titration data have been fitted by more sophisticated models (e.g. NICA–Donnan) by taking into account an assumed heterogeneity of the binding sites and the known polyelectrolytic properties of the biological surface (e.g. *Rhodococcus erythropolis*[24]).

Fish gills were one of the first experimental systems examined systematically in the context of the BLM.[29,58,59] Indeed, in line with the theoretical model above, gills were presumed to be the first point of contact between waterborne metals and fish.[60] Conditional affinity constants and the number of binding sites (per gram of tissue) have been determined using a Langmuir isotherm (see e.g. ref. [10]) or Scatchard analysis (see e.g. ref. [61]). For example, a Langmuir isotherm has been constructed by correcting the metal content of the gills for background gill concentrations and then plotting adsorption against free metal concentrations, as estimated by computer speciation programs and measured water chemistry parameters (see e.g. ref. [11]; Fig. 3).

It is important to note that the conditional adsorption constants (K_{NS}) and total site concentrations (R_{TOT}) determined in this manner (see Tables 2 and 3) take into account both physiologically sensitive sites and the non-specific sites that are unlikely to participate in the internalization process. Because most available data indicate that the numbers of non-specific sites greatly exceed the numbers of sensitive sites, often by an order of magnitude (see e.g. refs [43,62]), constants that are obtained are unlikely to represent adsorption to the 'biotic ligands'. In fact, constants for the specific sites will often be masked by the constants describing metal interactions with the non-specific sites. Although experimental approaches to distinguish between metal bound to the 'specific' and 'non-specific binding sites' are being developed,[9,43,46,63,64] techniques are thus far operational rather than mechanistic.

Toxicological End Points and Competition Bioassays

The conceptual framework of the BLM assumes that there is a direct relationship between the degree of toxic effect and the proportion of sensitive sites that are filled with metal, $f_{\text{M-R}_{\text{S}}}$, such that the observed severity of the biological response (as estimated by the 48-hour EC_{50}, 98-hour LC_{50}, 21-day EC_{50}, etc.) will increase progressively as the sensitive sites on the organism are filled[19] (assumption (h)). In such a case, a 50% effect could be expected for metal concentrations in solution corresponding to a 50% occupation of sensitive sites ($f^{50}_{\text{M-R}_{\text{S}}}$). Under such an assumption, it is possible to determine the binding constant for the metal of interest and those for the competing ions. In the presence of competing ions that (only) interact with the same site as the metal of interest, it is possible to deduce stability constants from a linear regression of the EC_{50}, expressed as the free metal concentration, as a function of the competing ion concentration $[C_i]$ (Eqn 15, Fig. 4). K_{S} and $f^{50}_{\text{M-R}_{\text{S}}}$ are then evaluated by extrapolating EC_{50} values to the y-intercept $f^{50}_{\text{M-R}_{\text{S}}}/(1-f^{50}_{\text{M-R}_{\text{S}}})K_{\text{S}}$, corresponding to the absence of competing ions (Eqn 16). Stability constants for competing ions, including H^+, may be determined from the ratio of the slope of the regression $f^{50}_{\text{M-R}_{\text{S}}}/(1-f^{50}_{\text{M-R}_{\text{S}}})K_{\text{S}}$ ($\sum_i K_{C_i}[C_i]$) and the intercept (Eqn 15):

$$\text{EC}_{50}(\text{M}_0) = \frac{f^{50}_{\text{M-R}_{\text{S}}}}{(1-f^{50}_{\text{M-R}_{\text{S}}})K_{\text{S}}} \tag{15}$$

$$\text{EC}_{50}(\text{M}) = \frac{f^{50}_{\text{M-R}_{\text{S}}}}{(1-f^{50}_{\text{M-R}_{\text{S}}})K_{\text{S}}}\left(1+\sum_i K_{C_i}[C_i]\right) \tag{16}$$

Using this methodology, the actual measurement of metal concentrations at the site of action (M-R_{S}) is not required for the determination of the binding constants. The method can therefore be applied to organisms where it is difficult or impossible to precisely determine concentrations of metal bound to a specific site. For example, in experiments involving *Daphnia magna*, the biotic ligand was considered to be a hypothetical ligand consisting of all external metal interaction sites that resulted in metal toxicity within a given exposure period.[44,65] Since one of the goals of the BLM is to provide a model that can be used to protect aquatic species against exposure to excessive metal concentrations, constants obtained in this manner will surely be the most relevant from a toxicological perspective. A disadvantage of the method is that it makes the likely unwarranted assumption that biological effect and concentrations of surface bound metal are directly related, namely that 50% effects are observed when 50% of the sites are filled. Another limitation comes from the fact that $f^{50}_{\text{M-R}_{\text{S}}}$ and K_{S} are coupled and cannot be derived unambiguously, that is, constants will be internally consistent among a series of metals but will depend upon the experimental protocol used. Finally, in the absence of competitors, the constant is conditional in that it depends on the concentration of the biotic ligand, namely the value of the constant

Table 2. Representative affinity constants for the binding of metal to biological surfaces
In the column 'Comments', information on the method of determination, the nature of the constants, and data treatment are given (see also the Glossary). Unless mentioned otherwise, values of total bound metal are generally given in moles per gram wet weight (mol g^{-1})

Organism		Metal	$\log K_{NS}$ [M^{-1}]	Comments	References
Bacteria	*Bacillus subtilis*	Cd	2.7, 4.2[A]	WC; IC; *I* 10^{-1} M; SCM; *I* 10^{-2} M also studied	[145]
		Cu	3.6, 4.9[A]		
		Pb	3.4, 5.1[A]		
	Bacillus licheniformis	Cd	3.9, 5.1[A]	WC; IC; *I* 10^{-1} M; SCM; *I* 10^{-2} M also studied	[145]
		Cu	4.7, 5.7[A]		
		Pb	4.4, 5.7[A]		
	Rhodococcus erythropolis	H	8.7, 5.7[A]	WC; IC; NICA interpretation, two binding sites	[24]
		Cd	5.3, 2.3[A]		
		Ca	5.3, 2.3[A]		
		Zn	4.9, 2		
Phytoplankton	*Chlamydomonas reinhardtii*	Cu	8.4–10.0[A]	WC; pH 5.0–6.5; $\{Cu\text{-}R_{TOT}\}_{max}$ 0.9–2.7 $\times 10^{-6}$	[146]
		H	4.9, 9.0[A]	WC; IC; CW-EPR; $\{H\text{-}R_{TOT}\}_{max}$ 9.1 $\times 10^{-4}$	[147]
		Cu	11.3	WC; CC; pH 6.9; LA; $\{Cu\text{-}R_{TOT}\}_{max}$ 6.2 $\times 10^{-6}$	
	Chlamydomonas var.	Zn	7.4	CC; pH 7.0; LA; $\{Zn\text{-}R_{NS}\}_{max}$ 3.5 $\times 10^{-5}$; EDTAW	[133]
	Chlorella kesslerii	Pb	5.5	CC; pH 6.0; LA; $\{Pb\text{-}R_{NS}\}_{max}$[B] 1 $\times 10^{-10}$; EDTAW	[43]
	Cyclotella cryptica	H	3.2, 9.8[A]	WC; IC; CW-EPR; $\{H\text{-}R_{TOT}\}_{max}$ 9.7 $\times 10^{-4}$	[147]
		Cu	11.9	CC; pH 6.9; LA; $\{Cu\text{-}R_{TOT}\}_{max}$ 7.6 $\times 10^{-7}$	
	Oedogonium sp.	Mg	3.4	CC; pH 4.5; LA; $\{Mg\text{-}R_{TOT}\}_{max}$ 1.1 $\times 10^{-4}$;	[23]
		Ca	3.6	$\{Ca\text{-}R_{TOT}\}_{max}$ 1.3 $\times 10^{-4}$;	
		Ba	3.3	$\{Ba\text{-}R_{TOT}\}_{max}$ 1.0 $\times 10^{-4}$;	
		Sr	2.9	$\{Sr\text{-}R_{TOT}\}_{max}$ 1.0 $\times 10^{-4}$	
	Scenedesmus subspicatus	Zn	6.9	CC; pH 7.0; LA; $\{Zn\text{-}R_{NS}\}_{max}$ 2.5 $\times 10^{-5}$; EDTAW	[133]
	Spirogyra sp.	Na	3.5	CC; pH 4.5; LA; $\{Na\text{-}R_{TOT}\}_{max}$ 0.9 $\times 10^{-4}$;	[23]
		K	3.3	$\{K\text{-}R_{TOT}\}_{max}$ 1.97 $\times 10^{-4}$;	
		Li	2.9	$\{Li\text{-}R_{TOT}\}_{max}$ 1.8 $\times 10^{-4}$;	
		Cs	2.9	$\{Cs\text{-}R_{TOT}\}_{max}$ 1.1 $\times 10^{-4}$;	
		Mg	3.7	$\{Mg\text{-}R_{TOT}\}_{max}$ 1.7 $\times 10^{-4}$;	
		Ca	3.7	$\{Ca\text{-}R_{TOT}\}_{max}$ 2.2 $\times 10^{-4}$;	
		Ba	3.1	$\{Ba\text{-}R_{TOT}\}_{max}$ 1.9 $\times 10^{-4}$;	
		Sr	3.4	$\{Sr\text{-}R_{TOT}\}_{max}$ 2.5 $\times 10^{-4}$	
	Vaucheria sp.	Mg	3.7	CC; pH 4.5; LA; $\{Mg\text{-}R_{TOT}\}_{max}$ 3.1 $\times 10^{-4}$;	[23]
		Ca	3.5	$\{Ca\text{-}R_{TOT}\}_{max}$ 3.0 $\times 10^{-4}$;	
		Ba	3.5	$\{Ba\text{-}R_{TOT}\}_{max}$ 1.4 $\times 10^{-4}$;	
		Sr	3.3	$\{Sr\text{-}R_{TOT}\}_{max}$ 1.5 $\times 10^{-4}$	
Fish[C]	*Pimephales promelas*	Cu	7.4	WC; IC; pH 6.2	[148]
		Cd	8.6		
		Ca	5.0		
		H	6.7		

[A] Corresponds to the determination of two different binding sites. [B] $\{Pb\text{-}R_{NS}\}_{max}$ in mol cm^{-2}. [C] Gills.

will change according to the density of organisms used for analysis.

In a similar approach, bioassays (phytoplankton[66] and fish[40,57]) have been performed in the absence and presence of organic ligands forming metal complexes with known stability constants. Ethylene diamine, picolinic, citric, oxalic, malonic, tartaric, and 2,6-pyridine-dicarboxylic acids are most often used.[40,57,66] The assumption in these experiments is that the metal of interest will reequilibrate among the ligands in solution and the biotic ligand (i.e. ion exchange predominates). In the presence of a competing ligand in solution, negligible metal accumulation and no biological effects are expected if a sufficient concentration of ligand is added such that $K_{ML}[L] >> K_S\{R_S\}$. On the other hand, for $K_{ML}[L] << K_S\{R_S\}$, no decrease in bioaccumulation or biological effects should be observed with respect to the control situation in the absence of added ligand. The value of the conditional stability constant with the biotic ligand is estimated by comparison with the stability constant of the organic ligand that decreases accumulation and/or gives survival rates near 100%. The detection window of this technique is defined by the affinity constants and concentrations of ligands required to maintain either low bioaccumulation/low effects or high bioaccumulation/high effects. The major disadvantage of this technique is that the stability constant depends on the concentration of sensitive sites available for binding. As observed above, the constants will change according to the organism densities used in the experiments and affinity constants can be compared among metals for a given experiment but cannot readily be compared to values obtained under a different set of experimental conditions.

General Comments on the BLM Model Parameters

As shown above, several techniques are available that allow for the determination of equilibrium constants describing

Table 3. Representative conditional affinity constants for the binding of metal to fish gills and invertebrates (log K_{NS}) and binding site concentrations

In the column 'Comments', information on the method of determination, important experimental details, treatment of the obtained data are given (see also the Glossary). Unless mentioned otherwise, values of total bound metal are generally given in moles per gram wet weight (mol g^{-1})

Organism		Metal	Log K_{NS} [M^{-1}]	Comments	References
Fish	*Oncorhynchus mykiss*	Ag	10.0	pH 7; GA; $\{Ag\text{-}R_{TOT}\}_{max}$ 1.2–1.5 $\times 10^{-8}$;	[149]
	(rainbow trout)	Ca	3.3	WW	[150]
		Na	4.7		
		H	5.9		
		Mg	3.0		
		Cd	7.6 (HW)	pH 6.5–7; 3 h ^{109}Cd GA; $\{Cd\text{-}R_{TOT}\}_{max}$	[151]
			7.3 (SW)	1.8×10^{-9}; SP	
		Cd	6.9 (LH)	pH 6.5–7; 3 h ^{109}Cd GA; $\{Cd\text{-}R_{TOT}\}_{max}$	[61]
			6.6 (MH)	7.0×10^{-9} (LH); 6.0×10^{-9} (MH);	
			6.3 (HH)	5×10^{-9} (HH); SP	
		Co	5.1	pH 6.5; GA; WW; $\{Co\text{-}R_{TOT}\}_{max}$	[11]
		Ca	4.7	8.8×10^{-8}; LA	
		Na	3.2		
		H	6.2		
		Cu	7.4	pH 6.7; 3 h GA; $\{Cu\text{-}R_{TOT}\}_{max}$ 2.0×10^{-9};	[74]
		Ca	3.4	LA	
		Na	3.0		
		Cu	6.4–7.2	pH 5.9–6.7; 5 d T; CB; $\{Cu\text{-}R_{TOT}\}_{max}$	[57]
			7.5	3.0×10^{-8};	
			7.6	24 h GA; SP; $\{Cu\text{-}R_{TOT}\}_{max}$ 2.9×10^{-8}; NLR	
		Cu	7.9 (SW)	pH 6.2; $\{Cu\text{-}R_{TOT}\}_{max}$ 1.9×10^{-9}; 3 h ^{64}Cu	[32]
			9.2 (HW)	GA; LA	
				pH 7.5; $\{Cu\text{-}R_{TOT}\}_{max}$ 6.0×10^{-11}; 3 h ^{64}Cu GA; LA	
		Pb	6.0	pH 6.4; $\{Pb\text{-}R_{TOT}\}_{max}$ 1.3×10^{-8}; 3 h GA;	[152]
		Ca	4.0	WW	
		Mg	4.0		
		Na	3.5		
		H	4.0		
		Zn	5.1–5.6	^{65}Zn; pH 7.5; $\{Zn\text{-}R_{TOT}\}_{max}$ 8.3×10^{-6}; GA	[120]
	Pimephales promelas	Ag	7.3	pH 6.2; $\{Ag\text{-}R_{TOT}\}_{max}$ 3.5×10^{-8}; 2–4 h; GA	[19]
	(Fathead minnows)	Cd	8.6	pH 6.2; $\{Cd\text{-}R_{TOT}\}_{max}$ 2.0×10^{-9};	[148]
		Ca	5.0	2–4 h GA; WW; LA	
		H	6.7		
		Cu	7.4	pH 6.2; $\{Cu\text{-}R_{TOT}\}_{max}$ 3.0×10^{-8};	[40]
		Ca	3.4	2–4 h GA; WW; LA	
		H	5.4		
		Cu	7.4	24 h GA;	[103]
		Ca	3.6	compared to T; 120 h LC_{50}	
		Na	3.0		
		H	5.4		
		Ni	4.0	pH 7.2–7.5; $\{Ni\text{-}R_{TOT}\}_{max}$ 1.0×10^{-9}; GA	[131]
		Ca	4.0		
		H	6.7		
		Na	3.0		
	Salmo gardneri	Zn	5.4	pH; 4.7–7.0; $\{Zn\text{-}R_{TOT}\}_{max}$ 5.4×10^{-9};	[153]
	(Steelhead trout)			comparison to T; 98 h and 168 h LC	
	Salmo salar	Al	6.5	pH 4.5; GA; $\{Al\text{-}R_S\}_{max}$ 5.6×10^{-7};	[154]
	(Juvenile Atlantic salmon)	HO-Al	14.5	EDTAW	
		H	4.5		
		H_2	9.1		
		Ca	3.7		
		F-Al	13.5		
	Salvelinus fontinalis	Cu	7.3	pH 5.9–6.7; 24 h GA; $\{Cu\text{-}R_{TOT}\}_{max}$	[57]
	(Brook trout)		7.1	6.0×10^{-8}; SP;	
				$\{Cu\text{-}R_{TOT}\}_{max}$ 6.3×10^{-8}; NLR	

(*Continued*)

Table 3. (*Continued*)

Organism		Metal	Log K_{NS} [M^{-1}]	Comments	References
Cladoceran	*Daphnia magna*	Ag	7.3	$\{Ag\text{-}R_{TOT}\}_{max}$ 3.5×10^{-9};	[105]
		Ca	2.3	calibration to LC_{50}	
		Na	2.3		
		H	4.3		
	Cerodaphnia dubia	Cu	7.4	$\{Cu\text{-}R_{TOT}\}_{max}$ 3×10^{-8} calibration to LC_{50}	[103]
	Hyalella azteca	Ni	4.0	pH 7.6–8.0; $\{Ni\text{-}R_{TOT}\}_{max} = 1.0 \times 10^{-9}$;	[155]
		Ca	4.0	calibration to 96 h T; LA_{50}	
		H	6.7		
		Na	3.0		
		Cd	8.6	$\{Cd\text{-}R_{TOT}\}_{max}$ 5.1×10^{-7}; tap water	[156,157]
		Cd	8.8	$\{Cd\text{-}R_{TOT}\}_{max}$ 5.1×10^{-7}; free ion tap water	[156,157]
		Cd	7.5	$\{Cd\text{-}R_{TOT}\}_{max}$ 3.7×10^{-6}; soft water	[156,157]
		Cd	7.8	$\{Cd\text{-}R_{TOT}\}_{max}$ 5.3×10^{-7}; tap water + 25 mg L^{-1} HA	[156,157]
		Cd	6.2	$\{Cd\text{-}R_{TOT}\}_{max}$ 1.6×10^{-6}; tap water + 0.5 μM EDTA	[156,157]
		Cd	8.8	$\{Cd\text{-}R_{TOT}\}_{max}$ 8.2×10^{-7}; free ion tap water + EDTA	[156,157]
		Hg	7.6	$\{Hg\text{-}R_{TOT}\}_{max}$ 1.8×10^{-6}	[157,158]
		Pb	6.9	$\{Pb\text{-}R_{TOT}\}_{max}$ 3.9×10^{-7}; sediment exposure	[157]
		Pb	6.9	$\{Pb\text{-}R_{TOT}\}_{max}$ 3.1×10^{-7}; water only, weekly water change	[157,158]
		Pb	5.8	$\{Pb\text{-}R_{TOT}\}_{max}$ 6.5×10^{-6}; water only, water change every 2 days	[157,159]
		Tl	5.6	$\{Tl\text{-}R_{TOT}\}_{max}$ 1.8×10^{-5}; tap water	[157,160]
		Tl	6.6	$\{Tl\text{-}R_{TOT}\}_{max}$ 9.3×10^{-6}; artificial medium without K	[157,160]
		Cu	6.5	$\{Cu\text{-}R_{TOT}\}_{max}$ 3.6×10^{-6}	[157,161]
		Zn	5.6	$\{Zn\text{-}R_{TOT}\}_{max}$ 3.6×10^{-6}	[157,161]

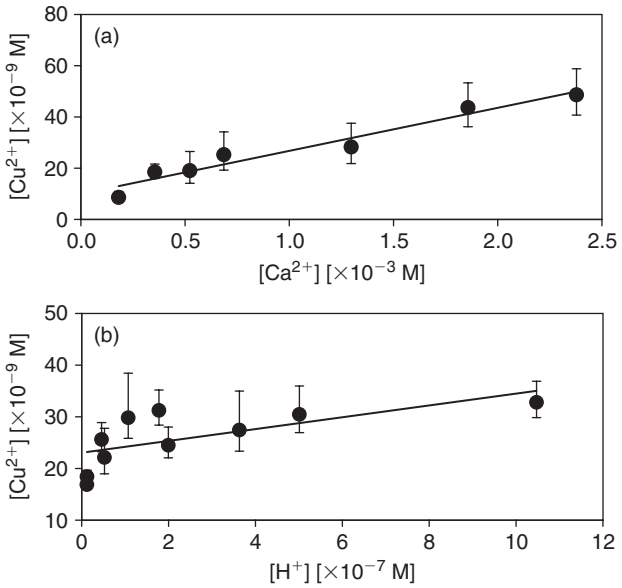

Fig. 4. Values of a 48 h EC_{50} expressed as free copper ion concentration for *Daphnia magna* as a function of (a) Ca^{2+} and (b) H^+ concentrations. Regressions were used to derive the conditional stability constants for competing ions. Reproduced with permission from *Environ. Sci. Technol.* **2002**, *36*, 48,[104] copyright the American Chemical Society.

the interaction of a given trace metal and sites representing the 'biotic ligand'. Nonetheless, in most cases, conditional constants are determined that are dependent upon pH, ionic strength, and the site density of 'biotic ligand'. Unfortunately, the concentration of 'biotic ligand' is rarely quantified, resulting in constants that will have little predictive value, except when comparing the interaction of several metals for a given set of conditions. In any case, as each of the techniques evaluates a specific metal–organism interaction, the constants are necessarily organism-dependent. Furthermore, non-specific effects, such as charge screening are largely ignored by neglecting the Coulombic contribution of the charge of the biological surface on the constants (Eqn 7). Finally, temperature is known to greatly influence bio-uptake and metal toxicity, yet the temperature dependence of the constants is rarely quantified.[67]

As discussed above, the BLM is an equilibrium model that assumes that the only role of ligands in solution is to complex metals; necessarily decreasing the equilibrium concentration of surface-bound metal, with a concurrent reduction in bioaccumulation and toxicity. This simple BLM does not take into account potential direct effects of the ligands (see e.g. ref. [68]), including, in the case of the humic substances, their adsorption to biological surfaces[69] due to their surface active properties.[33,70] Indeed, following the adsorption of humic substances on the biological surface, membrane permeability has been shown to increase[71] with a resulting increase of the metal uptake flux,[63,72] at least partly due to a more negative surface charge and corresponding increased Coulombic attraction. Both the adsorption of HS and the resulting increases in membrane permeability have been observed to be much more important at slightly acidic

Table 4. Representative conditional affinity constants (log K_S) for the binding of metal to fish gills and invertebrates
In the column 'Comments', information on the method of determination are given (see also the Glossary).

Organism	Metal	Log K_S [M^{-1}]	Comments	References
Oncorhynchus mykiss[A] (Rainbow trout)	Ag	7.6	Na^+/K^+-ATPase inhibition; comparison to T; LC_{50}; pH var.	[132]
	Ca	2.3		
	Na	2.9		
	H	5.9		
	Ag	8.0	pH 7.8–8.0; Na^+/K^+-ATPase inhibition; comparison to T; 3 h LC_{50}; 96 h LC_{50}; LA_{50}	[162,163]
Daphnia magna	Cu	8.0	pH 6.8; T; 48 h EC_{50}; $f^{50}_{M\text{-}R_S}$ 0.39	[104]
	CuOH	7.5		
	Ca	3.5		
	Mg	3.6		
	Na	3.2		
	H	5.4		
	Cu	8.0	pH 6.8; T; 21 d EC_{50}; $f^{50}_{M\text{-}R_S}$ 0.393	[50]
	CuOH	8.0		
	$CuCO_3$	7.4		
	Na	2.9		
	H	6.7		
	Zn	5.3	17 different media for model validation; 48 h EC_{50}	[164]
	Ca	3.3		
	Mg	3.1		
	Na	2.4		
Cerodaphnia dubia	Cu	5.7–6.2	pH 8.0; 24 h, CB with different synthetic ligands	[66]

[A] Physiological BLM.

pH values as compared to circumneutral pH values.[69,71] In addition to the direct adsorption of NOM, metal complexation in solution will depend greatly on the nature (or source) of the organic matter.[73] In order to improve the predictive power of the BLM, several authors have incorporated parameters that take into account the metal binding quality of the NOM.[50,74–76] Unfortunately, in spite of advances (e.g. *WHAM* program or NICA–Donnan models), these approaches are difficult to implement due to the absence of a single unifying parameter that represents NOM complexing capacity. A further difficulty is that, as seen for biological organisms, the binding of metals by NOM varies with the metal-to-ligand ratio and the charge of the complex.[34]

In spite of the above considerations, constants for a given metal and indeed ratios between different metals were remarkably similar (Tables 1–4), suggesting that the biotic ligand approach will nonetheless provide a useful construct with which it is possible to better understand the major factors determining the interaction of a given metal *for biological organisms that are at equilibrium with their surroundings*. In this respect, several of the important, documented exceptions to the BLM approach, especially those related to dynamic aspects of the uptake process, are examined in greater detail below.

Some (Dynamic) Limitations of the BLM Approach

As mentioned above, environmental processes, including trace metal bioaccumulation, are rarely at equilibrium. It is thus important to determine whether it is reasonable to apply an equilibrium model to the trace metal uptake process. Several documented exceptions where bio-uptake is not at chemical equilibrium are therefore documented below.

Diffusive Limitation of Metal Uptake

It is interesting to note that, unlike the equilibrium-based BLM, models that examine nutrient or organic pollutant uptake generally take into account the possibility of a rate-limiting mass transport.[77–79] If mass transport rather than biological internalization is rate-limiting, it will set an upper limit to the uptake flux that can be accessed by the organism. In that case, the size and charge of the metal ion, the size and shape of the organism, the position of the organism with respect to other cells (plankton, flocs, biofilms) and the nature of the flow regime will all be important factors that are required to describe uptake.

A mass-transport limitation has been shown to occur for both silver[80] and zinc[27] uptake by phytoplankton, under conditions of relatively high (metal) membrane permeability. Silver uptake was transport limited for *Chlamydomonas reinhardtii* but not for two more slowly (silver) accumulating species (*Pseudokirchneriella subcapitata* and *Chlorella pyrenoidosa*; Fig. 5).[81] For identical physicochemical conditions, labile silver–chlorine complexes were bioavailable to *C. reinhardtii* while they were not bioavailable to the other two species. In another example, zinc uptake by the unicellular green alga *Chlorella kesslerii* was shown to be diffusion limited for algae grown under starvation conditions. In that case, for low concentrations of zinc in the experimental medium, cells were able to induce dissociation of the zinc–NTA complexes, allowing those complexes to become effectively bioavailable. Diffusion limitation has also been postulated to occur for the bio-uptake of other trace metals[26] or compounds.[82–87]

Diffusion limitation on bio-uptake has been verified relatively rarely. However, it could potentially explain some of the

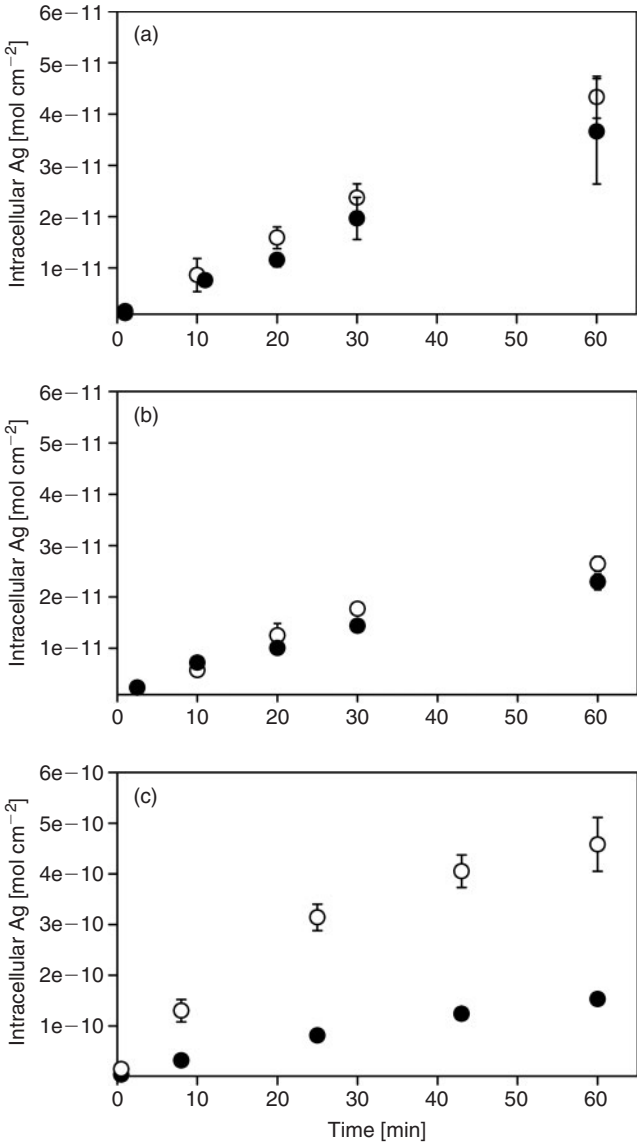

Fig. 5. Internalized silver as a function of time for three algal species: (a) *Pseudokirchneriella subcapitata*, (b) *Chlorella pyrenoidosa*, (c) *Chlamydomonas reinhardtii*. Free silver concentrations were maintained constant at 10^{-8} M. Open symbols represent silver uptake at high concentrations of total silver (1.04×10^{-7} M) and chloride (4×10^{-3} M) while closed symbols represent silver uptake at low concentrations of total silver (10^{-8} M) and chloride (5×10^{-6} M). Reproduced with permission from *Environ. Toxicol. Chem.* **2004**, *23*, 1012,[81] copyright Alliance Communications Group.

observed deviations from the FIAM (or BLM[13]) especially in cases where mass transport is slow (e.g. large compounds or restrained diffusion in soils or sediments) or where bio-uptake is rapid (e.g. nutrients, hydrophobic compounds). Indeed, due to a many-fold reduction of diffusion coefficients in restrained media,[2] diffusive limitation is more likely to occur in complex media such as sediments and soils than for microscopic organisms suspended in the water column. Furthermore, because radial diffusion to small (spherical) microorganisms is much more efficient than diffusion to large, planar surfaces, one would expect more cases of diffusion limitation to occur for macroscopic organisms that

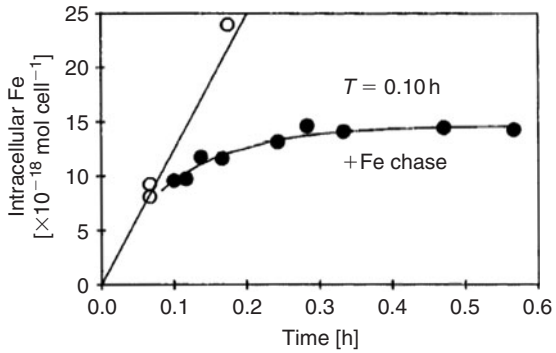

Fig. 6. Uptake of iron by 'iron-limited' *Thalassiosira weissflogii* in the presence of 10^{-6} M of unlabelled iron. Open symbols refer to iron uptake in the presence of a constant concentration of unchelated iron (2×10^{-8} M). Closed circles represent cells that were transferred to a chase medium. The iron uptake rate before transfer was 5×10^{-13} mol cm^{-2} min^{-1}. Reproduced with permission from *Limnol. Oceanogr.* **1990**, *35*, 1002,[95] copyright Society of Limnology and Oceanography.

for microorganisms. Indeed, for clams subject to variable dissolved oxygen content regimes, cadmium uptake was more reflective of the rate of water intake than the bulk solution [Cd^{2+}],[88] strongly suggesting a mass-transport limitation of cadmium uptake. Nonetheless, for larger organisms, a diffusive transport limitation may be overcome by swimming, sedimentation or an increased ventilation rate since the movement of the organism or of the surrounding water can enhance the supply of metal[85,89,90] by decreasing the thickness of the diffusion boundary layer.[86] Since unattached microorganisms move with the bulk fluid,[91] no uptake enhancement is expected to occur due to fluid motion for the uptake of typical (small) solutes by small, freely suspended microorganisms.[85,90,92–94]

Kinetic Limitation of Metal Internalization

To our knowledge, only a single group[6,7,95] has clearly demonstrated biological uptake of metal to be limited by its adsorption to sensitive sites on the surface of the organism. By performing transient uptake and pulse–chase experiments, Hudson and Morel were able to show that the rate of formation of the iron surface complex and the rate of internalization by *Thalassiosira weissflogii* were nearly equal and much larger than the rate of complex dissociation (the implications of these results have been discussed in refs [6,7,26]). The observation that the formation and dissociation rates of the surface complex were not equal was unambiguous evidence that the carriers were not in equilibrium with the solution iron, in contrast with the assumptions inherent in the BLM. Furthermore, pulse–chase experiments (initial exposure to radiolabelled iron followed by chase phase using non-labelled iron) demonstrated clearly that internalization occurred during the chase phase of the experiments, and was therefore not rate-limiting (Fig. 6).

For uptake rates that are limited by the formation kinetics of a metal–carrier complex, the forward rate constants (and metal and carrier concentrations) would be the most indicative of uptake rates. Indeed, for ocean surface waters, nutrient

availabilities appear to be better correlated with their kinetic lability rather than their complexation properties. Hudson and Morel[6] suggested that this might be because the essential micronutrients (with presumably faster internalization rates) are controlled by mass transport and uptake kinetics while non-essential metals could be thermodynamically controlled. For conditions where the metal uptake is kinetically limited, complexation by small inorganic ligands such as chloride, hydroxide, fluoride, or carbonate in solution would not be expected to decrease bio-uptake but rather could increase the rate of adsorption of the cations to surface sites,[96–98] potentially increasing uptake and toxicity.

Other potentially slowly reacting metals include chromium(III), aluminium(III), and nickel(II). Although the surface complexation kinetics of these metals have not been verified in detail, Al-F complexes have been shown to be bioavailable using solid state ^{19}F nuclear magnetic resonance spectroscopy (see e.g. ref. [99]) and bioassays.[16,62] Furthermore, based on toxicological results, several authors (see e.g. refs [100–102]) have postulated the formation of HO-Al-R_S complexes. Nonetheless, while the formation of ternary surface complexes would be consistent with a kinetic limitation, it does not preclude an equilibrium-based explanation in which ternary surface complexes could be taken into account in the BLM. Just such an explanation was proposed when describing the role of chloride and hydroxide on copper and silver acute toxicity to fish and *Daphnia*.[103–105] The non-equilibrium roles of complexation are examined further in the following Section.

Non-Equilibrium Transport of Metal Complexes

The biotic ligand model assumes that the metals form a surface-bound intermediate whose concentration is representative of the biological effects. Neutral complexes and complexes with ions that are essential for the organism are not considered in the model. In fact, hydrophobic metal complexes are thought to be transported directly across biological membranes by means of passive diffusion.[106–109] Although most work has examined the transport of hydrophobic metal complexes formed with 8-hydroxquinoline or dithiocarbamate,[106,109] the uptake and toxicity of mercury complexes are also well correlated with the $HgCl_2$ or CH_3HgCl complexes rather than equilibrium concentrations of the free ion.[108] Nonetheless, physiologically regulated mechanisms of mercury uptake might also be present for some organisms.[110,111] Finally, some metal complexes may be transported by the carriers meant for the ligands (e.g. Fig. 7). For example in the presence of cadmium and zinc complexes of citrate, glycine, and histidine (*Cyprinus carpio*[112]), magnesium, calcium, cobalt, and manganese phosphate complexes (*Escherichia coli*[113]), silver thiosulphate complexes,[114] and cadmium citrate complexes (*S. capricornutum*[115]), metal uptake fluxes were observed to be larger than expected based upon the concentration of free metal ion.

Biological Control of Trace Metal Uptake

While the biological internalization of non-essential metals is often a first-order, facilitated transport as assumed

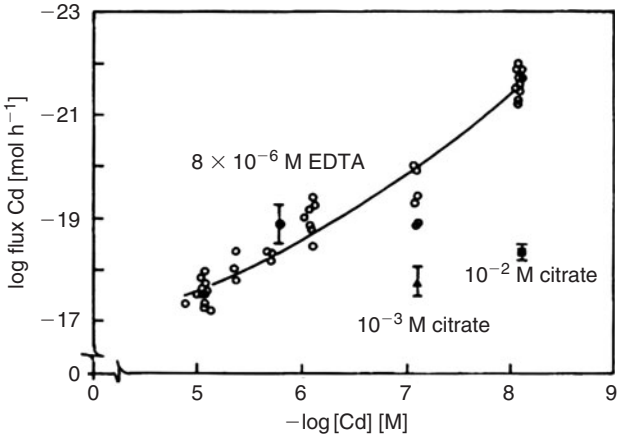

Fig. 7. Flux of cadmium through perfused rainbow trout gills as a function of $[Cd^{2+}]$ in the ventilatory water. Transfer values determined in the presence of 1 mM (▲) or 10 mM (○) citrate were below the regression line determined in the absence of organic ligands, indicating that influx was higher than predicted from the free $[Cd^{2+}]$. Modified from ref. [13]. Reproduced with permission from *Aquat. Toxicol.* **1984**, *5*, 277,[15] copyright Elsevier.

in the BLM (i.e. Eqn 3), active (energy-dependent) transport has been observed for several metals including iron,[36] manganese,[116] and zinc.[117] Deviations from the Scatchard-type plots or non-unity slopes of the non-saturated portion of the logarithmic Michaelis–Menten plots (see e.g. ref. [117]) have been explained by transport feedback mechanisms. For example, manganese has been shown to accumulate through a single high affinity transport system that is under negative feedback regulation by the cell. This enables the cell to maintain internalized manganese concentrations at optimal levels for growth in spite of variations in the external manganese concentration. In that case, the uptake rate was only directly proportional to $[Mn^{2+}]$ (cf. Eqn 4)[26,116] for Mn^{2+} concentrations that resulted in an undersaturation of the cellular carriers (i.e. $<10^{-8}$ M). Zinc uptake by a zinc-starved *Chlorella kesslerii* was also shown to be so tightly regulated that uptake fluxes were nearly independent of $[Zn^{2+}]$ in the bulk medium.[27] The importance of the organism in controlling uptake (and biological effects) has been demonstrated by experiments in which pre-exposure conditions were shown to increase (or decrease) metal uptake fluxes by nearly 1000-fold for metals including manganese,[26] zinc,[27,117] and cadmium.[118] In addition, the affinity of cadmium[61,119] and zinc[120] for the gills of rainbow trout also appeared to be reduced by a chronic sublethal exposure of the metals, although, in that case, the apparent number of binding sites increased. These results suggest that the BLM is best adapted for acute rather than chronic toxicity predictions. Indeed, the acute BLM could not serve as a basis to predict chronic copper toxicity to *Daphnia magna*, because in contrast to the acute BLM no significant calcium, magnesium, or combined competition effects were observed.[76]

Other unexpected responses have been observed for the normal environmental situation of multiple metals. For example, the observation of a decreased uptake due to competition for binding sites (see e.g. refs [47,121–124]) is the

expected direct *chemical* response. In contrast, iron uptake by *T. weissflogii* actually increased in the presence of zinc and aluminium,[125] presumably due to the stimulation of iron-binding siderophores. In another study, copper uptake was enhanced when nickel was added to solutions resulting in a concurrent reduction of the respiratory rate and chlorophyll *a* contents of *Scenedesmus quadricauda*.[126] Finally, copper and manganese have been shown to increase cadmium bio-uptake fluxes to the Gram-negative *Rhodospirillum rubrum*,[127] and copper has been shown to increase lead and zinc uptake fluxes to *C. kesslerii*.[27]

Conclusions and Future Research Directions

As demonstrated above, the BLM has been shown to be a useful construct both for predicting the effects of metals to aquatic biota and for increasing our mechanistic understanding of their interaction with biological surfaces (see also ref. [128]). Nonetheless, much remains to be done. Future research is clearly required to (a) better understand and quantify the relationship between bioaccumulation and toxicity; (b) better understand under what circumstances dynamic models are better suited to predict bio-uptake (i.e. mass-transport limitation, adsorption limitation, etc.); (c) to determine concentrations (and activities) of the biotic ligand; (d) to relate carrier-bound metal to uptake fluxes and to total accumulated metal; (e) to modify the BLM to take into account the more environmentally relevant case of low-level, chronic metal exposures;[39] and (f) to identify and quantify other important sources of metal uptake (such as dietary).[39,129]

With respect to this final point, it is often assumed that the contribution of waterborne metals to toxic effects predominates over metals obtained through dietary exposure.[130] For example, recent studies have revealed that although the exposure to dietary copper resulted in an increased total body burden for *Daphnia*, it did not contribute to toxicity as reflected by the 21-day effects concentrations when expressed as waterborne copper.[50] In this light, the biological compartmentalization of metals and the determination of metal fluxes is an important topic for future research. Internalization fluxes have often been monitored because they are a direct and dynamic means of quantifying potential metal effects that are closely related to several chemical speciation techniques. In contrast, it is precisely the complex biological response that is important to quantify when evaluating metal bioavailability in aquatic ecosystems. In the BLM, it is assumed that estimates of carrier bound metal, $\{M\text{-}R_S\}$, accurately reflect short-term uptake fluxes and that these are related to (acute) biological effects. Further research is clearly required in order to precisely interrelate trace metal speciation and concentrations in solution, uptake fluxes, various body burdens, and toxicity.[30] For example, recent work has related critical gill copper concentrations to LC_{50} values.[103] For copper, cadmium, and nickel, a strong relationship has been observed between metal accumulation on the gills of rainbow trout or fathead minnows and that predicted using acute toxicity data.[31,40,57,131] Furthermore, physiologically based log K values ($\log K_{\text{Ag-gill ATPase}} = 7.6$) have been shown to be useful for predicting the silver binding to specific toxic sites of the gills of rainbow trout.[132]

Future research will most certainly need to move beyond total body burdens and total bioaccumulation. Total body burdens have been shown to be a complex function of exposure time often composed of a rapid, initial phase dominated by adsorption that is followed by a slow (often linear) accumulation corresponding to the internalization (see e.g. ref. [133]). For fish, the majority of trace metal may be bound to mucopolysaccharides adhering to the body or gills[62] in a fraction that is clearly not bioavailable to the fish. One would expect that future improvements of the BLM will come when a better mechanistic, molecular understanding of the chemical, physical, and biological processes underlying trace metal uptake is acquired.

Glossary

C	Competitor ion
CB	Competition bioassay
CC	Conditional constant
CW EPR	Continuous wave electron paramagnetic resonance
EC_{50}	Median effective concentration at which 50% of a tested population shows a given effect
EDTAW	EDTA wash
F	Faraday constant
f	Metal fraction bound to a biotic ligand. The BLM constant corresponds to the reciprocal of the concentration that provokes a 50% effect (f^{50})
GA	Gill accumulation
HA	Humic acid
HH	High hardness
HS	Humic substances
HW	Hard water
I	Ionic strength
IC	Intrinsic constant
J	Uptake flux
$J_{\max}$	Maximum internalization flux
K_C	Stability constant of competing ions
K_{ML}	Stability constant of metal with ligand
$K_{S(NS)}$	Stability constant for the complexation of a given metal to the sensitive site (non-sensitive sites)
$k_{f,(d,int)}$	Formation (dissociation, internalization) rate constants
L	Ligand in solution
LA	Langmuir adsorption isotherm
LA_{50} (LC_{50})	Median lethal accumulation (lethal concentration) at which 50% of a tested population shows a lethal effect
LR	Linear regression
LH	Low hardness
M	Metal in solution
M_{int}	Metal that has been internalized, with a concurrent recycling of membrane carrier ligands

MH	Medium hardness
NICA	Non-ideal competitive adsorption
NLR	Non-linear regression
NOM	Natural organic matter
pK_a	Negative logarithm of the proton dissociation constant
R	Gas constant
R_S	Sensitive sites on the organism surface, i.e. biotic ligand
R_{NS}	Non-sensitive sites on the organism surface that are assumed not to be involved in a physiological response of the organism; binding sites on the organism cell wall, mucus, etc. would be considered a 'non-sensitive' site
R_{TOT}	Sum of R_S and R_{NS}
SCM	Surface complexation model
SP	Scatchard plot
SW	Soft water
T	Temperature
T	Toxicity data
V_{max}	Maximum internalization rate
WC	Whole cell titrations
WW	Deionized water rinse
ψ_0	Surface potential

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