

# Biotic Ligand Model, a Flexible Tool for Developing Site-Specific Water Quality Guidelines for Metals

SOUMYA NIYOGI AND CHRIS M. WOOD\*

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

The biotic ligand model (BLM) is a mechanistic approach that greatly improves our ability to generate site-specific ambient water quality criteria (AWQC) for metals in the natural environment relative to conventional relationships based only on hardness. The model is flexible; all aspects of water chemistry that affect toxicity can be included, so the BLM integrates the concept of bioavailability into AWQC—in essence the computational equivalent of water effect ratio (WER) testing. The theory of the BLM evolved from the gill surface interaction model (GSIM) and the free ion activity model (FIAM). Using an equilibrium geochemical modeling framework, the BLM incorporates the competition of the free metal ion with other naturally occurring cations (e.g.,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{H}^+$ ), together with complexation by abiotic ligands [e.g., DOM (dissolved organic matter), chloride, carbonates, sulfide] for binding with the biotic ligand, the site of toxic action on the organism. On the basis of fish gill research, the biotic ligands appear to be active ion uptake pathways (e.g.,  $\text{Na}^+$  transporters for copper and silver,  $\text{Ca}^{2+}$  transporters for zinc, cadmium, lead, and cobalt), whose geochemical characteristics (affinity =  $\log K$ , capacity =  $B_{\max}$ ) can be quantified in short-term (3–24 h) in vivo gill binding tests. In general, the greater the toxicity of a particular metal, the higher the  $\log K$ . The BLM quantitatively relates short-term binding to acute toxicity, with the  $\text{LA}_{50}$  (lethal accumulation) being predictive of the  $\text{LC}_{50}$  (generally 96 h for fish, 48 h for daphnids). We critically evaluate currently available BLMs for copper, silver, zinc, and nickel and gill binding approaches for cadmium, lead, and cobalt on which BLMs could be based. Most BLMs originate from tests with fish and have been recalibrated for more sensitive daphnids by adjustment of  $\text{LA}_{50}$  so as to fit the results of toxicity testing. Issues of concern include the arbitrary nature of  $\text{LA}_{50}$  adjustments; possible mechanistic differences between daphnids and fish that may alter  $\log K$  values, particularly for hardness cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ); assumption of fixed biotic ligand characteristics in the face of evidence that they may change in response to acclimation and diet; difficulties in dealing with DOM and incorporating its heterogeneity into the modeling framework; and the paucity of validation exercises on natural water data sets. Important needs include characterization of biotic ligand properties at the molecular level; development of in vitro BLMs, extension of the BLM approach to a wider range of organisms, to the

estuarine and marine environment, and to deal with metal mixtures; and further development of BLM frameworks to predict chronic toxicity and thereby generate chronic AWQC.

## Introduction

A comprehensive treatise on the biotic ligand model (BLM) has been provided by Paquin et al. (1), which serves as the introduction to a compendium of BLM-related papers occupying a whole journal issue (*Comp. Biochem. Physiol. C* 2002, 133). We ourselves have detailed the conceptual basis of the BLM and a number of organism-related matters (acclimation to different water hardness and sublethal waterborne metal levels, dietary ion, and metal levels) that affect the predictions of the BLM (2). The purpose of the present review is to complement rather than overlap these recent syntheses by here critically focusing on the development of the theoretical basis of the BLM and on the actual mechanics of how current BLMs work and, therefore, on their associated strengths and weaknesses.

Historically, ambient water quality criteria (AWQC) for metals have been based simply on total metal levels in the water or more recently on acid-extractable or dissolved metal levels, regardless of the water chemistry. AWQC were referenced to toxicity tests performed with highly soluble, completely dissociated metal salts in “clean” laboratory waters (i.e., low in organic substances, particulate matter, and salts) where most of the metal was present as the free ion, the form generally considered most toxic and a situation often very different from that in natural waters. This occurred despite a wealth of early physiological and toxicological research (e.g., refs 3–9), which demonstrated that the bioavailability and toxicity of metals to aquatic organisms were dependent on water chemistry factors such as hardness, salinity, specific ion levels, pH, alkalinity, complexing agents, and dissolved organic matter (DOM, earlier referred to as dissolved organic carbon, DOC). It was long believed that there existed too much uncertainty and difficulty to incorporate receiving water chemistry into regulations.

However, a conceptual breakthrough (i.e., acceptance that water chemistry mattered) occurred in the late 1970s and 1980s when European agencies and the U.S. EPA began developing ambient water quality criteria for metals that were different in freshwater and marine waters (i.e., salinity mattered) and which in freshwater were adjusted for hardness (i.e.,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  mattered) (10, 11). The latter adjustments were generally in the form of hardness-based equations, logarithmic regressions based on experimental  $\text{LC}_{50}$  data, which could be used to generate a site-specific AWQC based on the hardness of the receiving water. This approach was undoubtedly a vast improvement over previous practice, but

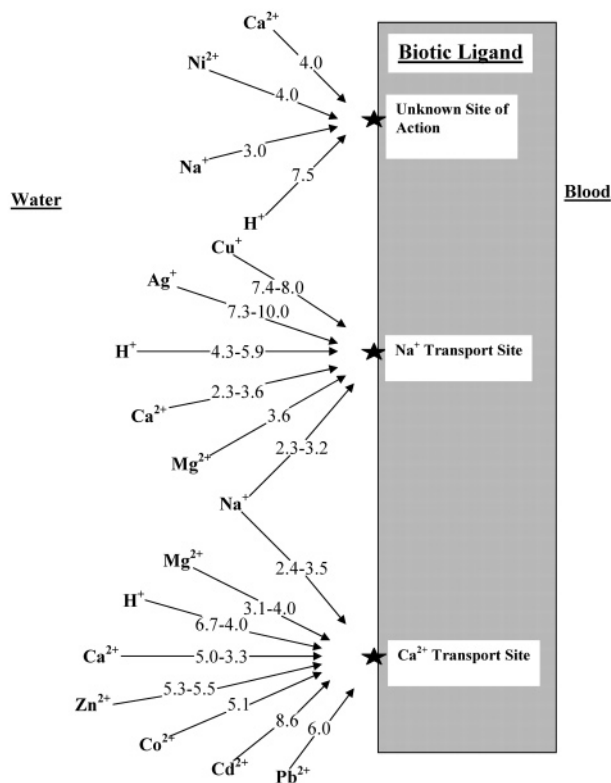
\* Corresponding author telephone: (905)525-9140, ext. 23537; fax: (905)522-6066; e-mail: woodcm@mcmaster.ca.

in hindsight, it is now clear that hardness was often really a surrogate for other water chemistry variables (e.g., alkalinity, specific ions, pH), which may have been equally or more important in the regression data sets (12–14), and that other critical water chemistry variables (e.g., DOM, sulfide) were overlooked. A second regulatory breakthrough came with the implementation of the water effects ratio (WER) procedure (15) for allowing site-specific deviations from the predictions of the hardness equations. The WER is a method for allowing the organism to tell the investigator and regulator whether local water chemistry modifies metal toxicity. The WER procedure involves side-by-side toxicity tests in the site-specific water of interest and in hardness-matched “clean” laboratory water. In practice, it is important to match  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ratios (16–18) of the site and laboratory water, which ideally should be referenced back to the  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ratio used in the original criteria development and to use organisms whose sensitivity is close to the criteria concentration in the laboratory water (19). The resulting WER (ratio of the  $\text{LC}_{50}$  values) thereby reflects the additional modifying influence of other, often unmeasured, water quality variables. The BLM can be viewed as a way of doing a WER test in a computer (with a great saving of time, effort, and money) when all important water quality variables have been measured, and indeed, experimental WER data have often been used to calibrate BLMs (20).

### Development of the Theoretical Basis of the BLM

Like all computer models, the BLM is only as good as the theory and the calibration data on which it is founded. The basic theory started in the 1970s with the realizations (i) that metal toxicity could be largely explained by the toxicity of the free ion (e.g., refs 6, 21, and 22); (ii) that complexation by inorganic and organic ligands and natural DOM would decrease metal toxicity by decreasing free ion activity (e.g., refs 6, 8, and 23–25); (iii) that a variety of water quality parameters affected metal toxicity by effects on both the organism and metal speciation (e.g., refs 9, 26, and 27); and (iv) that hardness cations were specifically protective because they competed with free metal ions for key binding sites on the organism, which had  $\log K$  values similar to those of known organic ligands (28). Shortly thereafter, Pagenkopf (29) proposed the gill surface interaction model (GSIM) while Morel (30) formulated the free ion activity model (FIAM), both of which brought together these ideas in a single framework. The GSIM and the FIAM are the theoretical ancestors of the modern BLMs.

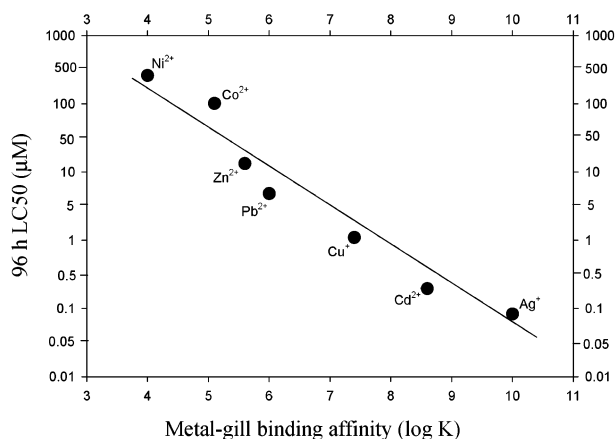
The GSIM (29) recognized that pH and alkalinity influenced metal speciation and that inorganic anions (the importance of DOM was curiously discounted) would complex metals, decreasing their availability, and it envisaged that toxicity to fish resulted from cationic metals binding to a finite number of anionic “interaction sites” on the gills in competition with protective cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{H}^+$ ). These reactions, including those at the gills, were assigned  $\log K$  values and were assumed to occur rapidly relative to the pathological response, allowing successful prediction of toxicity through equilibrium modeling. The FIAM (30) focused on cationic metal binding to critical sites on algae and made very similar assumptions to the GSIM, but additionally recognized the importance of DOM in complexation reactions, and accepted that other metal species in addition to the free cation might bind to the critical sites. Originally characterized as representing exceptions to the basic FIAM (31), the latter idea was later formalized mathematically in the extended FIAM (32). Again chemical equilibrium was assumed. In both the GSIM and the FIAMs, the degree of toxic response was thought to be related to the fraction of critical sites impacted by the reactive metal species.



**FIGURE 1.** Binding affinities ( $\log K$ ) of free ions of different metals (copper, silver, cadmium, cobalt, lead, zinc, and nickel) and environmental cations, interacting at different sites of toxic action on the biotic ligand. The data are taken from Tables 1–4 and reflect the lower and upper range in cases where multiple values are available.

While Pagenkopf (29) had assumed a “respiratory impairment” as the mechanism of acute toxicity of all metals in fish, he also noted that the mechanism was related to “salt and water balances within the gill tissue”. A series of mechanistic physiological studies in the 1980s and 1990s (cf. ref 33 for review) demonstrated that, at the levels of concern for acute toxicity (i.e., around 96 h  $\text{LC}_{50}$  levels), respiratory interference was minimal but many cationic metals had specific inhibitory effects on ion transport functions in fish gills. Thus  $\text{Cu}^{2+}$  (e.g., refs 34–37) and  $\text{Ag}^+$  (e.g., refs 38–40) specifically blocked active  $\text{Na}^+$  (and  $\text{Cl}^-$ ) uptake at the gills, while  $\text{Cd}^{2+}$  (e.g., refs 41–43),  $\text{Zn}^{2+}$  (e.g., refs 44–47), and  $\text{Co}^{2+}$  (48, 49) specifically blocked active  $\text{Ca}^{2+}$  uptake. More recently  $\text{Pb}^{2+}$  (50) has been added to the list of metals interacting at  $\text{Ca}^{2+}$  transport sites. This mechanistic information helped guide the subsequent development of the BLM framework, as illustrated in Figure 1. In many of these cases, at concentrations slightly above their thresholds for action on ion transport, these metals also caused general increases in diffusive losses of ions, probably by inducing inflammatory or oedematous reactions in the gills, reflecting the start of interference with respiratory gas exchange (51). It also became clear that the hardness cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (particularly the former) not only decreased metal action at transport sites by apparent competition but also decreased ionic losses associated with toxicant action by stabilizing the paracellular junctions in the gill epithelium (52, 53). For some metals,  $\text{H}^+$  not only changed speciation but also offered protection by competing with metals for binding sites (54, 55), although  $\text{H}^+$  itself at higher concentration (i.e.  $\text{pH} \leq 5.5$ ) directly inhibited active  $\text{Na}^+$  and  $\text{Cl}^-$  uptake (56, 57).

The next major breakthrough came from the development of gill metal binding models by Playle and colleagues (58–63). While the ultimate focus was toxicity prediction, these



**FIGURE 2. Relationship between gill–metal binding affinity (log  $K$ ) and acute toxicity (96 h LC<sub>50</sub>) in juvenile rainbow trout in the authors' standard laboratory water (moderately hard, from Lake Ontario) or similar water. The log  $K$  data for Ag<sup>+</sup>, Cu<sup>+</sup>, Cd<sup>2+</sup>, Pb<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, and Ni<sup>2+</sup> are taken from the cited literature: refs 61, 60, 60, 174, 72, 62, and 162, respectively. The 96 h LC<sub>50</sub> data for the same metals are taken from the cited literature: refs 13, 75, 73, 173, 77, 176, and 160, respectively. The Y-axis of the plot is in log scale.**

studies laid out simple practical methods for determining gill metal binding constants by short exposures (2–3 h) of live fish to low metal concentrations (i.e., around 96 h LC<sub>50</sub> levels) in ion-poor soft water where competition reactions would be minimized. Other workers have used 24-h exposures (64, 65); regardless, the key point is to measure metal binding before the physiology of the gill changes due to pathological reactions (i.e., to measure short-term, active binding before slower diffusion or nonspecific binding occurs). In general, workers have recognized that true equilibrium did not apply (i.e., had they waited longer, gill metal burdens would have increased) but have assumed like Pagenkopf (29) that chemical reactions at the gill occurred much faster than pathological response, such that equilibrium modeling principles could be used. In some cases, the fish were incubated with metal in the presence of organic ligands of known log  $K$  values so as to set up a competition between the ligand and the gill for metal binding, and standard geochemical speciation programs [e.g., MINTEQA2 (66) and MINEQL+ (67)] were used to calculate free cationic metal levels. Affinity constants (log  $K$ ) and binding site densities ( $B_{\max}$ ) on the gills were calculated by Langmuir adsorption or Scatchard analysis. Once these were known, the corresponding constants for unknown ligands in the water such as DOM were estimated indirectly by finding the concentrations necessary to keep metal off the gills.

With this approach, it became clear that different metals bound to different sites, as might be predicted from the physiological studies reviewed above. Thus Cd<sup>2+</sup> and Co<sup>2+</sup> bound to one set with very different log  $K$  values (for competing cations) and  $B_{\max}$  values from another set that preferentially bound Cu<sup>2+</sup> and Ag<sup>+</sup> (Figure 1) [While it may seem strange that divalent Cu<sup>2+</sup> competes with monovalent Ag<sup>+</sup> for a Na<sup>+</sup> site, copper generally crosses biological membranes as Cu<sup>+</sup> (68), and recent indirect evidence points to the presence of the reductases in the gills (69, 70).] Within a site, log  $K$  values for gill binding correlated with toxicity—for example, the more toxic Ag<sup>+</sup> had a higher log  $K$  (10.0; 61) than the less toxic Cu<sup>2+</sup> (7.4; 60) at the presumptive Na<sup>+</sup> transport site. It later became clear that there was a strong overall correlation between the log  $K$  values for gill binding and the acute toxicity of different metals as illustrated in Figure 2, even though different metals clearly bind to different sites (Figure 1). Most of the “biotic ligand” log  $K$  and  $B_{\max}$  values used in modern BLMs can be traced back to the original

determinations of Playle and co-workers on fathead minnows and juvenile rainbow trout, although they have undergone considerable modification in the process of “fitting” models to experimental toxicity data.

One limitation of the early gill metal binding studies was the relatively low sensitivity and precision of graphite furnace analysis of metal burdens on gills; this was especially true for nutrient metals (e.g., copper, zinc) when the gills already contained high endogenous levels under control conditions prior to metal exposure. Indeed it was not until the application of radiotracer technology that the first gill binding curves were determined for Zn<sup>2+</sup> (71, 72) or for fish that had been chronically exposed to sublethal waterborne metals and thereby built up greatly increased background levels in the gills prior to metal binding tests [e.g., Cd<sup>2+</sup> (73, 74), Cu<sup>2+</sup> (75, 76), Zn<sup>2+</sup> (72, 77)]. This more sensitive approach revealed that there was not just one discrete set of saturable binding sites but at least two or possibly more for most metals. The concept of different but overlapping high-affinity, low-capacity binding sites and low-affinity, high-capacity binding sites is now widely recognized. The latter appear to increase when fish are chronically acclimated to sublethal metal concentrations (2). However, the focus of the BLM is directed at the former. Nevertheless, recent evidence (cf. ref 2 for review) suggests that even the high-affinity, low-capacity sites may change with acclimation to hardness and sublethal metal levels in water (72–75, 77, 78), with dietary metal content (79, 80), and with dietary ion content (81–83). Furthermore, under any one condition, these sites may be heterogeneous; Grosell and Wood (70) identified separate Na<sup>+</sup>-sensitive and Na<sup>+</sup>-insensitive copper uptake sites in trout gills, both of which were of high affinity.

The final key step in BLM development, and the one where we still have the least information, was to demonstrate quantitative relationships between short-term (2–3 h or 24 h gill metal binding) and eventual toxicity (96 h+). Playle et al. (60) reported significant negative correlations between short-term (2–3 h) gill metal binding (at constant exposure concentration) and 96 h LC<sub>50</sub> values for both cadmium and copper in fathead minnow larvae exposed in a range of natural lake waters. However the real need is for calibration data relating short-term gill metal loads with percentage mortality at 96 h+, from which the LA<sub>50</sub> can be calculated. The LA<sub>50</sub> is the short-term gill metal burden that is predictive of 50% mortality at 96 h+. Such data have been provided in only a very few studies [Cu<sup>2+</sup> (64, 84), Ni<sup>2+</sup> (85), Ag<sup>+</sup> (65), Cd<sup>2+</sup> (86)]. In essence, test organisms must be exposed to a range of exposure concentrations, preferably in a range of different water chemistries, for measurement of percent mortality at 96 h+, with subsets sacrificed for gill metal analysis at 2–3 h to 24 h (before any mortality develops).

In a very elegant study on trout, MacRae et al. (64) not only provided LA<sub>50</sub> estimates by relating 24 h gill copper burden to percent mortality at 120 h but also demonstrated that log  $K$  values determined via Scatchard analysis of gill copper burdens versus water Cu<sup>2+</sup> concentrations were very similar to log  $K$  values (both around 7.2) estimated from 168 h mortality data obtained in the presence of competing organic ligands with known log  $K$  values. Marr et al. (87) provided additional supportive data relating copper toxicity to the log  $K$  values of the competing organic ligands, while MacRae et al. (88) devised a cocktail of organic acids that appeared to duplicate the copper binding characteristics and, therefore, the protective action of naturally occurring DOM in a particular stream. Morgan and Wood (65) determined good predictive relationships between 3 h or 24 h gill silver burden and percent mortality at 96 h in rainbow trout, as long as the silver exposures were done under flow-through conditions. MacRae et al. (64) concluded that “measurement of gill copper accumulation is an acceptable alternative for



determining a toxicity-based gill copper binding affinity". This conclusion (or in many cases its reciprocal) is central to most current BLMs, and these calibration data lie at the heart of the original copper BLM (20). Meyer et al. (85) provided similar data for nickel in 96 h toxicity tests with fathead minnows, demonstrating that 24 h gill nickel burden ( $LA_{50}$ ) was constant across a wide range of hardnesses, even though the 96 h  $LC_{50}$  varied 10-fold expressed as either total dissolved nickel or free  $Ni^{2+}$  ion concentration. Ma et al. (89) recently reported that sublethal copper toxicity (growth inhibition) in the freshwater alga *Scenedesmus subspicatus* appeared to correlate with a relatively constant extracellular copper accumulation when organic anions in the media were altered. For invertebrates, there are now several demonstrations of this same principle, using whole body metal burden rather than gill metal burden as the index for  $LA_{50}$  [cadmium in *Hyallela azteca* (90), copper in *Lumbriculus variegatus* (91)].

Despite this pioneering research of Borgmann and colleagues (90) on *Hyallela*, it is clear from the above history that the bulk of the original development work for modern BLMs has been done on fish, simply because their size makes them easy to work with and their gills provide sufficient tissues for analysis. Indeed the term biotic ligand model (BLM) was introduced so that concepts and results from "fish gill models" (63) could be generalized (85, 92). It is therefore somewhat ironic that the regulatory application of BLMs has been largely to replace or modify AWQC, which in general are driven by daphnids, the organisms that are the most sensitive to metals (often 5–10 times more sensitive than representative fish such as the rainbow trout). This sensitivity undoubtedly relates to their small size and the associated high ion uptake and leakage rates accompanying their high surface area-to-volume ratios (93, 94). Until very recently (cf. refs 95–97) there had been almost no mechanistic research on metal toxicity in daphnids. Therefore the approach adopted has been to recalibrate fish-based models directly to daphnid toxicity data—i.e., the reciprocal of the conclusion of MacRae et al. (64)—such that "measurement of toxicity is an acceptable alternative for determining a binding affinity based on gill metal binding". In general, this has been done on an entirely empirical basis without explicit consideration of mechanism, resulting in some interesting issues as detailed below.

Another issue of concern has been on how to deal with DOM in BLMs, in view of the importance of DOM in complexing metals. While the stability constants for most aspects of freshwater chemistry that affect metal speciation are well-described (and more or less identical) in standard geochemical modeling programs [MINTEQA2 (66), MINEQL+ (67), CHESS (98)], those for natural DOMs are lacking. One approach has been to "back-calculate" single-site log  $K$  values for DOMs from experimental observations of concentrations necessary to prevent gill metal binding or toxicity (e.g., refs 61, 88, and 99); the other (e.g., refs 20, 92, and 100) has been to make modifications to a dedicated multi-site modeling program, the Windermere humic acid model [WHAM-V (101)].

## Copper

To date, of all the BLMs that are being generated for different metals, the acute Cu-BLM is at the most advanced stage of development. At present, there are three available versions of the acute Cu-BLM and a single version of a chronic Cu-BLM, which for convenience we have termed A–D, respectively. Version A is the original version of the model developed for fish and adapted for daphnids by Di Toro et al. (92) and Santore et al. (20) (note: updates appear on the website <http://www.hydroqual.com/winblm>); version B is the acute Cu-BLM developed for *Daphnia magna* by De Schampelaere and Janssen (102); version C is the refined version

of model B for *D. magna* as described by De Schampelaere et al. (100); and version D is the chronic model for *D. magna* developed by De Schampelaere and Janssen (103), based on the same data set used in an earlier regression model approach (104).

In version A, the affinity constants (log  $K$ ) for biotic ligand–cation complexes were adopted from the gill-Cu binding model developed by Playle et al. (58–60) in fathead minnow (*Pimephales promelas*), the stability constants for  $Cu^{2+}$ -DOM interactions were implemented from the WHAM-V thermodynamic database (101), and other constants were taken from the CHESS modeling framework (98). In addition, an affinity constant for  $Na^+$  binding to the gill was added to the fathead minnow database, which was not part of the original gill-Cu binding model of Playle et al. (60), based on the fact that  $Na^+$  also has protective effects against copper toxicity in freshwater fish (105). This fits with knowledge that  $Cu^{2+}$  specifically targets branchial  $Na^+$  uptake sites (34–37, 70). The lethal gill accumulation predictive of 50% mortality ( $LA_{50}$ : 10 nmol  $g^{-1}$  wet wt above background) was determined by Di Toro et al. (92) using the gill-Cu load at 24 h versus 120 h percent mortality data of MacRae et al. (64) in rainbow trout (*Oncorhynchus mykiss*) and adjusted (6.3 nmol  $g^{-1}$  wet wt above background) for fathead minnow by Santore et al. (20). [Notably, recent direct determinations of 3 h  $LA_{50}$  for 96 h mortality in trout using radiotracer ( $^{64}Cu$ ) indicate that the true number may be much lower, around 0.2 nmol  $g^{-1}$  wet wt above background (84).] The  $LA_{50}$  for *Daphnia* sp. was obtained simply by fitting the fish-based BLM to available daphnid 48 h  $LC_{50}$  data sets while keeping the gill affinity constants the same as for fish.

In contrast, versions B and C of the model were based on new toxicity tests with *D. magna*, the affinity constants for the biotic ligand–cation complexes being estimated directly from the toxicity data.  $LA_{50}$  was not measured, so predictions were made based on fractional occupancy of the biotic ligand sites. In both versions B and C, the stability constants for Cu–inorganic and Cu–organic matter complexes were taken from Martell et al. (106) and the WHAM-V database (101), respectively, with calculations being performed in the original version A framework (20). Version D or the chronic Cu-BLM (103) was developed following the same approach as employed for versions B and C. The model constants for biotic ligand–cation complexes and fraction occupancy of the biotic ligand sites were derived using a large chronic data set (21-day  $EC_{50}$  for reproduction) across a wide range of water chemistry (taken from ref 104), and an additional experiment in which the individual effect of sodium on copper toxicity was investigated. Again, the stability constants for Cu–inorganic and Cu–organic matter complexes were employed from Martell et al. (106) and the WHAM-V database (101), respectively.

The primary differences between the three acute versions, discussed below, are as follows: (i) different affinity constants for some biotic ligand–cation complexes (Table 1); (ii) different assumptions about the forms of copper that may bind to the biotic ligand sites; (iii) different constants for inorganic copper complexes (Table 1); (iv) different assumptions with regard to the binding of  $Cu^{2+}$  by dissolved organic matter (DOM); and (v) different  $LA_{50}$  values for *Daphnia* sp.

First, a major difference between the three versions is that only versions B and C incorporate the protective effect of  $Mg^{2+}$  against acute copper toxicity and therefore have an affinity constant for biotic ligand– $Mg^{2+}$  complex unlike version A. Importantly, the affinity constant for the biotic ligand– $Cu^{2+}$  complex is considerably higher (0.62 log units) in versions B and C than in A (Table 1). Second, unlike version A, versions B and C interpret the increase in toxicity with increasing water pH as not so much due to decreasing  $H^+$  competition at the biotic ligand sites but rather to increased

**TABLE 1. Affinity Constants (log *K*) of BL–Cation and Inorganic Complexes Used in Different Versions of the Cu–BL Model<sup>a</sup>**

log <i>K</i>	version A (20, 92)	version B (102)	version C (100)	version D (103)
log <i>K</i> <sub>BL–Cu<sup>2+</sup></sub>	7.4	8.02	8.02	8.02
log <i>K</i> <sub>BL–CuOH<sup>+</sup></sub>	na <sup>b</sup>	7.45	7.45	8.02
log <i>K</i> <sub>BL–CuCO<sub>3</sub></sub>	na	na	7.01 <sup>c</sup>	7.44 <sup>c</sup>
log <i>K</i> <sub>BL–Ca<sup>2+</sup></sub>	3.6	3.47	3.47	na <sup>e</sup>
log <i>K</i> <sub>BL–Mg<sup>2+</sup></sub>	na <sup>d</sup>	3.58	3.58	na <sup>e</sup>
log <i>K</i> <sub>BL–Na<sup>+</sup></sub>	3.0	3.19	3.19	2.91
log <i>K</i> <sub>BL–H<sup>+</sup></sub>	5.4	5.4	5.4	6.67
log <i>K</i> <sub>CuOH<sup>+</sup></sub>	6.48	6.48	6.48	6.48
log <i>K</i> <sub>Cu(OH)<sub>2</sub></sub>	11.78	11.78	11.78	11.78
log <i>K</i> <sub>CuSO<sub>4</sub></sub>	2.36	2.36	2.36	2.36
log <i>K</i> <sub>CuCO<sub>3</sub><sup>2–</sup></sub>	6.55	6.77	6.77	6.77
log <i>K</i> <sub>Cu(CO<sub>3</sub>)<sub>2</sub></sub>	9.92	10.2	10.2	10.2
log <i>K</i> <sub>CuCl<sup>–</sup></sub>	0.4	0.4	0.4	0.4
log <i>K</i> <sub>CuHCO<sub>3</sub><sup>+</sup></sub>	14.32	12.13	12.13	12.13

<sup>a</sup> BL, biotic ligand; na, not available. <sup>b</sup> Version A considers that CuOH<sup>+</sup> does not bind to the biotic ligand. <sup>c</sup> Only versions C and D consider that CuCO<sub>3</sub> binds to the biotic ligand. <sup>d</sup> Version A does not consider the competition between Cu<sup>2+</sup> and Mg<sup>2+</sup> for the biotic ligand. <sup>e</sup> Version D does not incorporate the competition between Ca<sup>2+</sup> and Cu<sup>2+</sup> as well as Mg<sup>2+</sup> and Cu<sup>2+</sup> at the biotic ligand.

CuOH<sup>+</sup> formation that is bioavailable to these sites. Versions B and C therefore have an affinity constant for the biotic ligand–CuOH<sup>+</sup> complex. In addition, version C also allows binding of CuCO<sub>3</sub> to the biotic ligand of *D. magna* and has an affinity constant for the biotic ligand–CuCO<sub>3</sub> complex as well. However, the affinities of CuOH<sup>+</sup> and CuCO<sub>3</sub> for the biotic ligand are approximately 4–10-fold lower relative to Cu<sup>2+</sup> (Table 1). These adjustments have no mechanistic basis but better fit the observed toxicity data at high pH levels.

A third notable difference is that the stability constant for the formation of CuHCO<sub>3</sub><sup>+</sup> complex is much higher in version A as opposed to versions B and C (~100-fold; Table 1). According to simulations by Villavicencio et al. (107), this difference only yields importantly different copper speciation results and toxicity predictions at combinations of low DOM concentration (<1 mg of C L<sup>–1</sup>) and water pH between 7.0 and 8.5, where CuHCO<sub>3</sub><sup>+</sup> dominates copper speciation. Fourth, in version A, it is assumed that all DOM is reactive and consists of 10% humic acid and 90% fulvic acid. In contrast, versions B and C, based on the fitting of WHAM-V to measured Cu<sup>2+</sup> activities in the presence of natural dissolved organic matter (108), assume that only 50% of the DOM is reactive as fulvic acid and the other 50% is inert with respect to Cu binding. Version C furthermore evaluated Cu<sup>2+</sup>–DOM interactions in detail and, as a result, empirically modified the WHAM-V model by lowering the copper–proton exchange constant for humic acid to p*K* = 1.9 from p*K* = 1.5.

The final difference, in daphnid LA<sub>50</sub> values, is not one which necessarily affects the accuracy of prediction but rather influences how well the models duplicate reality. LA<sub>50</sub> values have never been directly measured in daphnids, so all result from manipulations of the original fish-based value [6.3 nmol g<sup>–1</sup> wet wt above background (20)]. The LA<sub>50</sub> values for daphnid species in version A (0.035–0.19 nmol g<sup>–1</sup> wet wt) are much lower relative to those which can be estimated from fractional occupancy in versions B and C (11.7 and 14.1 nmol g<sup>–1</sup> wet wt, respectively) for *D. magna*. This is because version A has a lower log *K*<sub>Cu<sup>2+</sup>–gill</sub>, it considers more of the DOM being reactive, and it calculates copper speciation with a higher log *K*<sub>CuHCO<sub>3</sub><sup>+</sup></sub>. The latter contributes to a lower concentration of free Cu<sup>2+</sup> in the media, which together with the lower log *K*<sub>Cu<sup>2+</sup>–gill</sub>, yields a lower estimated LA<sub>50</sub>. Interestingly, considering the total fish-based biotic ligand–Cu binding capacity of 30 nmol g<sup>–1</sup> wet wt, of which 10 nmol

g<sup>–1</sup> wet wt is endogenous background copper (92), it appears that version A predicts 50% mortality in *Daphnia* sp. when less than 1% of the total biotic ligand sites are occupied by copper, in other words, when copper binding is below background levels. In contrast, versions B and C predict 50% mortality in *D. magna* when 39–47% of the total biotic ligand sites are occupied by copper (100, 102). Realistically, it is difficult to conceive that such low occupancies of biotic ligand sites by copper, as predicted by version A, could result in such significant effects. However, it should be kept in mind that the adjustments of LA<sub>50</sub> values for daphnids from the LA<sub>50</sub> of fish in version A was a simple process for calibrating the BLM to address the differences in acute copper sensitivity between the two animals, in the absence of any specific information about the nature of the biotic ligands in the more sensitive daphnids. Depletion of internal Na<sup>+</sup> will occur at a faster rate in smaller animals (e.g., daphnids) as compared to large animals (e.g., fish) under acute copper exposures, even when the percent inhibition of Na<sup>+</sup> uptake (mechanism of acute copper toxicity) is the same, since smaller animals have higher Na<sup>+</sup> turnover rates (93, 94).

In contrast to the acute BLMs, no significant Ca<sup>2+</sup>, Mg<sup>2+</sup>, or combined competition effect was observed in the chronic Cu–BLM [version D (103), see also ref 104]. However, the affinity constants for Cu<sup>+</sup>–biotic ligand complex and the competitive effect of Na<sup>+</sup> in the chronic model are similar relative to the acute Cu–BLMs (versions B and C) (Table 1). In addition, an increase in proton competition and bioavailability of CuOH<sup>+</sup> and CuCO<sub>3</sub> complexes was observed (Table 1). It is important to note here that in the chronic model, the optimal percent FA assumption, derived from the estimation of different Cu<sup>+</sup> complexing capacities of different types of DOMs, was adopted instead of 50% reactive FA assumption used in versions B and C.

Version A of the Cu–BLM was calibrated against a comprehensive 96 h LC<sub>50</sub> data set for copper in fathead minnow (105). After fitting, the model predictions showed reasonably good agreement (within a factor of ± × 2) with the measured values, which varied from 10 to 1000 μg L<sup>–1</sup>, over a wide range of pH, water hardness (Ca<sup>2+</sup>:Mg<sup>2+</sup> = 2:1), Na<sup>+</sup> and DOM levels in synthetic water under static exposure conditions. Version A over-predicted LC<sub>50</sub> values only at high K<sup>+</sup> concentrations in the water, a factor that was not incorporated in the model. However, the model also over-predicted LC<sub>50</sub> values relative to measured LC<sub>50</sub> values in fathead minnow under flow-through exposures in Lake Superior water. This discrepancy was explained by the facts that it requires ≥24 h to achieve an equilibrium condition for copper speciation after mixing with natural organic matter (109), and Erickson et al. (105) allowed less than 1 h for equilibration in their flow-through exposures. It should be noted here that the current procedures for generating site-specific WERs do not require that toxicity tests be performed in such a way as to mimic any nonequilibrium conditions at a site. Moreover, the model predictions showed good correlation (± × 2) with measured values from WER studies using fathead minnow (96 h LC<sub>50</sub> values) and *D. pulex* (48 h LC<sub>50</sub> values) in two effluent-impacted field sites (110, 111).

Version B of the Cu–BLM was initially calibrated to a comprehensive 48 h EC<sub>50</sub> data set for *D. magna* (immobilization end point) in reconstituted water (with 24 h equilibration time) with a wide variation of pH, Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup> but constant low DOM levels (102) and, after fitting, predicted the data set extremely well (± × 1.5). However, version B was not evaluated with DOM variations, and furthermore, subsequent tests demonstrated that version B over-predicted EC<sub>50</sub> values at pH > 8 (100). This led to the adjustments outlined above which generated version C. With these changes, version C successfully predicted daphnid 48 h EC<sub>50</sub> values (± × 1.5) in 25 synthetic media used for model

calibration, including those with high pH and DOM levels (24 h equilibration). More importantly, it successfully predicted daphnid EC<sub>50</sub> values ( $\pm 2$ ) in 19 European natural waters, waters that had not been used in model calibration (100). In a recent study, Bossuyt et al. (112) used the version C of the acute Cu–BLM to predict 48 h EC<sub>50</sub> values in natural Cladoceran populations representing four different families and 11 different genera, both in laboratory reconstituted and natural surface waters. They reported that the model predicted 48 h EC<sub>50</sub> values for 27 of the 28 tested Cladoceran species within a factor of 2 of the observed values. However, the model was over-predictive for two acidic natural surface waters tested.

Very recently, Villavicencio et al. (107) evaluated all three versions against experimentally determined 48 h LC<sub>50</sub> values for three daphnid species in 35 natural waters of Chile with a wide range of pH, hardness, and DOM levels. Models were adjusted only for LA<sub>50</sub> values using the optimization procedure of Santore et al. (20). All three versions worked well (within  $\pm 2$  in 75%+ of cases), but rather surprisingly, the order of performance was version B > A > C. Gensemer et al. (113), working with *Ceriodaphnia dubia*, found good success with a slightly modified version A which had been “fitted” to literature toxicity data for this species and to which a log *K* for Mg<sup>2+</sup> binding to the biotic ligand was added, in predicting 48 h EC<sub>50</sub> values (agreement within  $\pm 2$  in all but one case) in synthetic water made up to unusually high hardness (>400 mg L<sup>-1</sup>). In contrast, Long et al. (114) had limited success (agreement in only 3 cases out of 10) with version A of the Cu–BLM in predicting copper toxicity to *D. magna* in synthetic waters of unusually low hardness (<50 mg L<sup>-1</sup>). On the basis of these results, it would be premature to recommend one Cu–BLM version over another for daphnids, though universally, all the BLMs worked better than the traditional “hardness equation” for copper (115). Clearly, version A is the only one validated for fish. An updated version of WHAM [model VI (116, 117)], which takes into account the few, higher affinity sites, will likely further improve the predictive capability of these BLMs. Recently, De Schamphelaere et al. (118) used a novel approach to address the differential protective effects of different types of DOM in the present acute Cu–BLM for daphnia. Using daphnid response as a “biological probe”, they derived a linear relationship between the UV absorbance coefficient at 350 nm ( $\epsilon_{350}$ ) and percent active fulvic acid (%AFA) using six different types of DOMs, collected from six different locations in Europe and North America, with a 6-fold difference between the lowest and highest in Cu<sup>+</sup> complexing ability among them. Linking this relationship to the version C of the acute Cu–BLM resulted in 90% of the predicted 48 h EC<sub>50</sub> values in daphnia, both in laboratory and natural surface waters, to be within a factor of 1.3 as opposed to a factor of 2 using the original version. This approach is certainly a very significant step toward making the current acute Cu–BLM approach applicable to a wide variety of natural aquatic environments. Additional supporting data suggesting that the UV absorbance coefficient may provide a convenient correction factor to describe DOM quality in acute Cu–BLM approaches for fish also has recently been produced by Luider et al. (119) and Ryan et al. (120). Interestingly, DOM quality appears to be much less important in chronic BLM approaches, at least with daphnids (103, 104).

The newly developed chronic Cu–BLM (version D) performed well in predicting 21-day EC<sub>50</sub> values and no-observed-effect concentrations (NOECs) on reproduction in daphnia in natural waters (103). Importantly, the prediction of the model did not appear to be appreciably altered by simultaneous dietary Cu exposure (121). About 79% of the toxicity threshold values were predicted within a factor of 2 of the observed values. However, the model had a number

**TABLE 2. Affinity Constants (log *K*) for BL–Cation Complexes Used in the Gill–Ag Binding Model and the Different Versions of the Acute Ag–BL Model<sup>a</sup>**

log <i>K</i>	gill–Ag binding model (61)	version A (124)	version B (99)	version C (125)
log <i>K</i> <sub>BL–Ag<sup>+</sup></sub>	10.0	7.3	7.6	8.88
log <i>K</i> <sub>BL–AgCl(aq)</sub>	na	6.7 <sup>b</sup>	na	na
log <i>K</i> <sub>BL–Na<sup>+</sup></sub>	4.7	2.3	2.9	2.9
log <i>K</i> <sub>BL–Ca<sup>2+</sup></sub>	3.3	2.3	2.3	2.3
log <i>K</i> <sub>BL–H<sup>+</sup></sub>	5.9	4.3	5.9	5.9
log <i>K</i> <sub>DOM–Ag<sup>+</sup></sub>	9.0	na <sup>c</sup>	9.0	7.5

<sup>a</sup> BL, biotic ligand; na, not available. <sup>b</sup> Only version A considers that AgCl(aq) binds to the biotic ligand. <sup>c</sup> Version A considers a different assumption for Ag<sup>+</sup>–DOM complexation (multiple binding site of DOM), see text for details.

of limitations discussed by the authors, and further research will be required to improve its predictive ability. Finally, it should be noted that there has also been one published attempt (122) to develop a chronic BLM for copper (based on growth inhibition) in the freshwater green alga *Pseudokirchneriella subcapitata*. This proved unsuccessful, primarily because of complications in interpreting the pH–toxicity relationship. Nevertheless, a multifactorial regression model was developed that worked reasonably well, constituting an important first advance for making chronic AWQC for copper, sensitive to site-specific conditions based on experimental data.

## Silver

The basic framework of the present acute Ag–BLM in aquatic animals originated from the gill–Ag binding model in rainbow trout developed by Janes and Playle (61) and later augmented by Schwartz and Playle (123). As yet there is no chronic Ag–BLM, but three versions of the acute Ag–BLM are available. Version A is the acute Ag–BLM developed in fish and later adopted for daphnids through downward adjustments of the LA<sub>50</sub> value (124). Version B is the physiologically based acute Ag–BLM for trout as described by McGeer et al. (99). Version C is the acute Ag–BLM for daphnids as proposed by Bury et al. (125).

Although conceptually all three versions of the model are quite similar to that of the gill–Ag binding model (61), the original affinity constants for gill–cation complexes derived by Janes and Playle (61) were found to be too high and thus over-predictive of acute silver toxicity in freshwater fish (99, 124). This is probably because in the experiments of Janes and Playle (61) not all of the silver bound to the gill, particularly Ag–thiosulfate species that probably contributed to total gill silver accumulation, may have resulted in toxicity (13, 39). As a result, a lower set of log *K* values for gill–cation complexes (summarized in Table 2) were adopted in all three versions of the acute Ag–BLM, derived primarily through the calibration of the models with available silver toxicity data. Major differences among three versions of the acute Ag–BLMs are as follows: (i) version B assumes that the actual sites of action for silver toxicity in fish gills are the Na<sup>+</sup>–K<sup>+</sup>–ATPase molecules that power Na<sup>+</sup> and Cl<sup>–</sup> uptake and relates acute silver toxicity to 85% inhibition of the gill Na<sup>+</sup>–K<sup>+</sup>–ATPase activity (a physiological endpoint associated with mortality; 126). In contrast, versions A and C, although recognizing the basic mechanism of silver toxicity (inhibition of Na<sup>+</sup> and Cl<sup>–</sup> uptake; 38, 40), relate acute toxicity to an assumed critical burden of silver on the biotic ligand (gill in fish); this has resulted in considerable differences in LA<sub>50</sub> values; (ii) while in all three versions the log *K*<sub>BL–Ag<sup>+</sup></sub> values have been adjusted downward from the original log *K* value of 10.0 for Ag<sup>+</sup> binding to the fish gill (61), the actual values differ considerably (Table 2); (iii) version A accounts for the



probable toxic effects of the neutral  $\text{AgCl(aq)}$  complex in a species-dependent manner and provides a log  $K$  value for  $\text{AgCl(aq)}$  binding to the gill epithelium in contrast to versions B and C (Table 2); (iv) version A employs WHAM-V (101) for incorporating  $\text{Ag}^+$ -DOM interactions and CHESS (98) for inorganic complexation reactions, whereas version B and C use the MINEQL+ database (127) for these purposes; and (v) like version A of the acute Cu-BLM, version A of the acute Ag-BLM employs a dual-binding site model (10% humic acid and 90% fulvic acid) for  $\text{Ag}^+$ -DOM interactions (101). In contrast, the other two versions use only a single ligand to account for  $\text{Ag}^+$ -DOM interactions, with a much lower log  $K_{\text{Ag}^+-\text{DOM}}$  value back-calculated from daphnid toxicity data (7.5, version C) than from gill-Ag binding database [9.0, version B, adopted from Janes and Playle (61); Table 2].

A shortcoming of version A recognized by its developers (124) is that it predicts acute toxicity of silver (96 h  $\text{LC}_{50}$ ) in fish based on an assumed critical gill silver burden, yet until recently, no measured relationship between short-term gill silver accumulation and 96 h percent mortality existed. This is a weakness in terms of biological reality, but not necessarily in terms of predictive accuracy. Silver accumulation in fish gills exhibited no relationship with toxicity when  $\text{Cl}^-$  was used as the modifying influence (126, 128). Furthermore, in several studies, gill silver burden appeared to be constantly changing over time, increasing and reaching a peak in the first few hours followed by a marked decrease with continued exposure (129–131). However, Morgan et al. (132) have now shown that this seemingly nonsaturating pattern of gill silver accumulation in juvenile trout results from the decreased bioavailability of silver under static exposures. In a separate but related study, Morgan and Wood (65) have demonstrated that short-term gill silver accumulation can indeed be correlated with percent mortality in trout at 96 h under flow-through exposure, as long as  $\text{Cl}^-$  is kept constant, thereby at least partially validating the assumptions of version A. However, the measured  $\text{LA}_{50}$  (1–2  $\text{nmol g}^{-1}$  wet wt) was only a fraction of the previously assumed  $\text{LA}_{50}$  [17  $\text{nmol g}^{-1}$  wet wt (124)]; the lower value seems more reasonable as it is close to the estimated number of  $\text{Ag}^+$  binding sites on  $\text{Na}^+$ - $\text{K}^+$ -ATPase molecules in the gills (1–4  $\text{nmol g}^{-1}$  wet wt), the presumed binding sites of toxicity (129). Furthermore, the estimated conditional log  $K_{\text{BL-Ag}^+}$  was 8.0, 5-fold higher than the originally fitted value of 7.3 in version A (Table 2), which had been less than log  $K_{\text{BL-Cu}^{2+}}$  (Table 1). In view of the generally accepted greater toxicity of  $\text{Ag}^+$  than  $\text{Cu}^{2+}$  (e.g., refs 33 and 72), this again seems reasonable.

In contrast, version B (99) does not predict acute toxicity in fish from the total gill silver burden but rather from quantifying the toxic mechanism (40), the percent inhibition of  $\text{Na}^+$ - $\text{K}^+$ -ATPase on the gill (thought to represent the key toxic sites) and therefore appears to be mechanistically more sound relative to version A. The log  $K_{\text{BL-Ag}^+}$  was estimated at 7.8 based on the concentration of  $\text{Ag}^+$  associated with 85% inhibition of branchial  $\text{Na}^+$ - $\text{K}^+$ -ATPase, which in turn was the degree of inhibition at the 96 h  $\text{LC}_{50}$  (129). This value was then adjusted downward to 7.6 (Table 2) based on fitting to experimental data (133). Recently, Bianchini and Wood (96) have demonstrated that inhibition of  $\text{Na}^+$  uptake by the blockade of  $\text{Na}^+$ - $\text{K}^+$ -ATPase is also the primary mechanism of acute silver toxicity in *D. magna* and derived a conditional log  $K_{\text{BL-Ag}^+}$  of 8.9 at key toxic sites on the biotic ligand, based on 90% inhibition of whole body  $\text{Na}^+$ - $\text{K}^+$ -ATPase at the 48 h  $\text{LC}_{50}$ . Whole body Ag accumulation was also directly measured, indicating an  $\text{LA}_{50}$  of 1–2  $\text{nmol g}^{-1}$  wet wt close to the  $\text{LA}_{50}$  for *D. magna* (2.3  $\text{nmol g}^{-1}$  wet wt) estimated in version A. Interestingly, this log  $K_{\text{BL-Ag}^+}$  is quite similar to that (8.88) derived in version C through the calibration of 48 h toxicity data for silver in daphnids by Bury et al. (125), who used an  $\text{LA}_{50}$  of 0.73  $\text{nmol g}^{-1}$  wet wt, thereby strongly

suggesting that version B could be extended from fish to daphnids mainly by changing the log  $K_{\text{BL-Ag}^+}$  at key toxic sites.

The role of  $\text{Cl}^-$  has been particularly troubling in the development of acute Ag-BLMs, because this moiety clearly protects trout against silver toxicity (126, 133–135) yet does not prevent silver accumulation on the gills (126, 128). Apparently the neutral  $\text{AgCl(aq)}$  complex, like Ag-thiosulfate complexes, can penetrate the epithelial cells (131) without inhibiting the  $\text{Na}^+$ - $\text{K}^+$ -ATPase (13, 126, 128). However  $\text{Cl}^-$  only slightly protects daphnids (125, 135, 136) and does not appear to protect fathead minnows at all (133, 135, 137). Version A therefore includes a conditional log  $K$  value for  $\text{AgCl(aq)}$  associated with toxicity in fathead minnow only, whereas this is not included in versions B and C (Table 2).

The other troublesome feature has been the protective role of DOM, which while widely agreed upon as the most important mitigating factor in silver toxicity, was dealt with by arbitrary adjustment of WHAM-V constants (101) for a two-site model in version A and assigned widely disparate single-site log  $K$  values for  $\text{Ag}^+$  binding in versions B (9.0) and C (7.5). Recent evidence suggests additional complexity may arise from the heterogeneous  $\text{Ag}^+$  binding characteristics of different DOMs (138).

Version A of the model (124) was calibrated and validated using the same data sets from fish (rainbow trout and fathead minnow; 133, 137) and *D. magna* (136), based on which it was originally developed. Not surprisingly, in all cases, the model satisfactorily predicted (within  $\pm \times 1.5$ –2) the variations in 96 h or 48 h  $\text{LC}_{50}$  values respectively due to different  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , and DOM levels. Version B was initially calibrated to eight trout toxicity data sets (133) and then evaluated over a wide range of water chemistries ( $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ , DOM) using an additional 23 toxicity measurements in trout from 10 different published studies. Approximately 55% of the data points fell within the reported error of measured  $\text{LC}_{50}$  values, and all but one point agreed with measured values (within  $\pm \times 2$ ). Similarly, Bury et al. (125) evaluated the predictive capabilities of version C by employing the toxicity data of *D. magna* and *D. pulex* at different  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ , and DOM levels in water from their own study as well as from Erickson et al. (137) and Karen et al. (135) and reported that more than 90% of the model predictions agreed with measured values (within  $\pm \times 2$ ). However, this daphnid model did not succeed in predicting acute toxicities for an amphipod, *Gammarus pulex*.

In summary, there remains considerable theoretical uncertainty in the present Ag-BLMs, though all three versions of the model show reasonably good success in predicting acute toxicity in laboratory waters. However, we note that none of these have been validated in the natural waters so far. In this regard, it will be important to modify the models to include stability constants for reduced sulfides that may play a major role in governing silver bioavailability in natural waters (139) but are generally absent from laboratory waters (140). These protective actions of sulfide have now been demonstrated in both *D. magna* (141, 142) and rainbow trout (143). Curiously, while highly protective against toxicity as might be predicted from a log  $K$  value around 13.6, in both cases, the presence of sulfide greatly augmented silver accumulation. It may also be important to include  $\text{Mg}^{2+}$  competition in certain desert and coastal freshwaters; log  $K_{\text{BL-Mg}^{2+}}$  has now been estimated for fish gill binding (123), though not for silver toxicity. Despite these uncertainties and gaps, the present acute Ag-BLM approach appears to be a far more reliable option for generating site-specific AWQC for silver relative to the current protocol of hardness-dependent adjustments (144). This is primarily because the real degree of protection by water hardness against acute silver toxicity in aquatic animals is negligible relative to other

**TABLE 3. Affinity Constants (log *K*) for BL–Cation Complexes Used in Different Versions of the Zn–BL Model<sup>a</sup>**

log <i>K</i>	version A (148)	version B (149)	version C (150)
log <i>K</i> <sub>BL–Zn<sup>2+</sup></sub>	5.50	5.31	5.50
log <i>K</i> <sub>BL–Ca<sup>2+</sup></sub>	4.80	3.34	3.6
log <i>K</i> <sub>BL–Na<sup>+</sup></sub>	na <sup>b</sup>	2.37	2.4
log <i>K</i> <sub>BL–Mg<sup>2+</sup></sub>	na <sup>b</sup>	3.12	3.1
log <i>K</i> <sub>BL–H<sup>+</sup></sub>	6.7	na <sup>c</sup>	6.3

<sup>a</sup> BL, biotic ligand; na, not available. <sup>b</sup> Version A does not consider the binding of Na<sup>+</sup> and Mg<sup>2+</sup> to the biotic ligand. <sup>c</sup> Version B does not incorporate the competition between H<sup>+</sup> and Zn<sup>2+</sup> at the biotic ligand.

factors. The acute toxicity of silver is largely related to the concentration of free Ag<sup>+</sup> ion in the media (134), for which the key determinants are Cl<sup>–</sup>, DOM, and reduced sulfide, and not the hardness cations Ca<sup>2+</sup> and Mg<sup>2+</sup> (145).

An important development that indicates the power and flexibility of the BLM approach is the recent work of Paquin et al. (146) integrating the BLM (version A) for rainbow trout with a physiologically based model of Na<sup>+</sup> homeostasis. By relating the formation of the Ag<sup>+</sup>–biotic ligand complex on fish gills to the inhibition of active Na<sup>+</sup> uptake, a sodium balance model (SBM) was derived that predicted the time required for plasma Na<sup>+</sup> stores to run down by 30%, the generally accepted threshold for fish death (38). The SBM successfully predicted survival time in a number of literature data sets. This approach holds great promise for the extension of the BLM to predict acute toxicity in new species depending on their Na<sup>+</sup> balance parameters (see also refs 93 and 94 for the interaction of body size here), to predict chronic toxicity, and to predict the outcome of pulse exposures. Furthermore, Zhou et al. (147) in a very recent study have studied the effects of Na<sup>+</sup>, Ca<sup>2+</sup>, Cl<sup>–</sup>, pH, and DOM on Ag<sup>+</sup> binding in reconstructed gill epithelia grown in primary culture from dispersed gill cells of freshwater rainbow trout (*O. mykiss*). With the exception of pH responses, all other effects were found to be qualitatively and quantitatively similar to in vivo BLM responses. These findings offer the exciting prospect of an in vitro BLM approach that may provide a simple and cost-effective way for evaluating the protective effects of site-specific waters.

## Zinc

The BLM approach for zinc is in a relatively less advanced state in comparison to copper and silver. At present, there are three versions. Version A is the acute Zn–BLM developed in fish and subsequently adopted for *D. magna* through downward adjustments of LA<sub>50</sub> (148), version B is the acute Zn–BLM for *D. magna* as described by Heijerick et al. (149), and version C is the chronic Zn–BLM for fish developed by De Schampelaere and Janssen (150). An unsuccessful BLM attempt to predict short-term Zn<sup>2+</sup> uptake by the green algae *Chlorella kessleri* was reported, though the experiments focused on fluxes rather than on metal binding or toxicity (151, 152).

In version A of the model, the measured strength of radiolabeled Zn<sup>2+</sup> binding in the gills of freshwater rainbow trout following 3 h exposure by Alsop and Wood (72) was slightly adjusted and then adopted as the affinity constant for biotic ligand–Zn<sup>2+</sup> complex (log *K*<sub>BL–Zn<sup>2+</sup></sub> = 5.5; Table 3). This value agreed reasonably well with earlier estimates of 5.1 by a similar gill binding approach (71) and 5.4 from 96 to 168 h LC<sub>50</sub> tests on trout in very soft water (153). However, the affinity constants for other gill–cation complexes (Ca<sup>2+</sup> and H<sup>+</sup>) were adopted from the gill–Cd binding model of Playle et al. (59, 60) and adjusted through the calibration to available gill–cadmium accumulation and toxicity data sets.

This adjustment was based on the assumption that the binding affinity of Ca<sup>2+</sup> and H<sup>+</sup> for the Cd<sup>2+</sup> and Zn<sup>2+</sup> binding sites at the biotic ligand should be similar since the latter are thought to share the same transport route in the fish gill, specifically the Ca<sup>2+</sup> uptake pathway (41–44, 46, 154, 155; Figure 1). The WHAM-V model (101) was used to incorporate the complexation of Zn<sup>2+</sup> with DOM and inorganic ligands, even though some inconsistencies in log *K* values for reactions of Zn<sup>2+</sup> with the latter were noted relative to other modeling frameworks. The origin of the LA<sub>50</sub> values (0.33–8.14 nmol g<sup>–1</sup> wet wt) used in version A is unclear; they were simply described as “calibrated to data sets”, with lower values being used for more sensitive organisms. Given that background levels of endogenous zinc in the fish gills are very high (~1000 nmol g<sup>–1</sup> wet wt; 72, 77), binding site numbers (*B*<sub>max</sub>) can only be measured directly by <sup>65</sup>Zn binding; the true 3 h values for *B*<sub>max</sub> in juvenile trout appears to be about 8.3–8.6 nmol g<sup>–1</sup> wet wt (71, 72).

In contrast, the affinity constants both for biotic ligand–Zn<sup>2+</sup> (log *K*<sub>BL–Zn<sup>2+</sup></sub> = 5.31, close to the version A value) and other biotic ligand–cation complexes in *D. magna* in version B were determined directly from toxicity data, a comprehensive 48 h EC<sub>50</sub> data set for zinc in *D. magna* (immobilization endpoint) in reconstituted water (with 24 h equilibration time). A wide variation of pH, Ca<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, and Na<sup>+</sup> but constant low DOM levels was employed, as described before in version B of the acute Cu–BLM (102). The WHAM-V model (101) was again used here to incorporate the complexation of Zn<sup>2+</sup> with organic and inorganic ligands, but DOM was not included as a variable in the battery of tests. All cations tested except K<sup>+</sup> and H<sup>+</sup> proved to be important in altering the 48 h EC<sub>50</sub>, so log *K* values were derived for Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup> competing at the biotic ligand. There appear to be inconsistencies in absolute values reported in various parts of the text of version B; values in Table 3 were taken from the summary table rather than the abstract or text of the original publication (149). As in De Schampelaere and Janssen (102), the model related toxicity to fractional occupancy of the biotic ligand sites, with the 48 h EC<sub>50</sub> occurring at 42% filled (absolute *B*<sub>max</sub> unstated).

The affinity constants for the chronic Zn–BLM (version C; 150) were derived following the same approach as employed in version B of the acute Zn–BLM and using a comprehensive 30 d chronic toxicity data set (end points: growth and survival) across a wide range of Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup> concentrations and pH in ion-poor soft water (Ca<sup>2+</sup>: 0.2 mM). The 30 d LA<sub>10</sub> and LA<sub>50</sub> values were estimated by incorporating the calculated log *K*<sub>Zn–BL</sub> and *B*<sub>max</sub>, 30 d LC<sub>50</sub> and LC<sub>10</sub>, water chemistry, and stability constants for the competing cations into the BLM software (windows version 1.0.0; Hydroqual, Mahwah, NJ). The stability constants for the inorganic Zn complexes in this model were adopted from Martell et al. (104). However, the effects of DOM on Zn toxicity were not taken into consideration in developing this model.

The main differences between the two versions of the acute model are as follows: (i) version A does not incorporate the competition of Mg<sup>2+</sup> and Na<sup>+</sup> ions with Zn<sup>2+</sup> for the biotic ligand and, therefore, assigns no affinity constants for them unlike version B (Table 3); (ii) version A considers that competition occurs between Zn<sup>2+</sup> and H<sup>+</sup> for the biotic ligand as opposed to version B (Table 3); and (iii) the assumed binding strength of Ca<sup>2+</sup> to the biotic ligand in version A is more than 100-fold greater than in version B (Table 3). These differences likely reflect differences in the origins of the model, version A from fish, and version B from daphnids. For example, Ca<sup>2+</sup> is effective while Mg<sup>2+</sup> is ineffective in protecting fish against acute zinc toxicity (156), whereas both protect in *D. magna* (149). Conversely, moderately low pH clearly protects fish (153, 157) but not daphnids (149). Interestingly, the chronic model (version C) for trout appeared



to be qualitatively similar to the version A of the acute Zn–BLM (fish-based model), with  $\text{Ca}^{2+}$  being the most protective cation against chronic Zn toxicity. The strength of the biotic ligand– $\text{Zn}^{2+}$  complexation is identical between the two versions, although the competitive effects of  $\text{Ca}^{2+}$  and  $\text{H}^+$  are slightly reduced in the chronic model (Table 3). Moreover, the competitive effects of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  are also similar between version C and version B (daphnid model) (Table 3).

Version A showed reasonable success in predicting acute toxicity in the literature data sets to which it was calibrated (particularly rainbow trout and fathead minnow) across a wide range of water hardness and pH; there was no independent validation. Although the overall trend of model predictions was similar to the observed  $\text{LC}_{50}$  values, version A either under- or over-predicted 96 h  $\text{LC}_{50}$  to fish at high pH ( $> 8.0$ ), probably because the solubility limit for zinc had been exceeded in the original tests at these pH values. It should also be noted that the water chemistry variables, particularly DOM levels, were not very well defined in the majority of the zinc toxicity studies used for the model validation, which possibly led to the incorrect estimation of  $\text{DOC–Zn}^{2+}$  complexation and thereby free  $\text{Zn}^{2+}$  ion estimation in the speciation calculations. In contrast, the version A predictions for *D. magna* corresponded with the measured 48 h  $\text{LC}_{50}$  values reasonably well, although the toxicity data available for this organism were very limited.

Heijerick et al. (149) evaluated the predictive capacity of version B using 17 toxicity data sets for *D. magna*, different from those used in the original calibration of the model, in synthetic media varying in pH (6.0–8.0), hardness (40–400  $\text{mg L}^{-1}$  as  $\text{CaCO}_3$ ), and DOM (2–40  $\text{mg L}^{-1}$  Aldrich humic acid). Overall, the model was able to predict zinc toxicity (48 h  $\text{EC}_{50}$ ) in all test media within a factor of 1.9, and about 88% of the predictions were within a factor of 1.3 from the observed values, despite the fact that DOM variation had not been employed in original model development.

At present, it appears that version A is preferable for fish and version B for daphnids, but for both acute models, further toxicity studies under well-defined water chemistry conditions are needed to confirm the effects of pH (especially at  $6.0 < \text{pH} < 8.0$ ), DOM, and  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  on acute zinc toxicity. Moreover, the predictive capability of both versions of the model has yet to be validated in natural waters.

The chronic Zn–BLM (150) showed reasonable success in predicting chronic effect (30 d  $\text{LC}_{50}$  and  $\text{LC}_{10}$ ) concentrations as most of the predicted values were within an error less than a factor of 2 of the observed values. However, further research should be carried out for further refinement of this preliminary chronic Zn model, particularly with respect to the effects of DOM on Zn toxicity and validation in natural waters. It should be noted here that BLM approaches have also been attempted to predict chronic zinc toxicity in the green algae *Pseudokirchneriella subcapitata* (72 h growth inhibition test; 158). Here, satisfactory multifactorial regression models were produced, but the basic BLM approach was unsuccessful. The BLM assumptions were apparently violated because the characteristics and numbers of the biotic ligand sites changed with pH. A surface response model for predicting chronic Zn toxicity in *D. magna* was also developed recently by Heijerick et al. (159), which showed reasonable success in predicting 21 d  $\text{EC}_{10}$  values and  $\text{EC}_{50}$  values for reproduction across a wide range of pH, hardness, and DOC levels in laboratory reconstituted water. From a regulatory point of view, these regression models constitute the first important advances in generating chronic AWQC for zinc toxicity in algae and daphnia based on experimental data that take into consideration site-specific water chemistry parameters.

**TABLE 4. Affinity Constants ( $\log K$ ) for BL–Cation and DOM–Cation Complexes Used in the Acute BL Model for Nickel and in the Gill Binding Models for Cadmium, Lead, and Cobalt<sup>a</sup>**

$\log K$	nickel (162)	cadmium (60)	lead (174)	cobalt (62)
$\log K_{\text{BL–M}^{2+}}$	4.0	8.6	6.0	5.1
$\log K_{\text{BL–Ca}^{2+}}$	4.0	5.0	4.0	4.7
$\log K_{\text{BL–Mg}^{2+}}$	na	na	4.0 <sup>b</sup>	na
$\log K_{\text{BL–Na}^+}$	3.0	na <sup>c</sup>	3.5	3.2
$\log K_{\text{BL–H}^+}$	7.5	6.7	4.0 <sup>d</sup>	6.2
$\log K_{\text{DOM–M}^{2+}}$	na <sup>e</sup>	7.4	8.4	5.1
$\log K_{\text{DOM–H}^+}$	na <sup>e</sup>	4.0	4.0	4.0
$\log K_{\text{DOM–Ca}^{2+}}$	na <sup>e</sup>	na	5.0 <sup>f</sup>	na

<sup>a</sup> BL, biotic ligand; M, metal; DOM, dissolved organic matter; na, not available. <sup>b</sup> Only the Pb–gill binding model (174) considers  $\text{Mg}^{2+}$  competition. <sup>c</sup> Cd–gill binding model (60) does not consider  $\text{Na}^+$  competition. <sup>d</sup> An increased value of 5.0 might be applicable for modeling other toxicity data sets (174). <sup>e</sup> Acute Ni–BLM (162) considers a different assumption for cation–DOM complexation (multiple binding site of DOM), see text for details. <sup>f</sup> Only the Pb–gill binding model (167) considers binding of  $\text{Ca}^{2+}$  to DOM.

## Nickel

At present, the acute Ni–BLM is at an early stage of development. The notion of extending the BLM approach to nickel originated from the demonstration by Meyer et al. (85) that 24 h gill–nickel accumulation was a constant predictor of acute toxicity (96 h  $\text{LC}_{50}$  values) in fathead minnow across a wide range of water hardness levels. This indicated that the fundamental principle of the BLM approach is applicable to nickel, even though recent evidence (160, 161) demonstrates that nickel is not a gill ionoregulatory toxicant in fish but rather a respiratory toxicant, in contrast to all other metals for which BLMs have been developed (33; Figure 1). The basic framework (affinity constants for gill–cation complexes as well as  $\text{LA}_{50}$ ; Table 4) of an acute Ni–BLM has been developed recently (162) based on the data provided by Meyer et al. (85). The affinity constants for  $\text{Ni}^{2+}$ –organic and  $\text{Ni}^{2+}$ –inorganic ligand complexation were adopted from WHAM-V (101). Notably, the  $\log K_{\text{BL–Ni}^{2+}}$  was low (4.0), and  $\text{LA}_{50}$  (240  $\text{nmol g}^{-1}$  wet wt) and  $B_{\text{max}}$  (1000  $\text{nmol g}^{-1}$  wet wt) in fathead minnows were both very high relative to values for other metals, reflecting the low acute toxicity of nickel relative to all other metals discussed here (Figure 2).

The predictive capacity of the provisional BLM was good for the Meyer et al. (85) data set on which it had been calibrated. However, it proved necessary to lower the  $\text{LA}_{50}$  values by 1 (28  $\text{nmol g}^{-1}$  wet wt) and 2 (3  $\text{nmol g}^{-1}$  wet wt) orders of magnitude respectively to satisfactorily predict two other fathead minnow data sets (163, 164) where hardness, pH, and DOM were variables. This approach was justified based on differences in age and size, with smaller fish being assumed more sensitive (94) to a postulated ionoregulatory toxicant. This phenomenon of markedly increasing nickel sensitivity with decreasing age and size was recently documented in fathead minnow by Hoang et al. (165). However, now it is known that nickel is actually a respiratory and not an ionoregulatory toxicant in fish (160, 161), so this probably reflects the higher weight-specific  $\text{O}_2$  demand of small fish. The nickel model was extended to daphnids with reasonable success by adjusting the  $\text{LA}_{50}$  value downward through the calibration of previously reported acute nickel toxicity data in *D. magna* and *C. dubia* ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and pH were the primary variables; 164, 166) and keeping all other parameters the same, the approach previously adopted in the version A's of the acute Cu–, Ag– and Zn–BLMs. The daphnid model was not evaluated on independent data sets.

A general phenomenon noted in this validation exercise was that the model over-predicted  $LC_{50}$  relative to measured values at high pH ( $>8.0$ ). Interestingly, applying the NIST database (167) instead of the WHAM-V database (101) for  $Ni^{2+}$ -inorganic ligand complexation greatly improved the predictive capacity of the model (both in fish and daphnids) at high pH although the discrepancy was not entirely eliminated. This is because the NIST database has a lower  $\log K$  value for the formation of  $NiCO_3$  species, the dominant nickel species at high pH ( $>8.0$ ), relative to the WHAM-V database [ $\log K_{NiCO_3}$ : 4.50 (NIST) and 5.78 (WHAM-V)] and therefore estimated higher free  $Ni^{2+}$  ion concentrations (the toxic nickel species) in the exposure water. This finding suggests that adoption of the NIST or MINEQL+ database ( $\log K_{NiCO_3}$ : 4.57) (67) for  $Ni^{2+}$ -inorganic ligand complexation could be an option for improving the predictive capability of the model.

Recently, a very detailed toxicity study on fathead minnow (165) allowed the development of a multiple regression model (with fish age, hardness, alkalinity, and DOM as variables) that satisfactorily predicted 96 h  $LC_{50}$  across a wide range of these variables. A provisional BLM (assumptions unstated) was also evaluated, but predicted  $LC_{50}$  values did not correspond very well with measured  $LC_{50}$  values at high or low alkalinity. Hoang et al. (165) suggested that this discrepancy could be resolved by considering  $NiCO_3$  to be bioavailable. Clearly, this is an option that should be investigated further since De Schamphelaere et al. (100) also resolved the discrepancy of toxicity predictions in the acute Cu-BLM (version C) at high water pH by rendering  $CuCO_3$  bioavailable to *D. magna*. In another recent study, Keithly et al. (168) used the provisional acute Ni-BLM constants (Table 4) but altered the  $\log K_{BL-H^+}$  (to 6.7) without explanation. They reported reasonably good agreement between the acute Ni-BLM predicted  $LC_{50}$  and measured 48 h  $LC_{50}$  values in both *D. magna* (data from ref 166) and *C. dubia* (data from their own study and 164), except for one data point in *C. dubia* corresponding with high pH (8.6). However, the authors noted that they were required to use a 2-fold higher  $LA_{50}$  value for *D. magna* and two different  $LA_{50}$  values for *C. dubia* (for modeling the two different data sets) that were 4–40-fold lower, relative to the  $LA_{50}$  value used in the original version of the model. Indeed they postulated that the lower  $LA_{50}$  value might be below endogenous background levels in uncontaminated daphnids. From the conceptual aspect of the present BLM approach, only one  $LA_{50}$  should exist for a given species and life stage, and the  $LA_{50}$  should be appreciably above background. However, it should be noted that the critical body burden for any metal (e.g.,  $LA_{50}$ ), even in the same life stage in daphnia, could vary from generation to generation due to interclonal differences (169) as well as acclimation to the waterborne (170) and simultaneous dietary metal exposures (121).

Toxicity data for nickel are far less extensive, for both fish and daphnids, than for other metals such as copper, silver, and zinc, probably because it is relatively less toxic (Figure 2) and was therefore considered to be not a metal of major environmental concern. Therefore, future research should focus on investigating the effects of various water chemistry parameters (particularly pH, alkalinity, DOM, and hardness), which will be very useful in refining the present model. The present version considers that hardness, pH, and alkalinity are far more important factors than DOM in determining bioavailability and toxicity of nickel. This is because the  $Ni^{2+}$  complexation capacity of organic matter is thought to approach saturation well before typical acute effect levels for nickel ( $0.1$ – $1.0$  mg  $L^{-1}$ ). However, it should be kept in mind that  $Ni^{2+}$ -DOM complexation may be an important factor in situations where effect levels are lower than  $<0.1$  mg  $L^{-1}$ . Moreover, Pane et al. (97) recently demonstrated

that the mechanism of acute toxicity in *D. magna* is ionoregulatory in nature (primarily due to the inhibition of  $Mg^{2+}$  uptake) and not respiratory as observed in fish (160, 161). This finding suggests that the  $Mg^{2+}$  component of hardness in the media could be a critical factor for nickel toxicity in daphnids (but probably not in fish) and should also be investigated. Overall, it is evident that more mechanistic information is needed to develop a technically sound acute Ni-BLM that will predict nickel toxicity over a wide range of water quality conditions. Nevertheless, progress to date suggests that the goal is achievable and provides encouragement to investigate the applicability of the BLM approach to other metals that are also respiratory toxicants in fish (e.g., Al; 33). It is important to note here that Gensemer and Playle (171) provided some preliminary modeling information on Al-gill interactions in fish gill, which could be useful for developing an acute Al-BLM in the future.

## Cadmium

To date, there is no published acute Cd-BLM, though extensive physiological research has established that it is a potent antagonist of  $Ca^{2+}$  uptake in fish gills (41–43, 78). However, the gill-Cd binding model for fish (fathead minnow), as proposed by Playle et al. (59, 60; Table 4), provides an excellent framework on which an acute Cd-BLM could be developed in future. In the absence of other information, it might be logical to take  $\log K$  values for competing cations from the acute Zn-BLM (148), based on similar mode of action and common uptake pathway of the two metals (154, 155; Figure 1). Indeed, Santore et al. (148) cite  $\log K$  values for an “unpublished” acute Cd-BLM, though the origin of added biotic ligand affinity constants for  $Na^+$  and  $Mg^{2+}$  [not measured by Playle et al. (59, 60)] was unstated. The original  $\log K$  values for gill-cation and  $Cd^{2+}$ -DOM complexation were derived using the measured gill-cadmium burden in fathead minnows exposed for 2–3 h to 0.05 mM of cadmium in the presence of different levels of  $Ca^{2+}$ , pH, or a variety of synthetic ligands of known binding strength (e.g., citrate, oxalate, glutamate) as well as natural DOM in ion-poor soft water (59, 60).  $B_{max}$  was about 2 nmol  $g^{-1}$  wet wt above background. The model considers that  $Ca^{2+}$  and  $H^+$  are the only cations that compete with the free  $Cd^{2+}$  ion for the binding sites at the fish gill, and the strength of  $Cd^{2+}$ -DOM binding is about 10 times weaker relative to that of gill- $Cd^{2+}$  binding. It is important to note here that a few recent Cd-gill binding studies (73, 74, 78, 79, 86) have reported  $\log K_{gill-Cd^{2+}}$  values (7.0–7.6) in rainbow trout which correspond to  $\geq 10$  times weaker affinity for  $Cd^{2+}$  to the fish gill relative to the gill-Cd binding model ( $\log K_{gill-Cd^{2+}} = 8.6$ ) of Playle et al. (59, 60). This difference could be due to the very low ionic concentration, particularly  $Ca^{2+}$ , in the exposure water used by Playle et al. (59, 60) as compared to all the other studies mentioned above, which probably produced very little competitive effect on  $Cd^{2+}$  for the binding at the gill and therefore higher affinity. Using a MINEQL+ modeling framework, Playle et al. (60) observed good agreement between measured and model-predicted gill-cadmium accumulation in synthetic laboratory water as well in natural waters of eight different lakes of Ontario, Canada. Using the same approach on rainbow trout, Hollis et al. (73, 74, 172) reported reasonable success in predicting the gill-cadmium accumulation but not in fish chronically exposed to waterborne cadmium.

Calibration of the present set of  $\log K$  values in the gill-Cd binding model with acute toxicity data sets in fish will be helpful in determining whether the present set of  $\log K$  values can be adopted or have to be modified for developing the future acute Cd-BLM. Although Playle et al. (60) reported a significant correlation between measured gill-cadmium accumulation and 96 h  $LC_{50}$  under different water chemistry

conditions, they did not determine the critical gill–cadmium burden that would be predictive of 50% mortality at 96 h (LA<sub>50</sub>). In this regard, Niyogi et al. (86) reported fairly low 3 h and 24 h LA<sub>50</sub> values (0.34–0.55 nmol g<sup>-1</sup> wet wt above background) predictive of 96 h LC<sub>50</sub> in rainbow trout. These corresponded to about 40% saturation of the high-affinity, low-capacity Cd<sup>2+</sup> binding sites in the gills of trout, indicating that the log  $K_{\text{gill}-\text{Cd}^{2+}}$  for binding corresponded very well with the log  $K_{\text{BL}-\text{Cd}^{2+}}$  for toxicity, though both were about 7.0. This is a significant step forward in the development of a provisional acute Cd–BLM.

From the regulatory angle, more research is required, particularly with respect to different water chemistry variables, to develop a technically reliable acute Cd–BLM to generate site-specific AWQC for cadmium in a wide variety of natural waters. In surveying different studies, Hollis et al. (78) noted that rearing conditions [water hardness of acclimation, diet; see also Niyogi and Wood (2)] had a large effect on gill–Cd<sup>2+</sup> binding parameters for trout (log  $K_{\text{gill}-\text{Cd}^{2+}}$ ,  $B_{\text{max}}$ ). Playle et al. (59, 60) studied the effects of only Ca<sup>2+</sup>, pH (4.8–6.3), and DOM on short-term gill–cadmium accumulation in developing the gill–Cd binding model (Table 4). Other important water chemistry factors such as alkalinity, Mg<sup>2+</sup> and Na<sup>+</sup> levels, and pH (>8.0) could have significant (yet unidentified) effects on gill–Cd<sup>2+</sup> binding and subsequently on acute cadmium toxicity and, therefore, could have important implications for the acute Cd–BLM. Moreover, efforts should be undertaken to extend the acute Cd–BLM approach to sensitive freshwater invertebrates such as daphnids to increase its applicability.

## Lead

At present there is no published acute BLM for lead. Recent research on fish suggests that Pb<sup>2+</sup> is primarily an antagonist of active Ca<sup>2+</sup> uptake like Zn<sup>2+</sup> and Cd<sup>2+</sup> (Figure 1), though the picture is complicated by additional effects on Na<sup>+</sup> and Cl<sup>-</sup> regulation (50, 173). McDonald et al. (174) recently proposed a gill–Pb binding model in rainbow trout that provides a basic framework for developing a future acute Pb–BLM. The log  $K$  values for gill–cation and DOM–cation complexation in this model (Table 4) were derived primarily from measured gill–lead accumulation data in rainbow trout exposed to 0.6–1.0 mM lead for 3 h in the presence of variable levels of competing cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>) or complexing ligands (citrate, ethylenediamine, and DOM) in ion-poor soft water. The  $B_{\text{max}}$  was 13.8 nmol g<sup>-1</sup> wet wt above background. The model considers that the free Pb<sup>2+</sup> ion competes with other cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, and H<sup>+</sup>) for the binding sites at the fish gill with  $\geq 100$  times greater affinity relative to other cations (Figure 1). The acute toxic mechanism of lead was largely unknown when this model was developed, so it is reassuring that the toxic action turned out to be inhibition of active Ca<sup>2+</sup>, Na<sup>+</sup>, and Cl<sup>-</sup> uptake at the gills (50, 173), thereby corroborating the basic assumptions in the model that Pb<sup>2+</sup> is capable of out-competing Ca<sup>2+</sup> and Na<sup>+</sup> for ion transport sites at the fish gill (Figure 1).

The log  $K_{\text{gill}-\text{Pb}^{2+}}$  estimated by McDonald et al. (174) was 6.0 (Table 4), greater than that for Zn<sup>2+</sup> (Table 3) but less than that for Cd<sup>2+</sup> (Table 4), in accord with the relative toxicities of the three metals (Figure 2). Rogers and Wood (50) estimated a comparable inhibitor constant ( $K_i = 6.3$ ) for the inhibition of active Ca<sup>2+</sup> uptake by Pb<sup>2+</sup>. Interestingly, Slaveykova and Wilkinson (175) reported that Ca<sup>2+</sup> competitively inhibits Pb<sup>2+</sup> uptake in unicellular algae, *Chlorella vulgaris*, as observed in fish (50). They derived log  $K$  values for Pb<sup>2+</sup> (5.5) and Ca<sup>2+</sup> (4.67) interactions with the transport sites (biotic ligand) in the algae, which are quite comparable to the values (6.0 and 4.0, respectively) obtained by McDonald et al. (174) in rainbow trout. This suggests that Pb<sup>2+</sup> binding sites at the biotic ligand are probably qualitatively similar in

nature irrespective of the aquatic organisms. However, Hassler et al. (152) later reported that Pb<sup>2+</sup> uptake data for *C. vulgaris* in the presence of competing Ca<sup>2+</sup> could not be simulated in a BLM framework, though the focus of their experiments was on flux rates rather than binding or toxicity.

McDonald et al. (174) reported good agreement between predicted and measured gill lead burdens across a wide concentration range of competing cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup>) and complexing ligands (citrate, ethylenediamine, and Aldrich humic acid), by incorporating the log  $K$  values for competition and complexation reactions of the model into the MINEQL+ modeling framework. They also simulated gill lead accumulation across a broad range of water pH (4–9) and assumed that accumulation would decrease at low (<6.0) and high (>7.0) pH due to increased competition with H<sup>+</sup> and complexation by OH<sup>-</sup> and CO<sub>3</sub><sup>-</sup>, respectively. The model was then validated by successful double-blind correlation ( $r = -0.86$ ) of predicted gill lead burden with LT<sub>50</sub> values (median lethal time) for trout exposed to 3.9 mM lead over 1 week in 19 natural water samples varying greatly in all parameters. Interestingly, they observed strong positive correlations of water Ca<sup>2+</sup> and Mg<sup>2+</sup> levels with measured LT<sub>50</sub> values, as well as a pH-dependent (range: 6.5–8.5) increase of LT<sub>50</sub>, thereby confirming the assumptions of the model. However, they did not experimentally assess pH values  $\leq 6.5$ , but speculated that a log  $K_{\text{gill}-\text{H}^{+}}$  (5.0) higher than that actually used (4.0; Table 4) might be required to explain other data sets.

The present gill–Pb binding model (174) considers that Pb<sup>2+</sup> has about >100 times greater affinity for DOM than does the fish gill (Table 4). Furthermore, the model also takes into consideration Ca<sup>2+</sup> competition in addition to Pb<sup>2+</sup> and H<sup>+</sup> for the binding sites on the organic ligand (DOM). Here in addition to their usual approach of estimating DOM–metal complexation based only on the assumption of single binding sites for DOM, Playle and co-workers adopted a novel approach which took into account DOM quality as indicated by a simple measure of DOM aromaticity (the specific absorbance coefficient) and observed an even better fit with the measured LT<sub>50</sub> values. Overall, these findings suggest that the present gill–Pb binding model has great potential to simulate the influence of wide variations of water chemistry variables on both gill–lead accumulation and acute toxicity of lead in fish in natural waters. However, to transform it into an acute BLM predictive of AWQC, there is a need for more toxicity testing in the form of LC<sub>50</sub> tests so as to quantify the critical gill–lead burdens (LA<sub>50</sub>), which could be used as a predictor of acute lead toxicity in any given water chemistry. There is also a clear need to extend these tests to model aquatic invertebrates such as daphnids.

## Cobalt

As for lead and cadmium, at present there is no published acute Co–BLM, but there is a gill–Co binding model in rainbow trout developed by Richards and Playle (62). The log  $K$  values for gill–cation and DOM–cation complexation in this model (see Table 4) were derived primarily using the gill–cobalt accumulation data in fish exposed to 7.5  $\mu\text{M}$  cobalt in ion-poor soft water for 2–3 h in the presence of variable levels of competing cations (Ca<sup>2+</sup> and Na<sup>+</sup>) and complexing ligands (nitrotriacetic acid and DOM) and a range of pH values (4.2–10.1).  $B_{\text{max}}$  was relatively high, about 88 nmol g<sup>-1</sup> wet wt above background, and the associated log  $K_{\text{gill}-\text{Co}^{2+}}$  value was 5.1. This indicates that Co<sup>2+</sup> binds to gill sites >1000 times more weakly than Cd<sup>2+</sup>, 10 times more weakly than Pb<sup>2+</sup>, and about 6 times more weakly than Zn<sup>2+</sup>; however, the binding strengths of Ca<sup>2+</sup> and H<sup>+</sup> at the same sites are more or less similar for the three metals (Tables 3 and 4). This is reasonable since increased Ca<sup>2+</sup> levels in the water decrease Co<sup>2+</sup> uptake and toxicity in fish (48, 176) as for the



other metals, and mechanistic analysis suggests that  $\text{Co}^{2+}$  is taken up primarily via the active  $\text{Ca}^{2+}$  transport pathway (49; Figure 1). The model also assumes that  $\text{Co}^{2+}$  has similar affinity for binding with DOM ( $\log K = 5.1$ ) as for binding to the fish gill, rather different from the situation with more toxic  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$ , where the greater affinity for DOM will play a greater protective role (Table 4).

After fitting the model to the experimental data using the MINEQL+ modeling framework, Richards and Playle (62) reported reasonable success in predicting gill–cobalt burdens in laboratory waters. More importantly, they were able to predict an absence of gill–cobalt accumulation in natural waters from nine different water bodies across Ontario, Canada, spanning a wide range of  $\text{Ca}^{2+}$  and  $\text{Na}^+$ , pH (4.2–7.6), and DOM levels. Overall, the model analysis indicated that  $\text{Ca}^{2+}$  competition and DOM complexation were the most important factors preventing  $\text{Co}^{2+}$  from binding at the gills in these natural water tests. Presumably the same would have been true had acute cobalt toxicity been measured. However, the model significantly under-predicted the gill–cobalt accumulations at the highly alkaline range (pH >9) in the laboratory soft water. Considering the fact that all cobalt in the media was in dissolved form, this finding raised the possibility of  $\text{CoOH}^+$  and/or  $\text{CoCO}_3(\text{aq})$  (the two dominant cobalt species under the existing conditions) also binding to the gill sites.

While this gill–Co binding model provides a basic framework, the same requirements as for the gill–Pb binding model will be needed to transform it into a technically sound BLM that can be used for environmental regulation. The most important area of future focus should be to correlate the model-simulated influence of water chemistry on gill–cobalt accumulations with measured acute toxicity (96 h  $\text{LC}_{50}$ ) in fish under well-defined water chemistry so as to quantify the critical gill cobalt burdens ( $\text{LA}_{50}$ ). Additional water chemistry variables such as alkalinity and  $\text{Mg}^{2+}$  should be investigated in this context. The approach should then be extended to model aquatic invertebrates such as daphnids.

## Future Directions and Recommendations

The scientific underpinnings of the present BLM approach are sound. The mechanistic framework that the model provides for predicting bioavailability and acute toxicity of metals in aquatic environments will lead to significant improvements over current regulatory protocols such as hardness-based adjustments or WERs. The empirical validations performed so far appear very promising, and are important steps toward establishing the BLM concept as a geochemically and biologically robust approach for incorporating bioavailability concepts into AWQC. Furthermore, the BLM can serve as a valuable predictive tool in environmental toxicology (i.e., to determine which experiments and which water quality variables are likely to be most important). In addition, to the further development needs for each metal-specific BLM that we have suggested above, further research or modifications of current approaches are required to enhance the potential of the BLM concept as a practical tool for site-specific ecological risk assessment for metals in the natural waters.

A fundamental principle of the BLM approach is to relate acute toxicity to the critical metal accumulation at the biotic ligand (i.e.,  $\text{LA}_{50}$ ). For mechanistic understanding, this remains essential, as well as for biological and geochemical correctness because this process defines the receptor site density. Operationally, in geochemical modeling the same endpoint can be achieved by a high  $\log K$  and a low  $\text{LA}_{50}$ , or by the opposite combination, but only one is biologically correct. However, in practice the actual measurement of this critical burden in the organism has proven difficult, and most reported  $\text{LA}_{50}$  values are probably just surrogate values. It is

now clear that a portion of the measured burdens on the apparent biotic ligand may be in nontoxic form and/or at nontoxic site of action. For example, silver complexed with chloride or thiosulfate may accumulate on the gill, yet may not cause acute toxicity in fish (129). Similarly, there are  $\text{Na}^+$ -sensitive (toxic site of action) as well as  $\text{Na}^+$ -insensitive (nontoxic site of action) components of the high-affinity Cu binding sites at the fish gill (70). The true  $\text{LA}_{50}$  is not necessarily a gill metal burden on a fish or a whole body metal burden in a daphnid but rather the actual amount of metal bound to a “receptor” protein such as an ionoregulatory enzyme or an ion channel. Therefore, incorporation of the true metal binding properties ( $B_{\text{max}}$ ,  $\log K_{\text{BL-Me}}$ ,  $\text{LA}_{50}$ ) of the key toxic site (e.g.,  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  for  $\text{Na}^+$  antagonists) instead of the gill as a whole, into the present acute BLMs remains an important and as yet unachieved goal. However a significant intermediate step is to quantify the functional inhibition of the protein in question, as demonstrated by McGeer et al. (99) in version B of the acute Ag–BLM, or its immediate consequence, as in the SBM of Paquin et al. (146). This approach could certainly improve current BLMs for other metals (copper, cadmium, zinc, cobalt, and lead) where key toxic sites of interference are now known or are strongly suspected. Future research should also focus on developing the in vitro BLMs as a practical tool for simple and cost-effective way for generating site-specific AWQC. The preliminary in vitro Ag–BLM (147) shows great promise and the same approach should be employed for developing in vitro BLMs for other metals as well.

This quest for mechanistic and geochemical accuracy need not and should not impede the regulatory implementation of BLMs, which work well despite an absence of information on critical metal burden. In practice, models calibrated directly to toxicity (which is the end point of regulatory interest) can be as effective as those which employ a measured  $\text{LA}_{50}$ . The single most important criterion for acceptance of a model should be its validation on natural water data sets different from those which were used to calibrate the model in the first place. Version C of the acute Cu–BLM (100) and version B of the zinc–BLM (149) are two good examples of toxicity-based BLMs that meet this standard.

The process of recalibrating fish based models to more sensitive invertebrates simply by reducing  $\text{LA}_{50}$  without altering the  $\log K$  values is questionable, irrespective of this issue as to the true  $\text{LA}_{50}$ . Toxic mechanisms, and therefore the nature of the toxic sites, may be fundamentally different. For example, nickel acts by a respiratory mechanism in fish (160, 161) but by an ionoregulatory mechanism, inhibition of active  $\text{Mg}^{2+}$  uptake, in daphnids (97), so  $\log K$  values for protective cations will probably differ. Buffering conditions for pH at the gill surface may also differ substantially between these very different organisms, changing the effective  $\log K_{\text{BL-H}^+}$  or even metal speciation (63). Furthermore, there is accumulating evidence that the protective effects of two major hardness cations differ between fish [where  $\text{Ca}^{2+}$  is more effective, probably because it regulates the permeability and stability of membrane proteins in the fish gills (53, 177, 178)], and daphnids [where  $\text{Mg}^{2+}$  appears to have equal or even greater protective effects than  $\text{Ca}^{2+}$  (16–18, 102, 113, 149)]. BLM constants should be worked out directly by experimentation on the organisms of interest.

In this regard, it is noteworthy that the present set of acute BLMs have been developed based only on studies with two fish (rainbow trout, fathead minnow) and assorted daphnids. There is a need for research to extend the BLM approach to a much wider range of freshwater organisms, including other families of fish, other invertebrates both pelagic and benthic, and representative algae. The goal here is 3-fold: (i) to determine the species-sensitivity distributions of BLM predictions for regulatory purposes [analogous to

those classically compiled as hardness-adjusted  $LC_{50}$  values (10, 11, 115, 144); (ii) to determine general principles by which BLMs can be recalibrated between related species (e.g., refs 84 and 86); and (iii) to detect those classes of organisms where BLM principles and/or constants fundamentally differ. For example, limitations of the present acute BLM approach in freshwater algae have now been reported (122, 151, 152, 158, 179). All these studies suggest that physiology of the biotic ligands in algae is probably somewhat different relative to fish and daphnids, perhaps related to oxygen generation and carbon dioxide consumption in plants versus oxygen consumption and carbon dioxide production in animals. There is also a need to extending the present BLM approach to the marine and estuarine environment where ion transport mechanisms (and therefore biotic ligands) and metal speciation are fundamentally different.

A fundamental but usually unstated assumption of the present BLM approach is that the properties of the biotic ligand are unchangeable regardless of water chemistry conditions. However, ion transport and permeability characteristics of the fish gill are known to be sensitive to water chemistry of acclimation, particularly water hardness and pH (e.g., refs 56, 57, 180, and 181). There is now ample evidence that these factors as well as chronic sublethal preexposure to the waterborne metal itself, dietary metal levels, dietary ions (2), and organism age (e.g., ref 165) can all lead to large changes in the metal binding properties ( $\log K$ ,  $B_{max}$ ,  $LA_{50}$ ) of the biotic ligand and significantly alter the toxicological sensitivity of the fish (see ref 2 for a review). Decreased sensitivity with acclimation to chronic metal exposures has been observed in daphnids as well (170, 182). These findings demonstrate that the properties of the biotic ligand are dynamic rather than fixed. If the goal is to make site-specific AWQC, these factors should be incorporated—i.e., regulations should be based on the BLM for the most sensitive life stages of organisms that occur in the area, acclimated to typical water chemistry, background metal concentrations, and diet at the site in question.

DOM character also tends to be site-specific, and one of the greatest areas of present uncertainty is how to deal with DOMs in current acute BLMs, let alone different DOMs. Given that the concentration range of interest for specific metals is limited for predicting acute toxicity, there exists no convincing evidence that multiple site models with multiple  $\log K$  values do any better than single site models (or vice versa) for dealing with DOM in acute BLMs. The more important need at present is to derive adjustment factors for the very different protective abilities of different types of DOM. Measurements of color (specific absorbance; 118–120, 174, 183) and reduced sulfide content (184) hold promise in this regard, though much more research is needed. Furthermore the action of reduced sulfides in oxic waters (either inside or outside DOM) in preventing toxicity has so far only been quantified directly for silver (140–143); this may prove to be an important protective factor that should be incorporated into acute BLMs for other metals.

Present acute BLMs have been developed so far for individual metals, but metals usually exist in mixtures in contaminated aquatic environments and are known to interact with each other (e.g., refs 185–187). The basic principles of the BLM approach appear to be ideally suited to analyze such phenomena, because once common sites of binding are identified (Figure 1), the biotic ligand constants can be used to predict the outcome of metal interactions. Metals interacting at common sites should follow principles of concentration additivity, and those acting at different sites should exhibit effects additivity (187, 188). Playle (189) recently simulated the former using two-to-six metal scenarios (silver, copper, cadmium, cobalt, lead and zinc) by combining  $\log K$  values of respective gill binding models

into the MINEQL+ V-4.5 framework (67) and using the toxic unit concept (188). The model simulations yielded greater than strict additivity at low metal concentrations, strict additivity at intermediate metal concentrations, and less than strict additivity at high metal levels, independent of metal combinations. Interestingly, Newman and McCloskey (190) reached similar conclusions based on their review of previously published literature dealing with acute toxic effects of metal mixtures on aquatic animals. Moreover, multiple metal–gill models behaved predictably against alterations of water pH and  $Ca^{2+}$ , although not against alteration of DOM. However, it should be noted that Playle (189) adopted a simplified assumption for simulation purposes—that both  $Ca^{2+}$  antagonists ( $Cd^{2+}$ ,  $Zn^{2+}$ ,  $Pb^{2+}$ , and  $Co^{2+}$ ) and  $Na^{+}$  antagonists ( $Cu^{+}$  and  $Ag^{+}$ ) were competing for the same binding sites (concentration additivity only). This not a true reflection of biological reality (Figure 1) where effects additivity should apply between the two sites. Nevertheless, it is a good beginning, and future research should assess the advantages and limitations of the BLM approach in assessing the toxicity of metal mixtures.

Finally, in many jurisdictions, AWQC are designed to protect against chronic toxicity, not just acute toxicity, so as to provide lifetime protection to the resident fauna. There is an urgent need for more research to derive BLMs that predict chronic toxicity in site-specific waters. It would be unfortunate if regulatory authorities apply the “acute-to-chronic ratio” (ACR), as done traditionally in hardness-based criteria (11) to acute BLM predictions to generate chronic AWQC. The strength of the BLM is its mechanistic foundation, and only in cases where the mechanism of chronic toxicity is the same as that of acute toxicity will the approach be valid. Already, a chronic Cu–BLM for daphnia has been developed very recently and is showing reasonable success in predicting chronic reproductive effects (103). Interestingly, the protective actions of  $Na^{+}$  seemed to be similar in the chronic model and the acute model, suggesting that the toxic mechanisms of acute and chronic copper toxicity are similar in nature. Moreover, the predictions of the chronic model did not seem to be altered by simultaneous dietary copper exposure (121). Furthermore, a chronic Zn–BLM has also been developed for fish (150), and its similarity to the acute Zn–BLM (148) in the dominant protective role of  $Ca^{2+}$  in predicting mortality (the most sensitive endpoint in the chronic history) again suggest that the acute and chronic toxic mechanisms of zinc in fish are similar (i.e., disruption of  $Ca^{2+}$  homeostasis). In case of silver also, the mechanism does appear similar ( $Na^{+}$  balance pathology; 191). However, the protective actions of some factors ( $Ca^{2+}$ ,  $Mg^{2+}$ , DOM, pH, and  $Cl^{-}$ ), which are effective against acute toxicity, are either lessened or non-existent during chronic exposure for copper and silver (103, 104, 192, 193). This may well be because the equilibrium assumptions of the present BLM are violated during prolonged exposures, pointing to a need to incorporate kinetic adjustments and to avoid ACR approach. Regardless, chronic BLMs should be based on chronic tests that, with skill and care, will be as useful as the present acute BLMs based on acute toxicity tests.

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