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Review

Metal bioavailability to phytoplankton—applicability of the biotic ligand model☆

Peter G.C. Campbell[a,*], Olivier Errécalde[b], Claude Fortin[c], Véronique P. Hiriart-Baer[d], Bernard Vigneault[e]

[a]*Université du Québec, INRS-Eau, 2800 rue Einstein, C.P. 7500, Ste-Foy, Que., Canada G1V 4C7*
[b]*Les Laboratoires Aeterna Inc., 456 rue Marconi, Ste-Foy, Que., Canada G1N 4A8*
[c]*Laboratoire de radioécologie expérimentale, IPSN, CE Cadarache, Bât. 186, 13108 St-Paul-Lez-Durance, France*
[d]*Department of Biology, University of Waterloo, Waterloo, Ont., Canada N2L 3G1*
[e]*Noranda Technology Centre, 240 boul. Hymus, Pointe Claire, Que., Canada H9R 1G5*

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Abstract

To elicit a biological response from a target organism and/or to accumulate within this organism, a metal must first interact with a cell membrane. For hydrophilic metal species, this interaction with the cell surface can be represented in terms of the formation of M–X-cell surface complexes, e.g. $M^{z+} + {}^{-}$X-cell $\leftrightarrow$ M–X-cell, where $^{-}$X-cell is a cellular ligand present at the cell surface. According to the free-ion model, or its derivative the biotic ligand model (BLM), the biological response elicited by the metal will be proportional to {M–X-cell}. In this paper, using freshwater algae as our test species, we examine some of the key assumptions that underlie the BLM, namely that metal internalization is slow relative to the other steps involved in metal uptake (i.e. the M–X-cell complex is in equilibrium with metal species in solution), that internalization occurs via cation transport, and that internalization must occur for toxicity to appear. Recent experiments with freshwater algae are described, demonstrating anomalously high metal accumulation and/or toxicity in the presence of a common low molecular weight metabolite (alanine), or in the presence of an assimilable inorganic anion (thiosulfate). The possible implications of these findings for the application of the BLM to higher organisms are discussed.

Keywords: Algae; Biotic ligand model (BLM); Ag; Cd; Uptake; Toxicity; Membrane transport

1. Introduction

The biotic ligand model (BLM) is designed to predict how (dissolved) metals interact with, and eventually affect, aquatic organisms. Many of the early insights in this area came from studies with marine and freshwater algae (see Campbell, 1995 for a review of these early papers); the purpose of the present paper is to update this earlier review and evaluate how well the BLM approach can explain metal uptake and toxicity in these eucaryotic organisms, and to consider the lessons that algae may teach us with respect to 'higher' organisms. Most of the examples used for this update have been taken from experiments in our own laboratory, both published and unpublished; some results have been drawn from the literature, but

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*Corresponding author. Tel.: +1-418-654-2538; fax: +1-418-654-2600.
E-mail address: campbell@uquebec.ca (P.G.C. Campbell).

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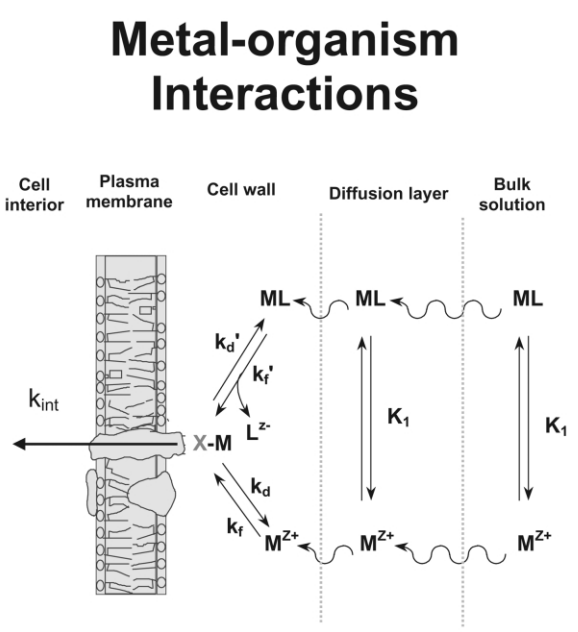

Fig. 1. Conceptual model of metal–alga interactions. M^{z+}, free-metal ion; ML, metal complex in solution; K_1, equilibrium constant for the formation of ML; M–X-membrane, surface metal complex; k_f, k_f', rate constants for formation of the surface complex; k_d, k_d', rate constants for dissociation of the surface complex; k_{int}, rate constant for 'internalization' or transport of the metal across the biological membrane. Charges on complex not shown for simplicity. (Modified from Campbell, 1995.)

there has been no attempt to perform a comprehensive review of all metal–alga papers published in the post-1995 period.

To accumulate within an algal cell and/or to provoke a biological effect, a metal must first interact with a biological membrane. In the bulk solution (Fig. 1, right-hand side), the metal may be present as the free-metal ion, or as one or more dissolved metal–ligand complexes. On approaching the surface of the algal cell, these metal forms will normally first encounter the cell wall. The macromolecules making up this highly porous external layer contain a variety of simple functional groups, dominated by O-containing donor groups ($-$COH; $-$COOH; $-P(O)(OH)_2$). At circumneutral pH values many of these functional groups will be ionized, affording a hydrophilic matrix of negatively charged sites through which the metal and its complexes must migrate, eventually reaching the plasma membrane (Fig. 1, left-hand side). The important features of this membrane barrier are its overall hydrophobic, phospholipidic character, the presence of proteins—some of which may traverse the lipid bilayer—and the existence of transport proteins and/or ion channels that facilitate the movement of ions across the membrane (Simkiss and Taylor, 1995).

The incoming metal will thus encounter a wide range of potential binding sites, which can usefully be divided into two classes: *physiologically inert* sites, where the metal may bind without obviously perturbing normal cell function, and *physiologically active* sites, where the metal affects cell metabolism. In the latter case, metal binding may affect cell metabolism directly, e.g. if the binding site corresponds to a membrane-bound enzyme, or indirectly, if the bound metal is subsequently transported across the plasma membrane into the cell. Once within the cell, the metal may interact with a variety of intracellular sites, resulting in positive or negative consequences.

The interaction of a metal with an algal cell will thus normally involve the following steps: (i) diffusion of the metal from the bulk solution to the biological surface; (ii) sorption/surface complexation of the metal at passive binding sites within the protective layer, or at sites on the outer surface of the plasma membrane; (iii) uptake or 'internalization' of the metal (transport across the plasma membrane). The biological 'end points' that are normally considered as indicators of 'bioavailability' for phytoplankton include metal bioaccumulation (sorption, uptake), as well as metal effects on such processes as photosynthesis, respiration, motility and growth; the goal of the BLM is to predict this bioavailability as a function of the metal's speciation in the bulk solution.

Within this construct, one normally makes a number of simplifying assumptions (Campbell, 1995; Van Leeuwen, 1999; Di Toro et al., 2001):

a. metal transport in solution, towards the membrane, and the subsequent surface complexation reaction occur *rapidly*, such that an equilibrium is established between metal species in the bulk solution and those at the biological surface ('rapid' = faster than metal uptake, faster than the expression of the biological response);
b. the plasma membrane is the primary site for metal interactions with living organisms (i.e. the 'physiologically active' sites referred to earlier are embedded in the plasma membrane), and this interaction occurs via a ligand exchange reaction, yielding M–X-cell, (Fig. 1);

c. the biological response, whether it be metal uptake, nutrition or toxicity, is dependent on the concentration of the M–X-cell surface complex, {M–X-cell}; in those cases where $^{-}$X-cell corresponds to a membrane transport site, metal internalization involves cation transport;
d. variations of {M–X-cell} as a function of $[M^{z+}]$ in solution follow a Langmuir-type adsorption isotherm; provided the concentration of free sites, $\{^{-}X\text{-cell}\}$, remains relatively constant in the range of metal concentrations of interest, variations in {M–X-cell} will follow those of $[M^{z+}]$ in solution (Appendix A, Eqs. (4) and (8));
e. during exposure to the metal of interest, the nature of the biological surface remains constant (i.e. the metal does not induce any changes in the nature of the plasma membrane or its ion transporters).

If these assumptions are valid, then at constant pH and constant hardness, the biological response of the alga should vary as a function of the free-metal ion activity in the exposure solution (Appendix A). Indeed, in the first formal presentation of this conceptual model of metal–organism interactions, Morel (1983) suggested that 'The most important result to emerge is the universal importance of the free-metal ion activities in determining the uptake, nutrition and toxicity of all cationic trace metals.' Recent results (some of which are discussed below) suggest the need to attenuate this statement, but nevertheless the importance of the free-metal ion activity as a predictor of metal bioavailability remains indisputable.[1]

In this paper, using freshwater algae as our test species, we re-examine the first three assumptions, namely that metal internalization is slow relative to the other steps involved in metal uptake (Fig. 1), that internalization occurs via cation transport, and that internalization must occur for toxicity to appear.

[1] In hindsight, the use of the expression 'Free-Ion Activity Model', or 'Free-Ion Model', to describe this approach to metal–organism interactions was perhaps regrettable, since it tended to focus attention on the free-metal ion alone, to the exclusion of other factors such as pH and water hardness ($[Ca^{2+}]$, $[Mg^{2+}]$) that are also known to affect metal bioavailability. One of the advantages of the BLM is that it subtly shifts the emphasis from the exposure solution to the biological receptor, i.e. from the free-metal ion activity in solution to the biotic ligand to which the metal binds (i.e. {M–X-cell} or {M–X-membrane} in the present context).

2. Materials and methods

2.1. General

Our metal uptake and toxicity experiments were carried out with unicellular algae grown axenically in the laboratory in defined culture media. Metals studied have included one essential element (Zn) and two non-essential ones (Ag, Cd); only the latter two metals are discussed here. Two algal species have been used: *Chlamydomonas reinhardtii* (University of Toronto Culture Collection, UTCC11) and *Pseudokirchneriella subcapitata* (Korshikov) Hindak (UTCC37, formerly known as *Selenastrum capricornutum* Printz). The normal experimental procedure involved the following steps: inoculating the algae into fresh growth medium and waiting until the culture had attained the mid-exponential growth phase; harvesting the algal cells by gentle filtration onto polycarbonate membrane filters; re-suspending the cells in fresh, defined medium containing the test metal and various test ligands (e.g. chloride, thiosulfate, citrate, or alanine). We selected these ligands on the basis of two criteria: (i) their assimilability, i.e. the presence in algal cell membranes of anion transporters capable of taking up each ligand; and (ii) their ability to form *hydrophilic* complexes with the metals of interest. Metal speciation in the exposure solutions was calculated with the chemical speciation model MINEQL+ (Schecher and McAvoy, 1994) with an updated thermodynamic database (available on our web site: http://www.inrs-eau.uquebec.ca/activites/groupes/biogeo/personal.htm) prepared from a reliable source of thermodynamic data (Martell et al., 1998). These chemical speciation calculations were based on the initial composition of the algal exposure media.

To determine the influence of the various test ligands on metal bioavailability, we monitored several biological end-points concurrently: metal uptake rates, ligand uptake rates, and algal growth rates. Metal uptake was followed with the use of the appropriate radioisotope (^{110m}Ag; ^{109}Cd); a similar approach was used to quantify ligand uptake rates. Unless otherwise noted, the term 'metal uptake' refers to intracellular metal; sorbed Cd was removed from the algal external surface at the end of the incubation period by extraction with excess ethylenediamine tetra-acetate (EDTA), whereas sorbed Ag was displaced by exchange

with non-radioactive metal. Detailed descriptions of the standard experimental procedures can be found in several recent papers from our laboratory (Errécalde and Campbell, 2000; Fortin and Campbell, 2000, 2001).

The effects of Cd on algal growth were determined in batch cultures. Exponentially growing *P. subcapitata* were gently harvested from the stock culture onto polycarbonate membrane filters as previously described (Errécalde et al., 1998). The cells were rinsed with sterile growth medium and re-suspended in the assay media containing added metal with or without a metal-complexing ligand. The inoculated cultures were maintained under the same light regime and with the same shaking as the original stock culture. Subsamples were removed every 12 h to follow algal growth over time for 4 days. Two growth parameters were used to monitor metal toxicity: exponential growth rate (μ) during the period of maximum growth and cell yield after 72 h (Nyholm, 1985). Measurements of metal concentrations at the beginning and end of the 4-day incubation procedure confirmed that metal losses from the metal-buffered exposure solutions were negligible.

Unfortunately this standard batch procedure could not be used for studying the effects of Ag on algal growth. In batch experiments run without a metal buffer, almost all the Ag disappeared from the exposure solution within the first 48 h (due to absorption by the algae). The normal remedy in such cases would be to buffer the solution with a metal-complexing ligand, but we were unable to find a suitable non-assimilable buffer for use with silver. To circumvent the problem, we opted to use continuous cultures (turbidostats). Exponentially growing *C. reinhardtii* were harvested from a batch stock culture and transferred to three replicate turbidostats for the toxicity experiments. Initially, the growth rate was determined for one day in control (Ag-free) media and this rate was used as the reference growth rate. Subsequently, at 18-h intervals, Ag was added to the culture vessel and the inflow media to obtain nominal Ag additions of 10, 20, 30, 50 and 80 nM (expressed as total silver). In a second suite of experiments, the toxicity of Ag in the presence of thiosulfate was monitored by successive step-wise additions of Ag (20, 30, 57, 80 and 104 nM) and thiosulfate (11.4, 25, 57, 85 and 114 nM), these additions having been chosen so as to maintain a free Ag^+ concentration of 10 nM. Growth rates were calculated on the basis of the dilution rate of the turbidostats.

2.2. Experimental design

Two complementary experimental approaches can be used to determine how metal availability is affected by the presence of assimilable ligands (Twiss et al., 2001):

Design I: A media series is prepared in which the concentration of the ligand is kept constant and the metal concentration is increased (i.e. a 'titration' of the complexing capacity of the ligand). Alternatively, one can hold the total metal concentration constant and vary the ligand concentration. In both such cases, the free-metal ion concentration can be calculated and the organism response plotted as a function of $[M^{z+}]$. This response curve can then be compared to that obtained by simply varying $[M^{z+}]$ in the absence of the test ligand to determine whether metal uptake/toxicity is 'enhanced' or 'suppressed' in the presence of the assimilable ligand.

Design II: A media series is prepared in which the concentrations of the test ligand and the metal are increased together, in proportions calculated to give a *constant* free M^{z+} concentration. In such an experiment, the BLM predicts that the biological response should be constant, i.e. that the response should be *in*sensitive to the increase in total metal and total ligand concentrations.

In both cases, it is critical that pH and water chemistry remain constant as the other experimental variables are changed. Such assumptions are more likely to be met in short-term experiments, which minimize the influence of the test species (algal cells) on the exposure medium.

3. Results and discussion

In the following section, we present experimental data to test BLM hypotheses (a), (b) and (c). For each hypothesis we present a brief background statement, describe the experiments, summarize the outcome and discuss the implications of the results. Particular attention has been accorded to these apparent 'exceptions' to the BLM, since such cases can be taken to define the limits of the model's possible application to the natural environment.

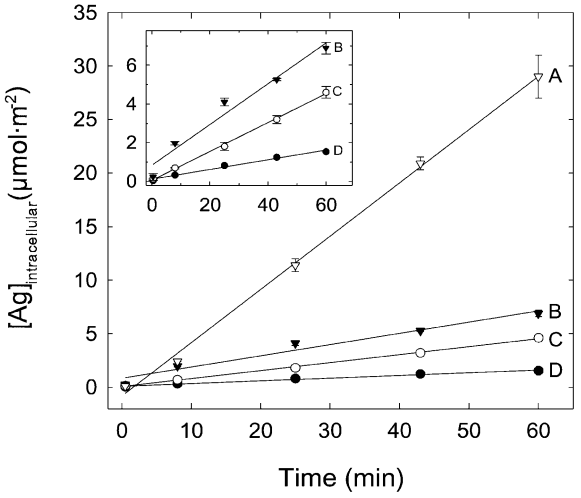

Fig. 2. Time course of silver uptake at constant [Ag^+] (10 nM). (A) Uptake at low [Cl] in the presence of thiosulfate but absence of sulfate. (B) Uptake at low [Cl] in the presence of both thiosulfate and sulfate. (C) Uptake at high [Cl] in the absence of thiosulfate. (D) Uptake at low [Cl] in the absence of thiosulfate. Error bars represent standard deviations from the average of three measurements. Inset shows uptake curves B, C and D on a smaller scale (modified from Fortin and Campbell, 2000, 2001). Note that plots A→D correspond to media A→D in Table 1.

3.1. Hypothesis (a): rate-limiting metal internalization

3.1.1. Background

Until quite recently (Hudson, 1998; Van Leeuwen, 1999), there has been surprisingly little discussion in the literature concerning the relative rates of the three steps illustrated in Fig. 1 (i.e. metal transport from the bulk solution to the biological surface, metal complexation at the plasma membrane, metal internalization). Recent results from our laboratory (Fortin and Campbell, 2000) provide an example of the role played by the first of these factors, diffusion from the bulk solution to the algal surface.

3.1.2. Influence of chloride on silver uptake by C. reinhardtii

Short-term silver uptake (e.g. 10–20 min) by the unicellular green alga *C. reinhardtii* was enhanced in the presence of chloride—e.g. for a fixed free Ag^+ concentration (10 nM), silver uptake increased markedly when the external chloride concentration was increased from 5 µM to 4 mM (Fig. 2, insert, compare curves C and D); over the first 25 min, silver uptake was 3.9× faster in the high-chloride medium than in the low-chloride medium, even though the free [Ag^+] was the same in both media. This experiment corresponds to Design II and the BLM would have predicted a constant silver uptake rate. We considered the possibility that the enhanced silver uptake in the presence of chloride was due to the passive diffusion of the neutral $AgCl^0$ complex across the algal cell membrane, or to the facilitated uptake of the anionic $AgCl_2^-$ complex, but neither explanation withstood close scrutiny (Fortin and Campbell, 2000).

In a second experiment corresponding to Design I, we maintained the total silver concentration constant at 10 nM but varied the chloride concentration from 5 µM to 50 mM to explore the whole range of silver chloro-complexes. Despite the major changes in silver speciation that occur over this concentration range, intracellular silver concentrations were relatively unaffected (Fig. 3). No trends were observed between intracellular silver accumulation and variations in the concentrations of the uncharged $AgCl^0$ complex or the free Ag^+ ion.

The enhanced uptake observed in the presence of chloride proved to be related to the very high silver uptake rates demonstrated by the test alga (e.g. ⩾1000× those observed for Cd^{2+} and Mn^{2+}), which led to diffusion limitation in the

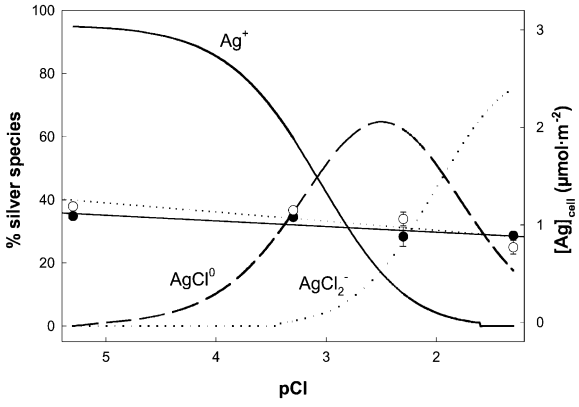

Fig. 3. Calculated distribution of silver species as a function of pCl using MINEQL+ (curves for Ag^+, $AgCl^0$ and $AgCl_2^-$ correspond to the left-hand axis). Short-term (15 min) intracellular silver uptake as a function of pCl for 10 nM total silver (right-hand axis). The two series of points (●, ○) correspond to two separate experiments. Straight dashed lines were drawn as linear regressions of the data (modified from Fortin and Campbell, 2000).

Table 1
Exposure conditions for the time-course silver uptake experiments (Fig. 2)

Medium	$[Ag]_T$ (nM)	$[Ag^+]$ (nM)	$[Cl^-]$ (mM)	$[NO_3^-]$ (mM)	$[SO_4^{2-}]$ (μM)	$[S_2O_3^{2-}]$ (μM)
A	104	10	0.005	5.23	0.0	0.114
B	104	10	0.005	5.07	81	0.114
C	104	10	4.0	1.07	81	0.0
D	10	10	0.005	5.07	81	0.0

boundary layer surrounding the algal cell (Fortin and Campbell, 2000). In such a situation, metal accumulation is proportional to the total metal concentration (i.e. to the concentration gradient between the bulk solution and the algal surface); in the high-chloride medium C, the total silver concentration was higher than in the low-chloride medium D (104 vs. 10 nM—see Table 1). At higher silver concentrations (e.g. $\geqslant 10^{-7}$ M), diffusion in the phycosphere is no longer rate-limiting, the chloride stimulation dissipates, and silver uptake becomes sensitive to the free-ion concentration. However, such a high concentration of silver is not likely to be encountered in the environment, even in wastewater effluents.

3.1.3. Discussion

In their thought-provoking analysis of the possible links among biological, thermodynamic and electrochemical 'availabilities', Whitfield and Turner (1979) estimated the diffusion layer thickness surrounding an algal cell and considered the possibility that transport of the metal across the diffusion layer could be the rate-determining process in metal uptake. These authors explicitly mentioned the dearth of data regarding metal internalization rates by unicellular micro-organisms (values needed to compare with the rate of diffusive supply of the metal from the external bulk solution), but their observation went largely unheeded and since that time it has generally been uncritically assumed that metal internalization is the rate-limiting step.

In a recent review of the marine phytoplankton literature, Hudson (1998) noted that diffusion constrains the uptake of the essential metals Fe and Zn in some species when their transport systems are up-regulated in response to metal depletion. Pinheiro and Van Leeuwen (2001) expanded on the original analysis by Whitfield and Turner, and developed a theoretical construct to predict under what circumstances diffusion in the boundary layer surrounding a unicellular organism and/or surface complexation kinetics might become rate-limiting. Very recently, using the Pinheiro and Van Leeuwen model as their starting point, Slaveykova and Wilkinson (2002) studied the uptake of Pb by the green alga *Chlorella vulgaris* in the absence or presence of nitrilotriacetic, iminodiacetic, malonic or citric acids; their results confirmed that lead uptake was governed by the free Pb^{2+} concentration, and that metal internalization was the rate-limiting step for Pb concentrations greater than ~20 pM.

In the present case, the combination of very high intrinsic silver uptake rates (favored by the presence of a Cu(I) transporter at the cell membrane surface—Weger, 1999) and the low total silver concentration in the ambient solution (yielding a relatively low concentration gradient between the bulk solution and the algal surface, and thus a relatively low rate of supply of the metal to the algal surface) led to a situation where the slow step was diffusion to the algal cell surface. Under such conditions, metal uptake proved relatively *in*sensitive to variations in the relative proportions of Ag^+, $AgCl^0$, and $AgCl_2^-$ (Fig. 3). Similar failures of the BLM to predict metal uptake could be anticipated under the following conditions: high intrinsic rates of metal uptake, k_{int}; low ambient metal concentration; presence of bulky ML species with significantly reduced diffusion coefficients, D_{ML} (see Van Leeuwen, 1999; Pinheiro and Van Leeuwen, 2001 for more details).

3.2. Hypothesis (b): metals enter algal cells by cation transport systems

3.2.1. Background

If the target of metal toxicity is not physically present at the algal surface (Section 3.3), then it follows that the expression of metal toxicity must involve the transport of the metal across the plasma membrane into the algal cell. Implicit in the BLM formulation is the idea that metals interact with cells as cations, and enter cells via *cation* transporters (e.g. cation channels or cation transport proteins—see Simkiss and Taylor, 1995 for a discussion of the membrane transport of cations). The cell membrane is however also the site of *anion* transporters (Stein, 1990). Relatively unselective uptake systems exist at the cell membrane surface both for inorganic nutrients (e.g. phos-

phate, sulfate) and for organic anions (e.g. carboxylate anions, amino acids). In some cases these assimilable anions are known to be able to form metal complexes. We reasoned that if the anion transport systems could be 'fooled' into binding and transporting the intact metal–anion complex, then the metal would find its way into the cell 'accidentally' via the anion transporter. Recent results from our laboratory provide examples of such piggyback transport for both inorganic and organic anions (thiosulfate and citrate, respectively: Fortin and Campbell, 2001; Errécalde and Campbell, 2000). The thiosulfate results are summarized below.

3.2.2. Influence of thiosulfate on silver uptake by C. reinhardtii

The design of these experiments was analogous to that described earlier for the silver/chloride system: for a fixed free silver concentration of ~10 nM, we followed silver uptake by *C. reinhardtii* over time in exposure media containing different anions (nitrate, chloride, thiosulfate). In the earlier experiments we had demonstrated that silver uptake by this alga was enhanced in the presence of chloride. A similar but even greater increase in silver uptake was observed in the presence of thiosulfate (Fig. 2, compare media A and B with medium C). In this case, however, changes in total silver concentration cannot be invoked to explain the enhanced metal uptake in media A and B, since both total and free silver concentrations were equal in all three media (104 and 10 nM, respectively—see Table 1).

Knowing that sulfate and thiosulfate might share a common transport system, and given that exposure medium B contained much more sulfate (81 μM) than thiosulfate (0.114 μM), we removed all sulfate from the medium (curve A; Fig. 2). Removal of sulfate led to a greater than 6-fold increase in silver uptake compared to the chloride exposure medium (curve C). This result clearly supports the idea that a sulfate/thiosulfate transporter is involved in silver uptake in the presence of thiosulfate. Progressive re-addition of sulfate resulted in a gradual decrease in silver uptake, as would be expected from sulfate/thiosulfate competition for a membrane transport system.

Changes in the sulfate concentration in the exposure medium only affected silver uptake if thiosulfate was present; silver uptake in the presence of 4 mM chloride was unaffected by the complete removal of sulfate. We conclude that in the absence of thiosulfate (media C and D) silver is taken up via a cation transporter (probably via a Cu(I) transport system: Fortin and Campbell, 2000) and that this transporter is unaffected by changes in ambient sulfate concentrations. In media A and B, however, a second parallel pathway for silver uptake is introduced, involving the accidental transport of silver–thiosulfate complexes via one or more sulfate/thiosulfate transporters. This conceptual model of silver uptake mechanisms is illustrated in Fig. 4.

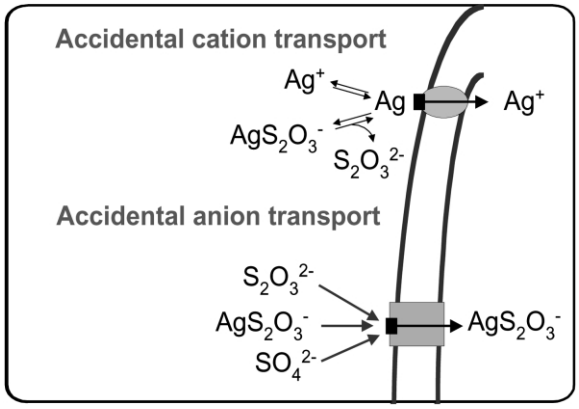

Fig. 4. Conceptual model of silver interactions with transport systems at the plasma membrane in the presence of sulfate and thiosulfate.

3.2.3. Influence of thiosulfate on silver toxicity to C. reinhardtii

Given the marked effect of thiosulfate on silver uptake rates, an obvious question arises: does the enhanced silver uptake in the presence of thiosulfate lead to increased *toxicity*? To circumvent the problem of silver loss from the exposure media, due to absorption by the algal biomass, we have used turbidostat cultures to determine the effect of silver and its thiosulfato-complexes on algal growth rates. In such systems fresh solution is pumped into the culture vessel as the cell population increases, therefore providing a constant influx of contaminant.

Using the turbidostat cultures we first determined the IC_{50} value for growth inhibition by silver in the absence of any complexing ligand. These experiments were run by transferring algae in their exponential growth phase into turbidostat culture vessels and allowing them to acclimate to their new growth conditions. Once the growth rate

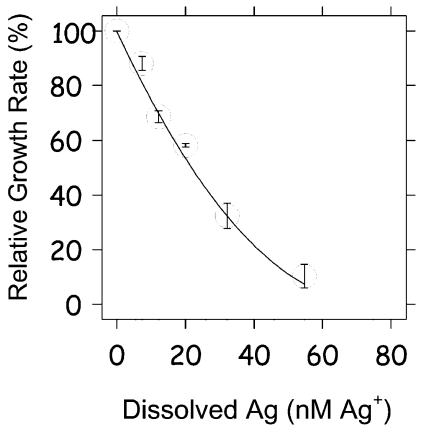

Fig. 5. Relative growth rate of *C. reinhardtii* (○) as a function of free Ag^+ concentration (growth rate of silver-free control = 100%). Exposure media contained no Ag binding ligands. Error bars represent ± standard error, $n = 3$.

had stabilized (~24 h), the concentration of free Ag^+ was increased in stepwise fashion and the growth rate determined at each new silver concentration; the IC_{50} for *C. reinhardtii* was ~21 nM, expressed as free Ag^+, or 23 amol/cell expressed as a cell quota (Fig. 5). We then chose a free silver concentration of ~10 nM, sufficient to cause approximately 10% growth inhibition (cf. Fig. 5). Once the algae were growing steadily at that concentration of free silver, we increased Ag and thiosulfate concentrations in concert, maintaining free $[Ag^+]$ constant. If the BLM applied under these conditions, the degree of growth inhibition should have remained constant at 10%, but as seen in Fig. 6, the growth rate declined progressively as [Ag]-T and $[AgS_2O_3^-]$ increased. Silver toxicity is clearly enhanced in the presence of thiosulfate. Note that the cell quota at the IC_{50} value was notably higher (100 amol/cell) than in the experiment without thiosulfate, suggesting that the silver entering the algal cells with thiosulfate was not entirely dissociated/available in the intracellular environment. This result also illustrates that algal metal quotas (and, by extrapolation, tissue metal concentrations/burdens in higher organisms) may not be unequivocal predictors of metal toxicity.

3.2.4. Discussion

A basic tenet in aquatic toxicology is that complexation of a metal will lead to a decrease in its bioavailability – in effect, most dissolved ligands that bind metals form hydrophilic complexes, $ML_n^{\pm}$, and in such systems metal uptake, nutrition and toxicity normally vary as a function of the concentration of the free-metal cation in solution. Exceptions to this simple model of metal toxicity generally involve ligands that form lipophilic complexes, ML_n^0, which can bypass normal metal transport mechanisms and cross algal membranes by simple diffusion (Florence and Stauber, 1986; Phinney and Bruland, 1994, 1997). The present example with the charged, hydrophilic silver–thiosulfate complexes (dominantly $AgS_2O_3^-$) clearly does not fall into this category. Here we have demonstrated a novel route of entry for trace metal ions into algal cells, where an assimilable (inorganic) ligand serves as a vector for metal uptake.

How widespread might this route of entry be? Sulfate transport systems in eucaryotic and procaryotic micro-organisms have been well studied (Ritchie, 1996; Perez-Castiñeira et al., 1998); both the use of thiosulfate as a sulfur source for growth and the transport of thiosulfate through sulfate transport systems have been clearly established (Hodson et al., 1968; Sirko et al., 1995). Given the universal need of algae to take up sulfate to satisfy their metabolic requirements for sulfur, and based on the frequent reports in the literature that thiosulfate and sulfate can compete for the same anion transport system, we anticipate that the bioavailability of Ag–thiosulfate complexes will prove to be a general phenomenon for phytoplankton.

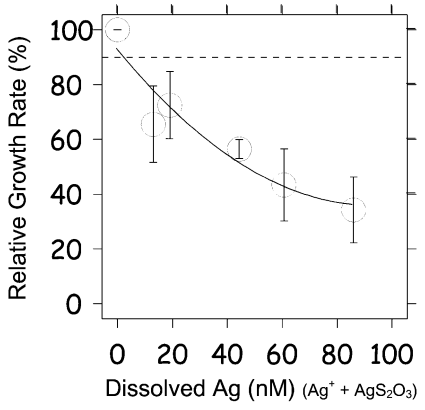

Fig. 6. Relative growth rate of *C. reinhardtii* (○) as a function of the $AgS_2O_3^-$ concentration (growth rate of silver-free control = 100%). The free Ag^+ concentration was held constant at 10 nM while the total Ag and the thiosulfate concentrations were increased concurrently. The dashed line represents the expected growth rate for a free Ag^+ concentration of 10 nM. Error bars represent ± standard error, $n = 3$.

The uptake of anionic metal–ligand complexes has also been suggested on the basis of work with metals in the presence of low molecular weight (LMW), assimilable *organic* metabolites. For example, in citrate-buffered systems the toxicity of Cu, Cd and Zn towards the green alga *P. subcapitata* was found to exceed that which would have been predicted on the basis of the free-metal ion concentration at equilibrium (Guy and Kean, 1980; Errécalde et al., 1998). Given that this alga can internalize and metabolize citrate, and that metal uptake (Cd) was markedly enhanced in the presence of citrate (Errécalde and Campbell, 2000), we suggested that the enhanced metal toxicity was due to the piggyback transport of the anionic metal–citrate complexes (i.e. the ligand is assimilated as a metal–ligand complex and the metal 'comes along for the ride').

The nature of the citrate transporter in *P. subcapitata* is unknown; indeed, a literature search failed to uncover any references to the existence of a specific membrane transport system for citrate in algae. However, it *is* known that algae can take up such LMW metabolites as amino acids and that their transport across the plasmalemma involves amino acid porters (Flynn and Butler, 1986); it is plausible that a similar transport mechanism exists in the case of citrate. The system responsible for citrate transport across the plasmalemma must preferentially transport the M–citrate complexes, since the contribution of these M–citrate complexes to overall citrate speciation is quantitatively minor.

The environmental relevance of these examples of piggyback transport is discussed in the final section of this paper.

3.3. Hypothesis (c): metal internalization must occur for toxicity to appear

3.3.1. Background

In its present formulation the BLM assumes that the interaction of M^{z+} or ML at the algal surface leads to the formation of a simple surface complex {M–X-cell}, i.e. the ligand may play a role in transporting the metal from the bulk solution to the algal surface, but thereafter it is uninvolved (i.e. it is freed back into solution). Recent experiments on the toxicity of Cd to *P. subcapitata*, analogous to those described above for citrate but with alanine as the LMW assimilable metabolite, yielded results that are inconsistent with this model and point to the involvement of ternary surface complexes in the expression of metal toxicity.

3.3.2. Influence of alanine on the toxicity of Cd to P. subcapitata

Two different media were used to determine the effects of Cd on algal growth and/or to follow algal metal uptake: (a) FRAQUIL growth medium (Price et al., 1988) buffered with nitrilotriacetate ($FRAQ_{NTA}$: 0.05 mM NTA); the free Cd^{2+} concentration was controlled by the $Cd^{2+} + NTA = Cd - NTA$ equilibrium (metal–NTA complexes are known to be unable to pass cell membranes: Mason et al., 1988); and (b) FRAQUIL growth medium buffered with D- or L-alanine ($FRAQ_{ALA}$: 0.5 mM alanine); the free Cd^{2+} concentration was controlled by the $Cd^{2+} + alanine = Cd - alanine$ equilibrium. In these two buffered media ($FRAQ_{NTA}$, $FRAQ_{ALA}$), the total Cd concentrations were adjusted to give the same range of free Cd^{2+} concentrations. For all experiments, the free-ion concentrations of the other essential trace metals (Cu, Fe, Mn, Co) and of Ca and Mg were maintained at constant levels as $[Cd^{2+}]$ was increased.

Comparison of the algal growth response in $FRAQ_{NTA}$ and $FRAQ_{ALA}$ media indicated that the toxicity of Cd was enhanced in the presence of 0.5 mM L-alanine (Fig. 7), as had been observed earlier with citrate. Parallel studies confirmed that *P. subcapitata* can accumulate alanine intracellularly (as expected—cf. Lewin and Hellebust, 1978). The stereospecificity of the enhanced toxicity was tested using $FRAQ_{ALA}$ media buffered by the two stereoisomers, L- and D-alanine. Since the membrane-bound transport systems designed to facilitate the assimilation of amino acids in micro-organisms are thought to be stereospecific for the naturally occurring L-configuration (Flynn and Butler, 1986), the D- and L-stereoisomers would be expected to have markedly different effects on Cd bioavailability if piggyback transport of Cd were occurring. To our considerable surprise, the enhancement of Cd toxicity was just as important for D-alanine as for the L-stereoisomer (Fig. 7). In both cases, at low free Cd^{2+} concentrations (<0.2 μM) the algal growth rate decreased abruptly to ~50% of that in the control culture, and then declined slowly from 50 to 10% for free Cd^{2+} concentrations higher than 0.2 μM (0.2–1.2 μM). The IC_{50} values for growth inhibition for the D-isomer (0.14 ± 0.09 μM Cd^{2+}) and the L-

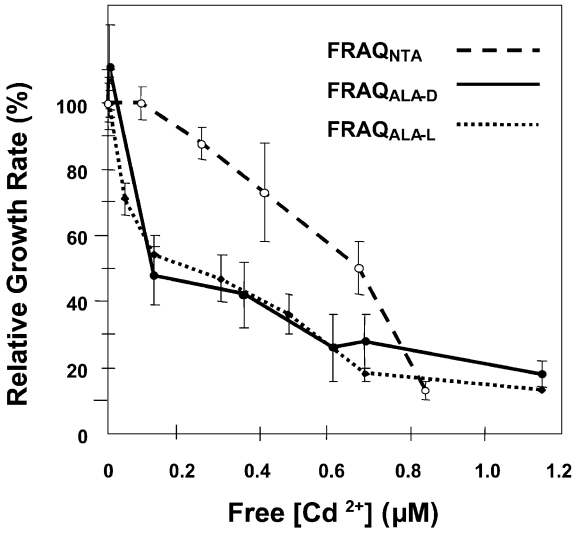

Fig. 7. Relative growth rate of *P. subcapitata* as a function of the free Cd^{2+} concentration in $FRAQ_{NTA}$ and $FRAQ_{ALA}$ media (growth rate of cadmium-free control = 100%). (○) medium buffered by NTA (dashed line); (♦) medium buffered by 0.5 mM L-alanine (dotted line); (●) medium buffered by 0.5 mM D-alanine (solid line).

isomer (0.19 ± 0.08 μM Cd^{2+}) were indistinguishable (Fig. 7). In the absence of alanine, the IC_{50} value was 3–4 times higher, 0.65 ± 0.06 μM, expressed as Cd^{2+}.

To clarify the mechanism of this surprising effect, we followed the short-term uptake (intra- and extra-cellular) of radiolabeled ^{109}Cd in the three media ($FRAQ_{NTA}$, $FRAQ_{D\text{-}ALA}$ and $FRAQ_{L\text{-}ALA}$); concentrations of the free Cd^{2+} ion were maintained at the same levels (0.5 μM) in each exposure medium. Cadmium accumulation was determined in cells that had simply been filtered and aspirated, and also in a parallel series where the cells were extracted briefly with excess EDTA (10^{-4} M) to remove the sorbed extracellular metal. Intracellular Cd was defined operationally as the Cd remaining after EDTA extraction; extracellular Cd was determined by difference (total Cd minus intracellular Cd—cf. Bates et al., 1982; Knauer et al., 1997). The time course of accumulation of intracellular Cd was the same in all three media (Fig. 8a), indicating that the enhanced toxicity of Cd^{2+} in the presence of alanine was *not* due to accelerated uptake of the metal (note that this observation contrasts markedly with the Cd–citrate system, where enhanced Cd uptake was observed—Errécalde and Campbell, 2000). However, binding of Cd to the algal surface *was* markedly increased (20-fold) in the presence of D- or L-alanine in comparison to that in $FRAQ_{NTA}$ medium (Fig. 8b). We conclude that this increased concentration of surface-associated Cd corresponds to the formation of ternary surface complexes, and that these surface complexes, {D-, L-alanine–Cd-X-cell}, are responsible for the additional toxicity observed in the presence of alanine.

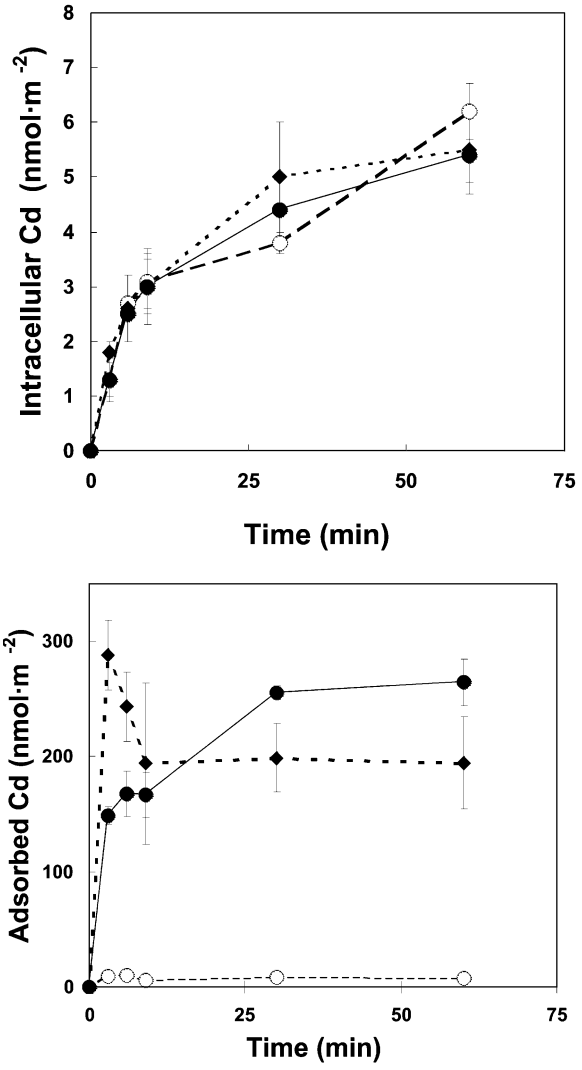

Fig. 8. (a) Intracellular cadmium taken up by *P. subcapitata* as a function of time, free $[Cd^{2+}] = 0.5$ μM in $FRAQ_{NTA}$, $FRAQ_{L\text{-}ALA}$ or $FRAQ_{D\text{-}ALA}$; (b) extracellular cadmium bound by *P. subcapitata* as a function of time. In both cases: (○) medium buffered by NTA (dashed line); (♦) medium buffered by 0.5 mM L-alanine (dotted line); (●) medium buffered by 0.5 mM D-alanine (solid line).

3.3.3. Discussion

Our interpretation of the Cd–alanine results involves two necessary conditions: (i) Cd can inhibit growth of *P. subcapitata* by binding to the algal surface, and (ii) this binding involves the formation of ternary surface complexes. The idea of surface-bound metal contributing to the inhibition of algal growth is not without precedent. In their pioneering studies on copper toxicity to algae, Steemann Nielsen and Wium-Andersen (1970) noted that during the first hours after metal addition Cu inhibited the growth of the green alga *Chlorella pyrenoidosa* but did not affect the rate of photosynthesis. They concluded that Cu did not at first penetrate into the cell but influenced the alga by blocking mechanisms in the cell membrane in such a way that no cell division took place. The decline of the rate of photosynthesis found after some hours was attributed to the accumulation of photosynthetic products which secondarily suppressed photosynthesis by negative feedback mechanisms.

Algal cell membranes play host to a variety of essential metabolic functions, including phosphatase activity (Dyhrman and Palenik, 1997), redox activity (e.g. $Fe(III) \rightarrow Fe(II)$; $Cu(II) \rightarrow Cu(I)$: Jones et al., 1987; Weger, 1999), as well as the cation and anion membrane transport functions that we have already considered. Given that these functions may well be metal-sensitive, it seems reasonable to postulate that metals may affect algal growth by inhibiting one or more of these essential processes, without actually crossing the plasmalemma into the cell interior. Referring to Fig. 1, if the binding site $^{-}$X-cell represents a physiologically active site at the cell surface, then the binding of metal M might induce a direct biological response. In a variation of this scenario, $^{-}$X-cell might correspond to a transport site normally used by an essential micronutrient; binding at the cell surface site by metal M would then inhibit the supply of the essential element and induce nutrient deficiency (e.g. Mn/Cu—Sunda and Huntsman, 1983; Fe/Cd—Harrison and Morel, 1983).

Consider now the suggestion that ternary metal complexes might form at the algal cell surface. This idea is also not without precedent. Morel (1983) noted that the reaction of EDTA metal complexes at the algal surface was 'surprisingly rapid' and speculated that ternary complexes of the type L–M–X-cell might form transiently, as intermediates in the reaction pathway leading to M–X-cell (where L = EDTA). However, the present results suggest that ternary surface complexes may be present not only as transient reaction intermediates, but also as equilibrium species that contribute to the biological response. Note that for such {L–M–X-cell} species to 'contribute to the biological response', one of two conditions must be satisfied: either $^{-}$X-cell must represent a metal-sensitive site at the origin of the biological response (i.e. the metal exerts its biological effect *without* crossing the plasma membrane), or L–M must be transported intact across the plasma membrane. Possible examples of this latter behavior were discussed in the preceding section (e.g. $Ag–S_2O_3$), but in the present case there was no evidence for the transport of the intact Cd–alanine complex (Fig. 8a) and thus we are led to conclude that $^{-}$X-cell must represent a metal-sensitive site.

4. Conclusions

In this review we have examined three of the hypotheses that underpin the BLM. The first of these, that metal uptake is under thermodynamic control (i.e. that internalization is slow relative to the transport of the metal from the bulk solution to the algal surface and its reaction at the algal surface), is rarely verified. Comparisons of calculated metal diffusion rates in the phycosphere with measured metal uptake rates, on a common (membrane) areal basis, suggest that under certain conditions (Table 2, right-hand column) the diffusive supply of the metal from the bulk solution may prove to be the rate-limiting step. Under such conditions, all labile diffusive species will contribute to metal uptake, and metal uptake will prove *in*sensitive to changes in the distribution of the metal among the different labile forms (cf. Fig. 3). This result is 'politically incorrect', as it flies in the face of the dictum that metal bioavailability should be sensitive to changes in metal speciation. It clearly will be important to determine for which metals and under what conditions diffusive supply of the metal may be the rate-limiting step. In their review of trace metal interactions with marine phytoplankton, Sunda and Huntsman (1998) argued that cases of diffusion limitation are most likely for essential metals with rapid ligand exchange kinetics, such as Zn, and for larger phytoplankton cells, since diffusion limitation is proportional to the inverse square of the cell diameter. Pinheiro and Van Leeuwen (2001) have

Table 2
Test of three BLM hypotheses—comments and environmental relevance

BLM hypotheses	Case for algal cells	Environmental relevance	Possible extrapolation to higher organisms
(a) Metal internalization is the slow, rate-determining step (cf. Fig. 1)	Assumed but rarely demonstrated Demonstrated not to be the case for silver uptake by *C. reinhardtii* (also not the case for Fe nutrition of *Thalassiosira weissflogii* (Hudson and Morel, 1990)) Metals may also enter algal cells via *anion* transporters, designed to transport either inorganic or organic anions[a]	Conditions where metal transport to the biological surface may be rate-limiting: low ambient [M] and thus low concentration gradient at biological interface; high degree of metal complexation, high [ML]/[M^{z+}] ratio; bulky ligand, low aqueous diffusion coefficient, D_{ML}; high intrinsic metal uptake rates, k_{int}	Metal internalization may also not be the rate-determining step for metal uptake at respiratory epithelial surfaces (where ventilation rates may vary—Tran et al., 2001)
(b) The X-membrane site (cf. Fig. 1) is a *cation* transporter		Conditions where metals may enter algal cells by piggyback transport via anion transport sites: high concentrations of *assimilable* ligands, favoring formation of M–L complex (e.g. for L = LMW organic ligand, receiving waters downstream from outfalls of organic-rich effluents; for L = thiosulfate, mine tailings ponds)	Possibility of piggyback transport of metals at respiratory epithelial surfaces has not been investigated. Some evidence of anomalous high uptake of water-borne Ag–thiosulfate via rainbow trout gills 'Internal' membrane surfaces perhaps more likely candidates for piggyback transport
(c) Surface complexation yields simple complex, M–X-cell	Demonstrated not to be the case for Cd and *P. subcapitata*, where the ternary surface complex, alanine-Cd-X-cell, contributed to sorption and toxicity	Formation of ternary surface complexes only important if $^{-}$X-cell corresponds to a metal-sensitive binding site If this condition is satisfied, and if ternary surface complexes form, then the biological response will depend on exposure concentration of both [M^{z+}] and [ML] Ternary complex formation favored if ligand concentrations are high, and if metal M has a strong tendency to form coordinate bonds and an ability to form stable ternary complexes	Ternary F–Al–gill complexes formed when fish gill cells were exposed to Al and F simultaneously, at pH ~5.5; the ternary complexes contributed to the ionoregulatory perturbation responsible for fish mortality

[a] *Note:* As mentioned in the text, metals may also enter algal cells by passive diffusion of lipophilic ML_n^0 species.

recently developed a model describing metal interactions at the surface of a generic micro-organism, and have used the model to define under what conditions diffusive supply or the reaction kinetics of the metal with X-cell might be expected to limit the metal uptake rate.

These questions of metal supply from the ambient water may also be important for higher organisms, where metal uptake across respiratory epithelia could in theory be limited by the advective and/or diffusive supply of metal to the gill surface. The BLM in its current formulation assumes that metal uptake across the gill is slow relative to metal influx with the inspired water, and that the gill surface achieves equilibrium with the inspired water; the BLM thus does not consider the possible influence of gill ventilation rates on metal uptake or toxicity. Recent studies on Cd uptake by *Corbicula fluminae*, a filter-feeding mollusc, suggest that this assumption may not stand up to scrutiny. The animals were subjected to different dissolved oxygen regimes, leading to very different pumping rates, and Cd uptake proved sensitive to the rate of water intake even though the ambient free Cd^{2+} concentrations were identical in all exposures (Tran et al., 2001).

The second hypothesis, that metals can only enter algal cells via cation transport pathways, is clearly wrong. We have presented evidence for the uptake of Ag as the Ag–thiosulfate complex, and Cd as the Cd–citrate complex, via anion transport systems (in addition, there is unequivocal evidence in the literature that neutral lipophilic metal forms can cross algal membranes by passive diffusion—Florence and Stauber, 1986; Phinney and Bruland, 1994, 1997). However, one can legitimately question the environmental relevance of these results: are they of academic interest only, or does piggyback uptake of metal–ligand complexes really occur under realistic environmental conditions?

Normally the concentrations of *organic* assimilable ligands in natural waters are maintained at sub-micromolar levels by the natural heterotrophic micro-organisms (Button, 1985), these levels being too low to complex trace metals significantly. However, LMW metabolites such as citrate, malate and glycolate are known to be excreted as extracellular products of photosynthesis by healthy phytoplankton cells (Sundh, 1992; Rosenstock and Simon, 2001). Given that many of these potentially assimilable metabolites have the ability to complex di- and tri-valent metals, their presence in the micro-environments close to the biological interface, e.g. in the ⟨phycosphere⟩, raises the possibility that the enhanced metal uptake/toxicity observed in the present experiments may well occur under natural conditions.

LMW metabolites are also naturally present in animal digestive tracts, as breakdown products of the digestion process. Since the epithelial membrane in the gut is naturally rich in transport systems designed to assimilate these molecules, it seems logical that the piggyback metal uptake observed with algae in the present experiments may well occur in the gut. For example, in cell culture experiments with human enterocytes, Jumarie et al. (2001) demonstrated enhanced uptake of Cd in the presence of glutathione.

In principle, the assimilation of intact hydrophilic metal–ligand complexes could also occur with *inorganic* ligands such as phosphate, sulfate, carbonate or chloride. Uptake systems for these anions exist at many biological interfaces; if these transport systems could be 'fooled' into binding and transporting the intact metal–anion complex, then the metal would find its way into the cell via anion transport. For most metals, the formation of complexes with phosphate or sulfate anions is thermodynamically unfavorable; in freshwater environments and at environmentally realistic metal and anion concentrations, these complexes exist at levels so low as to rule out a significant role for piggyback transport. However, if the metal and the inorganic ligand form very stable complexes, this thermodynamic argument would no longer apply.

To our knowledge, only one example of piggyback transport of a metal with an inorganic ligand is known for algae (Ag–thiosulfate). Thiosulfate is not common in natural waters, but is present in effluents from photo-finishing plants (Purcell and Peters, 1998) and has also been detected in the interstitial waters of suboxic sediments (Luther et al., 1986) and in mine tailings ponds (Wasserlauf and Dutriac, 1982; Edwards et al., 2000). As indicated earlier, assimilatory sulfate reduction is ubiquitous in algal cells, and thus we would predict that the Ag–thiosulfate pathway will prove to be widespread in algae (in vitro). In natural waters, however, reduced sulfur species are likely to outcompete thiosulfate for binding to silver (Smith et al., 2002), rendering this pathway inoperative. Could other metals enter algal cells with thiosulfate? Thermodynamic stability constants for M–

thiosulfate complexes are very scarce in the National Institute of Standards and Technology (NIST) standard reference database (Martell et al., 1998) and it is thus difficult to judge which other metals may form sufficiently stable complexes under environmentally realistic conditions. Cadmium (and other 'soft' or Class B metals) would be possible candidates.

Extrapolation of the thiosulfate example from algal cells to higher organisms is difficult, since little is known about sulfate transport mechanisms in epithelial cells (gills, intestine). However, in laboratory water-borne exposure experiments, Ag accumulation by rainbow trout, *Oncorhynchus mykiss*, was inexplicably enhanced in the presence of thiosulfate (Hogstrand et al., 1996; Wood et al., 1996). It is tempting to explain this greater-than-expected silver accumulation by generalizing our conceptual model of silver accumulation, but thiosulfate uptake by fish remains to be demonstrated. Interestingly, preliminary data with fathead minnow larvae (*Pimephales promelas*) exposed to Cd in the presence of thiosulfate showed greater metal accumulation than could be explained on the basis of the calculated free-metal concentrations (D.G. Dixon, Department of Biology, University of Waterloo, personal communication).

Finally, should we consider piggyback transport of metals with inorganic ligands other than thiosulfate? A search of the animal physiology literature turned up several suggestions that Cd, Cu and Zn carbonate/chloride complexes may be taken up by red blood cells (Alda and Garay, 1990; Kalfakakou and Simons, 1990; Lou et al., 1991). The postulated transported species (e.g. $[Cd(OH)(HCO_3)Cl]^{-1}$; $[Zn(OH)(HCO_3)Cl]^{-1}$) are entirely speculative; no thermodynamic data exist to suggest that such mixed-ligand complexes actually exist in solution, let alone at the transport sites. However, it is possible to explain the erythrocyte experimental data not in terms of the transport of intact ternary complexes, but rather as the *co-transport* of the carbonate anion and one or more metal species (Stein, 1990). If such co-transport were widespread for metals and anions, then the whole BLM construct would crumble! The fact that, on the contrary, the BLM does a good job of predicting (acute) toxicity, suggests that co-transport cannot be a widespread phenomenon at *external* epithelial membranes (respiratory structures; integument). The case with *internal* epithelial membranes (intestine; erthyrocytes; hepatocytes; lymphocytes; etc.) may differ, however; ligand concentrations are much higher in the internal environment, and as a consequence it is perhaps more likely to encounter ternary complexes and co-transport phenomena.

With respect to the final hypothesis examined, that metal interactions at the biological surface lead to the formation of a simple M–X-cell complex, and that metals must be internalized before they can exert their toxic action, we presented evidence suggesting that metals can in fact exert their toxic action by binding to sites present at the membrane surface (i.e. that membrane transport is *not* a necessary condition for metal-induced toxic effects to occur), and that under such conditions both {M–X-cell} and {L–M–X-cell} can contribute to metal toxicity. In such cases, the concentration of the surface species {M–X-cell} and {L–M–X-cell}, and the biological response, will be influenced by variations of both $[M^{z+}]$ and [ML] in the exposure solution; if the response is additive, then the metal will appear to be more bioavailable than predicted on the basis of the BLM.

Of the three 'exceptions' to the BLM discussed in this review, this last one remains the least well documented. If ternary complexes *are* going to be involved in the toxicity response, then the first necessary condition is that there be a metal-sensitive site present at the algal surface, readily accessible. Examples of such sites have been discussed earlier. The second necessary condition is that the conditions at the algal surface favor the formation of ternary complexes. Based on equilibrium considerations, these conditions will include reasonably high ligand and high metal concentrations together with the presence of a metal with a strong tendency to form coordinate bonds and an ability to form stable ternary complexes. In the present case we are talking about a ternary *surface* complex (cf. Charlet and Karthein, 1990; Schindler, 1990); perhaps in this case the biological surface can act as a template to increase the effective metal concentration.

There are already several indications in the literature that ternary surface complexes may be involved in the expression of metal toxicity in higher organisms such as fish. In earlier work on the toxicity of Al–fluoride complexes to juvenile Atlantic salmon, Wilkinson et al. (1990, 1993) demonstrated that ternary F–Al–gill complexes formed when fish gill cells were exposed to Al and F simultaneously, at a pH of approximately

5.5, and that these complexes contributed to the ionoregulatory perturbation that was responsible for fish mortality. Similarly, in their refinements to the copper BLM for freshwater fish, Santore et al. (2002) have recently suggested that the ternary complex HO–Cu–X–gill may contribute to the greater-than-expected toxicity of copper at alkaline pH values.

As has been pointed out in this review and in many other papers in this special issue, the roots of the BLM can be traced back to pioneering work published in the late 1970s and early 1980s (Shaw and Brown, 1974; Sunda and Guillard, 1976; Andrew et al., 1977; Anderson et al., 1978; Pagenkopf, 1983). The name of the model has changed, and it has graduated to software status, but the underlying concepts have remained essentially unchanged. As Di Toro has said, 'Those early workers got it right!' (D.M. Di Toro, Manhattan College, personal communication, January 2001).

In the present review, we have tended to focus on those areas where the BLM may not yield accurate predictions (e.g. when assimilable ligands are present, or where the metal-sensitive site is present on the outer surface of the target organism), with the idea that by emphasizing such 'exceptions' we are helping to establish the limits of application of the model. However, the reader should not lose sight of the fact that these 'exceptions' are far less numerous than are the cases where the BLM performs credibly. For example, in reviewing experiments performed at constant pH and hardness, and in the presence of synthetic ligands forming hydrophilic metal complexes, Campbell (1995) reported that the biological response consistently varied as a function of the concentration of the free-metal ion, as predicted by the BLM (52 of 59 cases examined).

The true measure of a model's success is its ability to explain a wide body of empirical information 'better than any other model'. Some 20 years after the original groundwork was laid for the BLM, it seems clear that the model has passed this test. One of the remaining challenges will be to demonstrate its general applicability in the presence of natural ligands such as humic and fulvic acids and reduced sulfur species.

Acknowledgments

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Appendix A:

In the simplest case, where the hydrated free-metal ion is the species reacting at the cell surface, one can envisage the following reactions:

(1) Solution equilibrium

$$M^{z+} + L \overset{K_1}{\leftrightarrow} ML \tag{1}$$

$$K_1 = [ML]/([M^{z+}][L]) \tag{2}$$

(2) Surface reaction of M^{z+}

$$M^{z+} + {}^{-}X\text{-cell} \overset{K_2}{\leftrightarrow} M-X\text{-cell} \tag{3}$$

$$\{M-X\text{-cell}\} = K_2\{{}^{-}X\text{-cell}\}[M^{z+}] \tag{4}$$

where K_1 and K_2 are conditional equilibrium constants, L is a ligand in solution, charges on complex species are omitted for simplicity, and molecules of water held in the metal's coordination sphere are not indicated. Note that {} and [] refer to concentrations of surface and dissolved species respectively. The biological response is assumed to be proportional to the concentration of the surface complex, {M–X-cell} (see text). Provided the concentration of free $^{-}$X-cell sites remains approximately constant, Eq. (4) indicates that the biological response will vary directly as a function of $[M^{z+}]$, i.e. the linear portion of a Michaelis–Menten type uptake curve.

A similar situation prevails if a metal complex (ML) is the species reacting at the cell surface, provided that the reaction proceeds by ligand-exchange (Eq. (5)).

Surface reaction of ML

$$ML + {}^{-}X\text{-cell} \overset{K_3}{\leftrightarrow} M-X\text{-cell} + L \tag{5}$$

$$\{M-X\text{-cell}\} = K_3\frac{\{{}^{-}X\text{-cell}\}[ML]}{[L]} \tag{6}$$

By rearranging Eq. (2), one obtains

$$\frac{[ML]}{[L]} = K_1[M^{z+}] \quad (7)$$

Substitution of Eq. (7) into Eq. (6) then yields

$$\{M-X\text{-cell}\} = K_3K_1\{^-X\text{-cell}\}[M^{z+}] \quad (8)$$

which shows the same dependency on M^{z+} as Eq. (4).

In the original formulation of the model the interaction of metal M with the plasma membrane was described as a ligand exchange reaction (Eq. (3) or Eq. (5)), such that in the resulting surface complex metal M was bound only to $^-$X-cell (M–X-cell) and water molecules. If one allows one or more of the ligands originally bound to M to remain in the metal's coordination sphere (Eq. (9)),

$$ML + {}^-X\text{-cell} \overset{K_4}{\leftrightarrow} L-M-X\text{-cell} \quad (9)$$

i.e. the formation of a mixed-ligand complex, then the concentration of the surface complex, L–M–X-cell, is proportional to [ML] rather than to $[M^{z+}]$ alone.

$$\{L-M-X\text{-cell}\} = K_4\{^-X\text{-cell}\}[ML] \quad (10)$$

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