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Characterizing Dissolved Cu and Cd Uptake in Terms of the Biotic Ligand and Biodynamics Using Enriched Stable Isotopes

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The biotic ligand model considers the biological and geochemical complexities that affect metal exposure. It relates toxicity to the fraction of physiological active sites impacted by reactive metal species. The biodynamic model is a complementary construct that predicts bioaccumulation and assumes that toxicity occurs when influx rates exceed rates of loss and detoxification. In this paper we presume that metal influx rates are mechanistically the resulting processes that characterize transmembrane transport. We use enriched stable isotopes to characterize, both in terms of the biotic ligand and biodynamics, dissolved metal uptake by a freshwater snail at water hardness varying up to 180-fold. Upon 24 h exposure, metal uptake was linear over a range encompassing most environmental concentrations; although saturation kinetics were observed at higher concentrations. Cadmium influx rates correlate with changes in the affinity of the biotic ligand, whereas those of Cu correlate with changes in both site affinity and capacity. A relationship between metal influx rate and ligand character asks whether toxicity is the result of accumulation at the biotic ligand or the rate at which metal is transported by that ligand.

Introduction

The risk of metal toxicity is linked to exposure, but the nature of that linkage is a subject of discussion. The biotic ligand model (BLM) brings metal speciation in solution and key biological processes into estimates of bioavailability and toxicity (*1*, *2*). Specifically, the BLM incorporates complexation together with competition of the free metal ions with environmental ligands for physiological sites of action (biotic ligand). According to the BLM, toxicity occurs when a critical amount of metal is bound to sites of toxic action on the organism. Toxicity also correlates with the biotic ligand affinity to bind a particular metal (*3*).

The biodynamic model is a complementary construct that predicts bioaccumulation from the balance of fluxes between uptake and loss rates under specific geochemical conditions (*4*−*6*). Although the model does not directly establish the link between bioaccumulation and toxicity, it assumes that toxicity occurs when influx rates exceed combined rates of loss and detoxification (*7*). The physiological processes controlling the inward and outward fluxes are quantified by species-specific and metal-specific rate constants empirically derived under different environmental conditions (e.g., speciation) (*8*).

The BLM and the biodynamic model have physiological frameworks, rooted in principles derived from enzyme kinetics and membrane transport of nutrients and major ions. Both approaches assume that very short exposures can be used to define metal uptake characteristics that eventually can influence toxicity. Proponents of both models call for the expansion of their approach to a variety of species to better develop general principles (*3*, *8*). Although the two models link exposure to metal toxicity differently; this does not seem a subject of much discussion. In fact, the two models seem to exist in different worlds; both literatures largely ignore the other. In this paper we address that omission. Specifically, we characterize copper (Cu) and cadmium (Cd) uptake both in terms of the biotic ligand and biodynamics by a freshwater gastropod.

The common pond snail *Lymnaea stagnalis* is a good biomonitor for metal pollution given its capacity to accumulate metals, its suitable size for metal analysis and its wide distribution (*9*). The species has been used to investigate the effects of toxic substances on physiological processes (e.g., ref *9*), but its bioaccumulation characteristics are not characterized. Here we presume that metal influx rates are mechanistically the result of processes that characterize membrane transport, i.e., the affinity of each transport site on the membrane for a metal and the number of transport sites. We use a novel isotope tracer approach to quantify Cu and Cd unidirectional influx rates from solution (*10*). We expose snails to various concentrations of enriched stable ^{65}Cu and ^{106}Cd isotopes for 24 h at water hardness varying up to 180-fold. We use simplified calculations to determine the amounts of accumulated tracers in each organism (rather than in pooled samples) and thus take advantage of the sensitive detection limits of the methodology.

Radiolabel tracers are the traditional way to sensitively measure changes in ion fluxes in and out of an organism. For example, ^{45}Ca has been used to measure unidirectional influx kinetics in rainbow trout gills (*11*), and zinc accumulation in the gill of juvenile rainbow trout has been determined using ^{65}Zn (*12*). Gill-binding characteristics for metals, including Cd (*13*), Cu (*13*), Ag (*14*), Pb (*15*), and Co (*16*), are also determined using non-radioactive metals. But large background metal concentrations must be subtracted from small increases in concentration in the exposed organisms or tissues. This reduces the sensitivity and precision of uptake estimates, requiring relatively high exposure concentrations to detect differences, especially for essential elements like Cu and Zn (*12*, *17*). Recently, enriched stable isotope methodology was used to trace Cu biodynamics and to delineate accumulation pathways in a freshwater bivalve (*10*, *18*). This methodology directly traces unidirectional fluxes without the handling issues of radioisotopes, the insensitiveness that result from the need to account for pre-existing metal concentration (e.g., refs *13*, *17*) or the use of elevated exposure concentrations (e.g., ref *12*).

Materials and Methods

Exposure Media. Acute waterborne exposures to ^{65}Cu and ^{106}Cd were conducted in deionized (DI), soft (SO), moderately hard (MH), and hard water (HA) at 15 °C (Table S1, Supporting Information). In this experiment, the major ions associated with the carrier systems for both Cu (Na^+) and Cd (Ca^{2+}) varied together in the different treatments. Our goal was not to separate the influences of Na and Ca since a great deal of work on that question already exists (e.g., refs *17*, *19*). We

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rather aim to systematically contrast Cu and Cd uptake with changes in water chemistry typical of nature (i.e., when both major ions vary together).

Experimental Organisms. *Lymnaea stagnalis* were reared in the laboratory in MH water at 15 °C. Snails of a restricted size range (mean shell size of 13.4 ± 0.4 mm 95% CI, $n = 183$) were acclimatized to the experimental conditions in aerated 10 L glass aquaria (50 snails per tank) for 1 week. Lettuce was fed to snails during the acclimation period. Three days prior to the experiment, snails were randomly transferred to 1 L acid-washed polypropylene jars (8 snails per jar) filled with unspiked media. Food was withheld during this period.

^{65}Cu and ^{106}Cd Uptake from the Dissolved Phase. Acclimated snails were transferred to acid-washed 1 L HDPE containers filled with media spiked with different dissolved concentrations of ^{65}Cu and ^{106}Cd (Table S2, Supporting Information). We used commercially purchased standards (Trace Sciences International) isotopically enriched in ^{65}Cu (99.4%) and ^{106}Cd (96.5%). Exposure concentrations ranged from 0.6 nM to 4.8 μM for Cu and from 0.004 nM to 2.7 μM for Cd. The lowest total tracer concentration (see eq 2 below) was 4−8 times lower than typical concentration in pristine lakes (*20*). Over the lower range of concentrations, uptake rates are likely to be a linear function of concentrations (i.e., first-order kinetics). Short exposures (24 h) were employed to minimize the influence of efflux, allowing measurement of gross influx rates (*21*) and inferences about membrane transport processes from whole organism data. Snails were not fed during the exposure period to minimize fecal scavenging. The 24 h exposure was sufficient to ensure enough tracer accumulation for accurate detection. After exposure to both metals, snails were removed from the experimental media, rinsed in ultrapure water, and frozen. Before and after the exposure phase, water samples (5 mL) were taken from each vial, filtered through a 0.45 μm Millex-HV filter (after exposure only) and acidified with concentrated nitric acid (Baker Ultrex II grade, 2% final concentration).

Sample Preparation and Analysis. To minimize inadvertent metal contamination, labware, vials, and Teflon sheeting were soaked for at least 24 h in acid (15% nitric and 5% hydrochloric), rinsed several times in ultrapure water, and dried under a laminar-flow hood prior to use.

Partially thawed *L. stagnalis* were dissected to remove soft tissue, placed individually on a piece of acid-washed Teflon sheeting, and allowed to dry at 40 °C for 3 days. Dried snails were weighed and digested at room temperature in Teflon vials with concentrated nitric acid (100 μL mg dry weight sample^{-1}) for 7 days (*22*). Hydrogen peroxide (Baker Ultrex II grade, 40 μL mg dry weight sample^{-1}) was added prior to final dilution with ultrapure water (860 μL mg dry weight sample^{-1}). Samples of similar weight from the certified reference material NIST-2976 (mussel tissue from National Institute of Standards and Technology) were submitted to the same digestion procedures during each analytical run. Cu and Cd concentrations measured in NIST-2976 were within the certified range.

Water samples and digested snails were analyzed for all the naturally occurring stable isotopes of Cu and Cd by inductively coupled plasma−mass spectrometry, as described by Croteau et al. (*10*). Certified reference riverine water samples (National Research Council Canada; SLRS-4) were analyzed for Cu and Cd during each analytical run. Metal concentrations derived from signal intensities for both Cu stable isotopes and from the most abundant Cd isotope (e.g., ^{114}Cd) were within the certified range. To account for instrument drift and change in sensitivity, internal standardization was performed by the addition of germanium (^{74}Ge) to all samples and standards but the calibration blanks. We also reanalyzed one of our standards after every 10 samples.

Calculation of Accumulated Tracer Concentrations. The accumulated tracer concentrations were determined using equations derived from Croteau et al. (*10*) (see example for ^{106}Cd in the Supporting Information). Briefly, the relative abundance of each tracer (e.g., ^{65}Cu and ^{106}Cd) was determined using the signal intensities of each isotope in the standards used to calibrate the ICP−MS. That is,

$$p^{\mathrm{i}} = \left(\frac{\text{Intensity } {}^{\mathrm{i}}\mathrm{E}}{\sum_{\mathrm{j}}^{\mathrm{jj}} \text{Intensity } {}^{\mathrm{j}}\mathrm{E}} \right)_{\text{Standard}} \quad (1)$$

where p^{i} is the relative abundance of the natural isotope $^{\mathrm{i}}$E (the tracer), E is the element (metal), j and jj are the lightest and heaviest isotopes of the element E, respectively. Concentrations of tracer in the experimental organisms ($[^{\mathrm{i}}E]_{\hat{\mathrm{e}}}$) were then calculated as the product of p^{i} and the total metal concentrations inferred by the ICP−MS software from tracer intensity ($[\mathrm{T}^{\mathrm{i}}\mathrm{E}]$):

$$[{}^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}} = p^{\mathrm{i}} \times [\mathrm{T}^{\mathrm{i}}\mathrm{E}] \quad (2)$$

Total metal concentrations inferred from the intensity of the most abundant isotope were then used to derive the original load of tracer ($[^{\mathrm{i}}\mathrm{E}]^0_{\hat{\mathrm{e}}}$) that occurred in each sample in the absence of a spike, i.e.,

$$[{}^{\mathrm{i}}\mathrm{E}]^0_{\hat{\mathrm{e}}} = p^{\mathrm{i}} \times [\mathrm{T}^{\mathrm{k}}\mathrm{E}] \quad (3)$$

where k is the most abundant isotope of the element E. Finally, net tracer uptake ($\Delta[^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}}$) were then $[^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}}$ as derived from the total experimental metal inferred from tracer signal (eq 2) minus the pre-existing load of tracer (eq 3),

$$\Delta[{}^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}} = [{}^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}} - [{}^{\mathrm{i}}\mathrm{E}]^0_{\hat{\mathrm{e}}} \quad (4)$$

Biodynamic Calculations and Membrane Transport Characterization. Membrane transport characteristics were characterized by fitting a Michaelis−Menten equation to plots of accumulated metal concentrations in the snail ($\Delta[^{\mathrm{i}}\mathrm{E}]_{\hat{\mathrm{e}}}$ in nmoles g^{-1}, eq 4) versus free metal ion concentrations (nM), as estimated by the speciation model WHAM 6.0. The nonlinear regression fit was used to determine the saturation point of the curve, which is reflective of binding site capacity ($B_{\max}$ in nmol g^{-1}), and the ionic concentration at half-saturation ($K_{\text{metal−snail}}$ in nM), the inverse of which is indicative of site affinity (log K).

Rate constants for dissolved metal uptake (k_{u}) were determined from the slope of the regression between the amount of tracer accumulated by the snail soft tissue (data for the linear uptake only) at each concentration for each treatment, and the waterborne exposure concentrations (total dissolved concentrations, Table S2 Supporting information). k_{u} is typically expressed as nmoles g$^{-1}{}_{\text{tissue}}$ d^{-1} per nmoles l$^{-1}{}_{\text{water}}$, or l g$^{-1}{}_{\text{tissue}}$ d^{-1}.

The assumption that whole body tissue concentration can be used to coarsely characterize membrane transport processes and unidirectional fluxes does not differ much from the BLM approaches where tissue-specific uptake is used to characterize membrane transport processes (*2*). Biodynamic forecasts of bioaccumulation agree well with observations from nature, confirming that this is a reasonable assumption (*8*). It is important to note that site affinity and capacity are traditionally inferred from rates, which should be independent of exposure time (K_{m} and $J_{\max}$, e.g., ref *11*). Directly characterizing transport on the basis of concentrations (K_{D} and $B_{\max}$), in the metal literature, is conditional on

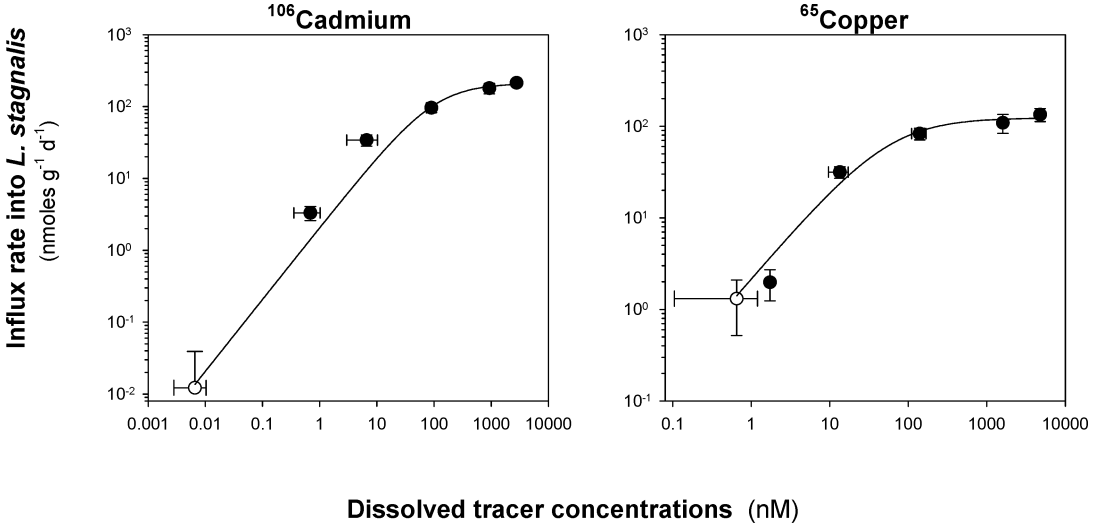

FIGURE 1. Metal uptake rates (nmoles g^{-1} d^{-1}) in *L. stagnalis* (soft tissue) exposed to waterborne ^{106}Cd and ^{65}Cu for 24 h in DI water. Each symbol represents mean metal concentrations of eight individuals and six water samples (±95% confidence interval). ○ are for controls; ● are for the experimental snails. Curves represent nonlinear regression fits to Michaelis−Menten equation. Metal uptake rates at different water hardness (DI, SO, MH, and HA) are shown in Figure S2 (Supporting Information).

the time of exposure (in our case 24 h). Different conditional constants should be expected if the exposure time is changed (*3*, *23*).

Statistical Analysis. Metal concentrations are expressed as means ±95% confidence intervals. Standard errors are reported for the metal binding characteristics and rate constants of metal uptake. We used *t* tests to compare metal concentrations, binding characteristics and rate constants of uptake between metals and among water hardness levels.

Results

Tracer Uptake from the Dissolved Phase. One of the questions in using stable metal isotopes is whether the tracer can be detected in the short exposures necessary to characterize unidirectional fluxes or membrane processes. Snails exposed for 24 h to low ^{106}Cd concentrations (0.87 ± 0.13 nM) accumulated significant amounts of ^{106}Cd compared to controls (control water had 0.014 ± 0.016 nM of ^{106}Cd; e.g., Figure 1, open circles, $p < 0.05$). Similarly, significant ^{65}Cu bioaccumulation occurred upon 24 h exposure to dissolved ^{65}Cu concentrations 7-times higher than that for controls, i.e., 15 ± 1.3 nM as opposed to 2.2 ± 1.9 nM, respectively. The preexisting load of tracer occurring in the absence of a spike was 2 orders of magnitude higher for ^{65}Cu compared to that for ^{106}Cd (i.e., $[^{65}Cu]^0_{\hat{e}} = 168 \pm 8$ nmoles g^{-1} and $[^{106}Cd]^0_{\hat{e}} = 0.40 \pm 0.03$ nmoles g^{-1}, $n = 183$, data not shown). Concentrations of Cu in organisms in nature are always higher than those for Cd because Cu is more abundant in the environment than Cd. Furthermore ^{65}Cu has a greater relative abundance than ^{106}Cd (i.e., ^{65}p and ^{106}p averaged 0.328 ± 0.003 and 0.011 ± 0.0001, respectively). The high background concentration of ^{65}Cu means that higher exposure concentrations are needed to achieve detectable ^{65}Cu than ^{106}Cd accumulation, although those concentrations are still considerably lower than if enriched stable isotopes were not used.

The uptake of both ^{106}Cd and ^{65}Cu was linear over a range that would encompass most environmental exposures; <98 nM for ^{106}Cd and <146 nM for ^{65}Cu. The k_u for Cd was significantly higher than that for Cu in DI water ($p < 0.001$), but the opposite was found in the hardest water (i.e., k_u was significantly higher for Cu than for Cd, $p < 0.001$, Figure 2A). On the basis of total metal, the k_us for Cd and Cu in DI water were 0.98 and 0.55 L g^{-1} d^{-1}, respectively, compared to 0.39 L g^{-1} d^{-1} for Cd and 0.78 L g^{-1} d^{-1} for Cu in hard water (Table 1). Thus the k_us for Cu increase with hardness, in contrast to the decrease in the k_u, for Cd with increasing hardness cation concentrations. If k_u is calculated on the basis of free ion, then no differences occur with Cd because a high proportion of Cd is free ion (73−90%). The k_u for Cu is much higher, however, and the influences of hardness are amplified because only a small proportion of Cu occurs as free ion (1.7−6.9%). That is, the changes in free ion Cu with increasing hardness are proportionally bigger than for Cd (Table S1, Supporting Information).

Snails ultimately demonstrate saturation uptake kinetics for both ^{106}Cd and ^{65}Cu when exposed for 24 h to a wide range of metal concentrations (Figure 1). Saturation kinetics is observed at all hardness cation concentrations (Figure S1, Supporting Information). The shape of the curves is typical of the Michaelis−Menten kinetics that suggest uptake via a finite number of carrier proteins or ion channels (*24*). For both metals, saturation occurred at metal concentrations in the range of what has been described as a high-affinity, low-capacity transport system in fish gills (reviewed in ref *25*).

Saturation kinetics were not the result of impairment of membrane processes at high metal concentrations. Dissolved uptake of ^{106}Cd and ^{65}Cu by *L. stagnalis* also reached saturation after shorter (12 h) exposures, although there were some differences in the conditional constants, especially for Cd (Croteau and Luoma, unpubl. results). Thus, saturation uptake kinetics found upon 24 h exposure to tracers likely reflect physiological characteristics of Cd and Cu waterborne uptake by this species.

Metal-Binding Characteristics. The saturation point of each curve increases by 59% for ^{65}Cu and by 37% for ^{106}Cd with water hardness (Figure 2B). Transport capacity (B_{max} in nmol g^{-1}) increased from 211 to 289 for ^{106}Cd, and from 124 to 196 for ^{65}Cu when Ca concentrations increased from 0 to 768 μM ($p < 0.001$) (Table 1). For each hardness level, B_{max} was significantly higher for Cd compared to that for Cu ($p < 0.001$). For instance in DI water, site capacity for Cd is almost twice that for Cu, i.e., B_{max} of 211 compared to 124 nmol g^{-1} (Table 1). Thus Cu uptake sites, which presumably include those for Na^+ transport, are less abundant than those for Cd, which can use Ca^{2+} transporters (*26*).

Transporter affinity was affected differently for each metal by water hardness (Figure 2C). For example, site affinity for Cu was 25-times higher in hard water (log *K* of 8.6) compared to that in DI water (log *K* of 7.2). In contrast, sites affinity for Cd decreased steadily with water hardness, i.e., log *K* for Cd

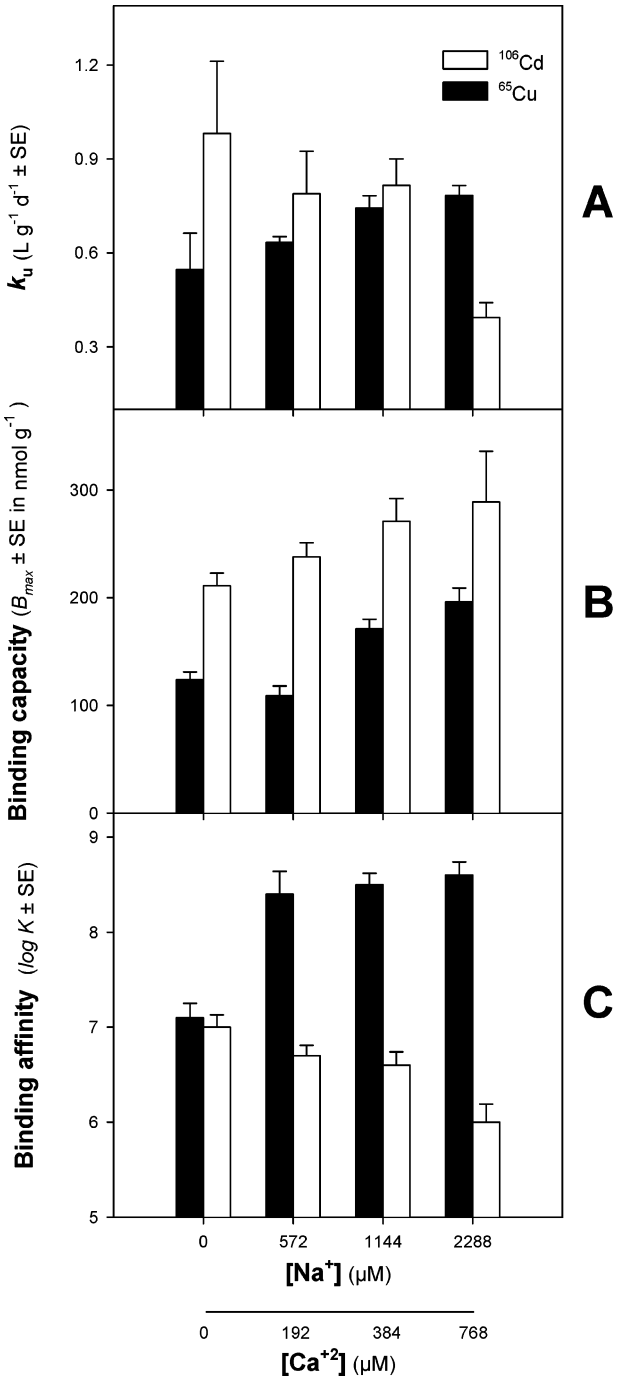

FIGURE 2. (A) Rate constants of dissolved metal uptake (k_u ± S.E. in l g^{-1} d^{-1}); (B) Number of binding sites (B_{max} ± S.E. in nmol g^{-1}); (C) Binding affinity (log K ± S.E.).

was 7.0 in DI water and declined to 6.0 in hard water. Both metals bind to sites with a comparable strength in DI water (i.e., similar log K values). However, Cu binds 400 times better than does Cd in hard water (Table 1).

As shown in Figure 3, site affinity correlates strongly with changes in the dissolved Cd influx rate constant ($r^2 = 0.97$, $p < 0.05$). However, for Cu, both binding site affinity and capacity need to be taken into account to accurately predict dissolved Cu influx rate constants ($k_u = 0.07\log K + 0.0016B_{max}$-0.13, $r^2 = 0.99$, $p < 0.05$).

Discussion

Metal influx rates result from mechanistic processes that characterize membrane transport, although water quality conditions can also play a role. Metal influx rate from solution is defined by an uptake rate constant (k_u) multiplied by the metal concentration in water. The uptake rate constant reflects the physiological characteristics of the biotic ligand. Specifically, affinities of transport sites for metals (log K) are indicative of binding strength and reflect competitive effects on ligand binding (*11*). Transport site capacity (B_{max}) predicts the quantity of metal that can bind to transport sites and thus represents a limit to the amount of metal accumulation. In general, greater metal affinity, or higher transport capacity, or both, make faster metal influx possible.

Biotic Ligand Characteristics and Rate Constants of Uptake. The decrease in k_u with water hardness for Cd in *L. stagnalis* probably reflects the inhibitive influence of Ca ions on Cd uptake and bioaccumulation (*24*, *25*). The inhibition of Cd uptake rates by Ca varies among species and also between saltwater and freshwater invertebrates (*27*, *28*). Binding site strength for Cd in *L. stagnalis* also gradually decreases in concert with water hardness. The strong correlation found between log K and the Cd influx rate constant (Figure 3) suggests that binding site affinity, not capacity, is the most influential mechanistic transport aspect determining dissolved Cd influx rates for this freshwater gastropod.

The positive relationship between Cu uptake rate constants and water hardness is somewhat surprising given the "protective effect" of Ca, Mg, and Na ions found elsewhere (*29*). Copper taken up by fish gills inhibits Na^+/K^+-ATPase, which causes osmoregulatory disturbance, as Na^+ is lost (*30*). If this involves faster Na influx (to make up for greater loses of Na) then it might be accompanied by faster Cu influx (as Cu can cross membranes using the Na^+ transport system). However, Cu can also inhibit Na uptake at high concentrations (*30*). But in multi-metal exposures, metals such as Cd could potentially offset the inhibition by stimulating Na influxes (*31*).

Although affinity for Cu in *L. stagnalis* increases with water hardness, both affinity and capacity must be considered in the relationship between log K and k_u. Stepwise multiple regressions suggest that affinity has a stronger influence on k_u than does capacity ($r^2 = 0.72$), although capacity is still influential ($r^2 = 0.99$).

Both metals bind to sites with comparable strength in DI water (i.e., similar log K values). They have thus a similar "true" affinity (i.e., "true" log K are determined without competitive ions). The slightly higher influx rate of Cd than Cu in the absence of competitive ions appears to result from more binding sites for Cd. However, the "conditional" affinities, as hardness increases, are 50−400 times higher for Cu than Cd. If Ca has an inhibitive effect on the affinity of Cd transporters, but neither Na nor Ca affect the affinity Cu transporters, these results are as expected. The greater affinity of Cu for the biotic ligand influences influx rates more than does the greater number of Cd binding sites in the harder waters.

Comparison with Other Studies. The k_us for Cd in *L. stagnalis* (Table 1) are within the wide range reported in a variety of freshwater bivalves (i.e., 0.37−3.2 L g^{-1} d^{-1}: *4*, *32*) and marine gastropod species (i.e., 0.029−0.056 l g^{-1} d^{-1}, *33*). Similarly, the k_us for Cu are similar to those reported in the only two available studies on Cu biodynamics (i.e., 0.22 and 5.1 L g^{-1} d^{-1}; *10*, *34*). The estimates of k_u for *L. stagnalis* are also reflective of its biology. This pulmonate snail breathes air. Thus, it likely assimilates waterborne metals from swallowed water or across its integument. Therefore, dissolved metal uptake is at the lower end of the ranges, compared to purely aquatic species.

The basic biology of a species is also essential to consider when making assumptions about similarities in ligand character among species. Transport capacity for *L. stagnalis* is at least 2 orders of magnitude higher than reported in fish

TABLE 1. Metal Binding Characteristics[a] and Rate Constants of Dissolved Metal Uptake for *L. stagnalis* Exposed for 24 h to a Range of ^{106}Cd and ^{65}Cu in DI and Synthetic Water of Different Hardness

water type	metal	B_{max}[b] (nmol g^{-1})	log K[b]	P	R^2	N[c]	k_u^{bc} (l g^{-1} d^{-1})
deionized	Cd	211 ± 12	7.0 ± 0.13	<0.001	0.98	6	0.98 ± 0.23 (n = 4)
	Cu	124 ± 7	7.2 ± 0.15	<0.001	0.97	6	0.55 ± 0.12 (n = 4)
soft	Cd	238 ± 13	6.8 ± 0.10	<0.001	0.99	5	0.79 ± 0.14 (n = 3)
	Cu	109 ± 9	8.4 ± 0.24	0.004	0.96	5	0.63 ± 0.02 (n = 3)
moderately hard	Cd	271 ± 21	6.6 ± 0.12	<0.001	0.98	6	0.82 ± 0.08 (n = 4)
	Cu	171 ± 9	8.5 ± 0.12	<0.001	0.98	6	0.74 ± 0.04 (n = 4)
hard	Cd	289 ± 47	6.0 ± 0.15	<0.001	0.98	6	0.39 ± 0.05 (n = 4)
	Cu	196 ± 13	8.6 ± 0.14	<0.001	0.97	6	0.78 ± 0.03 (n = 4)

[a] Metal binding characteristics were determined by fitting a Michaelis−Menten equation to plots of accumulated metal concentrations in the snail versus free metal ion concentrations (nM) [b] ±S.E. [c] N and n represent the number of data points used to determined metal-binding characteristics and rate constants of dissolved metal uptake, respectively

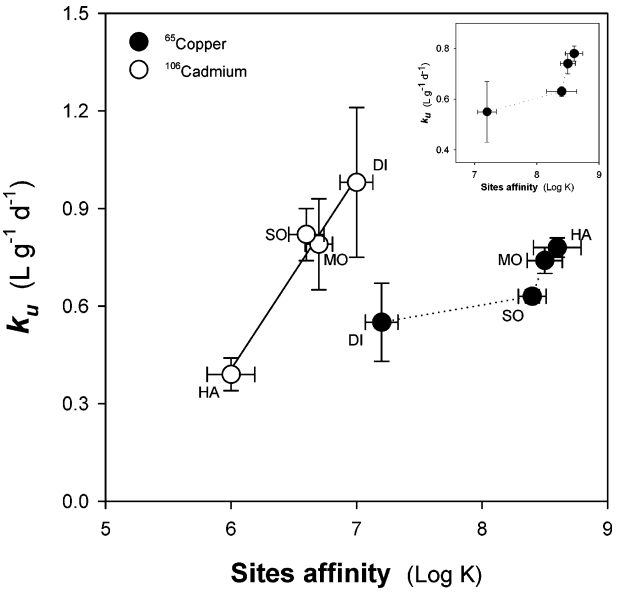

FIGURE 3. Relationship between rate constant of uptake (±SE) and binding sites affinity (log K ± S.E.) for Cd (○) and Cu (●) at various water hardness; HA for hard water, MO for moderately hard water, SO for soft water, and DI for deionized water.

gill studies. B_{max} (in nmol g^{-1}) for Cu in rainbow trout can vary from 0.6 (*17*) to 3.6 (*35*) (if a wet weight-dry weight conversion factor of 10 is used; see also Table S3 in the Supporting Information). The higher number of binding sites for Cd in *L. stagnalis* may reflect the extremely high Ca requirements for shell formation for this species; Cd uses Ca^{2+} transporters to enter the cells (*26*). Such significant differences among species raise serious questions about using binding site densities determined from one species to calculate ligand characteristics or metal concentrations at the ligand in another species (*1*). Although differences among species might have large effects on BLM calculations, it appears that the number of binding sites is not very influential in determining uptake rates for *L. stagnalis*. The large number of metal binding sites found in *L. stagnalis* does not yield exceptional metal uptake rates, highlighting the importance of site affinity as a determinant factor to exposure and thus toxicity.

Metal binding affinities for *L. stagnalis* were also within the range of those found in fish gill studies. For example, log K for Cu in rainbow trout can vary from 7.4 to 9.9 (Table S3, Supporting Information). The influence of hardness on metal binding characteristics in *L. stagnalis* also follows trends reported for fish. For instance, binding site affinity for rainbow trout exposed for 3 h to both metals showed a 20−32-fold increases for Cu (*17*, *35*) and a 2-fold decrease for Cd (*19*), similar to that for snails. Differences in binding affinity occur between metals, however, but more so for some species than others. For example, Cd binds to sites 16 times stronger than Cu in the minnows *Pimephales promelas* (*13*). Extensive comparisons are, however, difficult to make because a very limited number of studies have determined log K for more than one metal at the time.

The BLM predicts toxicity from the amount of metal accumulated at the biotic ligand, assuming that the accumulation of metal to the biotic ligand determines the toxic effect (*2*). Higher log K implies better binding to uptake sites, and greater acute toxicity (*3*). If that is the case, then waterborne exposure to Cu in freshwater systems would be more toxic for *L. stagnalis* than exposure to Cd. Fish, however, have a higher log K for Cd than for Cu (e.g., ref *13*). Relative (Cd vs Cu) toxicity predictions from log K in fish would not be an accurate reflection of relative toxicity in this snail.

The correlations of metal influx with the characteristics of the biotic ligand are logical extensions of transport physiology. In particular, the correlation between metal influx rate constants and site affinity is of interest. If site affinity is indicative of acute toxicity from dissolved sources (within the BLM framework, refs *3*, *25*), then influx rate constants could be similarly indicative, and might be similarly used to predict toxicity (in toxicity tests). Neither, however, gives evidence for which conceptual model for toxicity is the closest to reality. The BLM concept is an equilibrium model in which concentration at the site of effect is correlated with toxicity. But if a primary ingredient in that correlation is log K, then influx rates will also correlate with toxicity. The biodynamic concept, that toxicity results when the rate of internalization exceeds the rate of loss plus the rate of detoxification, cannot be separated from the equilibrium/BLM construct. This could add some confusion to the expanding regulatory use of the BLM construct, on one hand. On the other hand, it could offer opportunities to overcome some of the inherent limitations of the BLM results to date (e.g., species differences, dietary uptake).

Risks pose by metals in nature depend on exposure. Predicting the effects of metal contamination is difficult because biological responses differ among exposure routes (and likely among metals, species and environmental conditions). Dietary uptake of metals is increasingly recognized as an important pathway for metal accumulation (*5*). Complementary use of the biodynamic and BLM modeling approaches, centered around the relationship between transport affinities and influx rates, might offer opportunities to quantify such sources of exposure (see also Table S4, Supporting Information). Merging knowledge of transport physiology and biodynamics may provide key insights to understand metal bioaccumulation and likely, help to better predict metal toxicity. Understanding the linkage between the BLM and biodynamics is, however, just beginning. Much more is left to learn from their connection, which would greatly benefit both conceptual worlds.

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Note Added after ASAP Publication. The assignment of copyright for the version published ASAP on March 20, 2007 was in error. The corrected version was published ASAP April 10, 2007.

Supporting Information Available

Chemical composition of the experimental media, metal-binding characteristics derived from fish gill studies, calculations of accumulated ^{106}Cd concentrations, differences between the BLM, and the biodynamic model as well as figures of metal uptake rates at different water hardness are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Di Toro, D. M.; Allen, H. E.; Bergman, H. L.; Meyer, J. S.; Paquin, P. R.; Santore, R. C. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ. Toxicol. Chem.* **2001**, *20*, 2383−2396.

(2) Paquin, P. R.; Gorsuch, J. W.; Apte, S.; Batley, G. E.; Bowles, K. C.; Campbell, P. G. C.; Delos, C. G.; Di Toro, D. M.; Dwyer, R. L.; Galvez, F.; Gensemer, R. W.; Goss, G. G.; Hogstrand, C.; Janssen, C. R.; McGeer, J. C.; Naddy, R. B.; Playle, R. C.; Santore, R. C.; Schneider, U.; Stubblefield, W. A.; Wood, C. M.; Wu, K. B. The Biotic Ligand Model: A historical overview. *Comp. Biochem. Physiol.* **2002**, *133C*, 3−35.

(3) Niyogi, S.; Wood, C. M. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environ. Sci. Technol.* **2004**, *38*, 6177−6192.

(4) Wang, W. X.; Fisher, N. S.; Luoma, S. N. Kinetic determinations of trace element bioaccumulation in the mussel *Mytilus edulis*. *Mar. Ecol. Prog. Ser.* **1996**, *140*, 91−113.

(5) Schlekat, C. E.; Lee, B.-G.; Luoma, S. N. Dietary metals exposure and toxicity to aquatic organisms: Implications for ecological risk assessment. In *Coastal and Estuarine Risk Assessment*; Newman, M., Ed.; CRC Press: Boca Raton, FL, 2002.

(6) Riggs, D. S. *The Mathematical Approach to Physiological Problems*; Williams & Wilkins: Baltimore, MD, 1963.

(7) Rainbow, P. S. Trace metal concentrations in aquatic invertebrates: why and so what? *Environ. Pollut.* **2002**, *120*, 497−507.

(8) Luoma, S. N.; Rainbow, P. S. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. *Environ. Sci. Technol.* **2005**, *39*, 1921−1931.

(9) Gomot, A. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring. *Ecotox. Environ. Saf.* **1998**, *41*, 288−297.

(10) Croteau, M.-N.; Luoma, S. N.; Topping, B. R.; Lopez, C. B. Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve *Corbicula*. *Environ. Sci. Technol.* **2004**, *38*, 5002−5009.

(11) Hogstrand, C.; Wilson, R. W.; Polgar, D.; Wood, C. M. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. *J. Exp. Biol.* **1994**, *186*, 55−73.

(12) Alsop, D. H.; Wood, C. M. Kinetic analysis of zinc accumulation in the gills of juvenile rainbow trout: Effects of zinc acclimation and implications for biotic ligand modeling. *Environ. Toxicol. Chem.* **2000**, *19*, 1911−1918.

(13) Playle, R. C.; Dixon, D. G.; Burnison, K. Copper and cadmium binding to fish gills: estimates of metal-gill stability constants and modelling of metal accumulation. *Can. J. Fish. Aquat. Sci.* **1993**, *50*, 2678−2687.

(14) Janes, N.; Playle, R. C. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* **1995**, *14*, 1847−1858.

(15) MacDonald, A.; Silk, L.; Schwartz, M.; Playle, R. C. A lead-gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhyncus mykiss*). *Comp. Biochem. Physiol.* **2002**, *133C*, 227−242.

(16) Richards, J. G.; Playle, R. C. Cobalt binding to gills of rainbow trout (*Oncorhynchus mykiss*): An equilibrium model. *Comp. Biochem. Physiol.* **1998**, *119C*, 185−197.

(17) Taylor, L. N.; McGeer, J. C.; Wood, C. M.; McDonald, D. G. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: Evaluation of chronic indicators. *Environ. Toxicol. Chem.* **2000**, *19*, 2298−2308.

(18) Croteau, M.-N.; Luoma, S. N. Delineating copper accumulation pathways for the freshwater bivalve *Corbicula* using stable copper isotopes. *Environ. Toxicol. Chem.* **2005**, *24*, 2871−2878.

(19) Hollis, L.; McGeer, J. C.; McDonald, D. G.; Wood, C. M. Effects of long term sublethal Cd exposure in rainbow trout during soft water exposure: implications for biotic ligand modeling. *Aquat. Toxicol.* **2000**, *51*, 93−105.

(20) Hare, L.; Tessier, A. The aquatic insect *Chaoborus* as a biomonitor of trace metals in lakes. *Limnol. Oceanogr.* **1998**, *43*, 1850−1859.

(21) Luoma, S. N.; Fisher, N. S. Uncertainties in assessing contaminant exposure from sediments. In *Ecological Risk Assessments of Contaminated Sediments*; Ingersoll, C. G., Dillon, T., Biddinger, G. R., Eds.; SETAC Special Publication Series: Pensacola, FL, 1997.

(22) Croteau, M. N.; Hare, L.; Tessier, A. Influence of temperature on Cd accumulation by species of the biomonitor *Chaoborus*. *Limnol. Oceanogr.* **2002**, *47*, 505−514.

(23) MacRae, R. K.; Smith, D. E.; Swoboda-Colberg, N.; Meyer, J. S.; Bergman, H. L. Copper binding affinity of rainbow trout (*Oncorhyncus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: Implications for assessing bioavailable metal. *Environ. Toxicol. Chem.* **1999**, *18*, 1180−1189.

(24) Simkiss, K.; Taylor, M. G. Transport of metal across membranes. In *Metal Speciation and Bioavailability in Aquatic Systems;* Tessier, A., Turner, D. R., Eds.; Wiley: New York, 1995.

(25) Niyogi, S.; Wood, C. M. Effects of chronic waterborne and dietary metal exposures on fill metal-binding: Implications for the Biotic Ligand Model. *Hum. Ecol. Risk Assess.* **2003**, *9*, 813−846.

(26) Verbost, P. M.; Van Rooij, J.; Flik, G.; Lock, R. A. C.; Wendelaar Bonga, S. E. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *J. Exp. Biol.* **1989**, *145*, 185−197.

(27) Stephenson, M.; Mackie, G. L. A laboratory study of the effects of waterborne cadmium, calcium, and carbonate concentrations on cadmium concentrations in *Hyalella azteca* (Crustaea: Amphipoda). *Aquat. Toxicol.* **1989**, *15*, 53−62.

(28) Wang, W.-X.; Fisher, N. S. Effects of calcium and metabolic inhibitors on trace element uptake in two marine bivalves. *J. Exp. Mar. Biol. Ecol.* **1999**, *236*, 149−164.

(29) De Schamphelaere, K. A. C.; Janssen, C. R. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: The effects of calcium, magnesium, sodium, potassium and pH. *Environ. Sci. Technol.* **2002**, *36*, 48−54.

(30) Laurén D. J.; McDonald, D. G. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can. J. Fish. Aquat. Sci.* **1986**, *43*, 1488−1496.

(31) Reader, J. P.; Morris, R. Effects of aluminium and pH on calcium fluxes, and effects of cadmium and manganese on calcium and sodium fluxes in brown trout (*Salmo trutta* L). *Comp. Biochem. Physiol.* **1988**, *91C*, 449−457.

(32) Roditi, H. A.; Fisher, N. S. Rates and routes of trace element uptake by zebra mussels. *Limnol. Oceanogr.* **1999**, *44*, 1730−1749.

(33) Wang, W.-X.; Ke, C. Dominance of dietary intake of cadmium and zinc by two marine predatory gastropods. *Aquat. Toxicol.* **2002**, *56*, 153−165.

(34) Chang, S. I.; Reinfelder, J. R. Relative importance of dissolved versus trophic bioaccumulation of copper in marine copepods. *Mar. Ecol. Prog. Ser.* **2002**, *231*, 179−186.

(35) Taylor, L. N.; Wood, C. M.; McDonald, D. G. An evaluation of sodium loss and gill metal binding properties in rainbow trout and yellow perch to explain species differences in copper tolerance. *Environ. Toxicol. Chem.* **2003**, *22*, 2159−2166.

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