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Binding of Nickel and Copper to Fish Gills Predicts Toxicity When Water Hardness Varies, But Free-Ion Activity Does Not

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Based on a biotic-ligand model (BLM), we hypothesized that the concentration of a transition metal bound to fish gills ($[M_{gill}]$) will be a constant predictor of mortality, whereas a free-ion activity model is generally interpreted to imply that the chemical activity of the aquo ("free") ion of the metal will be a constant predictor of mortality. In laboratory tests, measured $[Ni_{gill}]$ and calculated $[Cu_{gill}]$ were constant predictors of acute toxicity of Ni and Cu to fathead minnows (*Pimephales promelas*) when water hardness varied up to 10-fold, whereas total aqueous concentrations and free-ion activities of Ni and Cu were not. Thus, the BLM, which simultaneously accounts for (a) metal speciation in the exposure water and (b) competitive binding of transition-metal ions and other cations to biotic ligands predicts acute toxicity better than does free-ion activity of Ni or Cu. Adopting a biotic-ligand modeling approach could help establish a more defensible, mechanistic basis for regulating aqueous discharges of metals.

Introduction

For several decades, water hardness (the sum of the normalities of the divalent cations in solution) has been known to affect toxicity of transition metals to aquatic organisms, with mortality at a specified total dissolved metal concentration decreasing as hardness increases (*1*). The U.S. Environmental Protection Agency (USEPA) method to compensate for the hardness effect in site-specific discharge permits has been to calculate a hardness-adjusted LC50 (median lethal concentration at a specified exposure time) using a regression equation of the form

$$\ln(\mathrm{LC50}) = a\cdot\ln(H) + b \qquad (1)$$

where H = water hardness and a and b are regression coefficients fitted to toxicity data (e.g., refs *2*, *3*). However, hardness is the only water-quality parameter for which such a procedure is used. Other water-quality parameters that modify metal toxicity (e.g., pH, alkalinity, dissolved organic carbon (DOC), total suspended solids (TSS)) can be accounted for only by using costly and time-consuming, site-specific toxicity testing and tortuous calculations that still have no underlying mechanistic basis (e.g., water-effects ratios (*4*)).

To provide a more mechanistic basis for predicting toxicity of metals to aquatic biota, the free-ion-activity model (FIAM) was proposed (*5*, *6*). Based mainly on research in which toxicity correlated with free-ion activity as the concentrations of organic ligands and dissolved organic matter were varied in exposure solutions (Tables 1 and 7 in ref *6*), the FIAM has generally (but incorrectly) been interpreted to imply that a constant degree of biological effect (e.g., mortality) will occur at a constant chemical activity of the aquo ("free") ion of a metal ($\{M^{2+}\}$ for divalent transition-metal cations such as Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}), independent of other water-quality parameters. However, free-ion activity does not appear to be a good predictor of toxicity across some water-quality conditions (*6*), including when water hardness varies.

Building on that concept, a recently resurrected surface-interaction model (*7*) of metal binding to fish gills not only incorporates an equilibrium between $\{M^{2+}\}$ and the concentration of metal accumulated at binding sites on the gill surface (*8*) but also incorporates competition of M^{2+} with other cations (e.g., Ca^{2+}, H^+) for binding at the receptor sites. Historically, Zitko and Carson (*9*) suggested the cation-competition concept prior to Pagenkopf's (*7*) formal presentation of a chemical-speciation model that incorporated a biotic ligand. And although Morel (*5*, p 303) stated in his explanation of the FIAM, "... the free metal ion activity is the parameter that determines the physiological effect of the metal ...", he later specifically mentioned (a) the importance of cation competition for binding sites on the surfaces of biotic ligands (*5*, p 307) and (b) the relationship between measurable physiological effects and the degree of complexation of metals with reactive ligands on (or in) organisms (*5*, p 303). According to this type of model, which we refer to as a biotic-ligand model (BLM) to distinguish it from the current general interpretation of the FIAM, free-ion activity is a necessary but not sufficient component to describe metal accumulation and, presumably, toxicity. Thus, bioavailability of metals can be decreased in two ways: (1) by decreasing $\{M^{2+}\}$ and, thus, decreasing the potential for M to bind to receptor sites (e.g., as increasing [DOC] does through equilibrium partitioning among the dissolved ligands) or (2) by increasing the concentrations of competing cations and, thus, decreasing the amount of M bound to receptor sites (e.g., as increasing $\{Ca^{2+}\}$ does).

We hypothesized that, if the BLM is correct, the amount of a specified metal accumulated on fish gills ($[M_{gill}]$, expressed as mol M·g tissue^{-1}) will be a constant predictor of mortality, independent of other water-quality parameters (except when $\{H^+\}$ becomes high enough to cause acid toxicity). Borgmann (*1*) and Pagenkopf (*7*) alluded to but did not directly test this concept for fish exposed to transition metals, although it later was tested with Atlantic salmon (*Salmo salar*) exposed to Al and Zn as pH varied (*10*). Herein we present the first published evidence that $\{M^{2+}\}$ is not a constant predictor of metal toxicity to fish as water hardness increases, but $[M_{gill}]$ is.

Experimental Section

Ni Toxicity Tests. We exposed subadult (1−6 g) fathead minnows (FHM; *Pimephales promelas*) to $NiSO_4$ in four 96-

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h, continuous-flow toxicity tests. In each test, a control and 11 serially diluted concentrations of Ni (25% decrease in $[Ni_{total}]$ at each serial dilution) were tested in a mixture of well water and reverse-osmosis-treated, deionized (RO-DI) well water at the University of Wyoming's Red Buttes Environmental Biology Laboratory. Exposure waters contained a different concentration of Ca and a different series of $NiSO_4$ concentrations in each test, but temperature, pH, alkalinity, and [DOC] remained constant. Water-quality parameters (average or range of values) were as follows: temperature, 20 °C pH, 7.3 (range = 7.2−7.5); alkalinity, 0.5 mN; DOC, <0.3 mg C·L^{-1}; and major ions (mN) [Ca^{2+} − 0.3−4.8 (varied among tests by adding 0, 1.0, 2.6, or 4.5 mN of $CaCl_2$ to the base mixture of well water and RO-DI water to adjust hardness); Cl^-, 0.02−4.7 (varied among tests as addition of $CaCl_2$ varied); K^+, 0.03; Na^+, 0.04; Mg^{2+}, 0.21; NO_3^-, 0.02; SO_4^{2-}, 0.03−6.7 (varied within and among tests as addition of $NiSO_4$ varied)].

For each Ni concentration and the control, 20 FHM were placed in one aquarium, and 10 or 15 FHM were placed in a second aquarium. Fish in the first aquarium were monitored for survival through 96 h, and the $[Ni_{total}]$ LC50 was calculated by linear regression of the logit transformation of mortality ($\ln(m/(1-m))$, where m is the mortality proportion) on ln-($[Ni_{total}]$). The LC50 was the $[Ni_{total}]$ at which the predicted logit(mortality) equalled zero.

Fish in the second aquarium were removed in groups of 5 at 24 h, and their gills were excised, rinsed in control water for 10 s, blotted dry, wet-weighed, and digested at 86 C for 6−8 h in an equivolume mixture of 70% trace-metal-grade HNO_3 (prepared in ultrapure water) and H_2O_2 (30%). The gill digestates were then diluted to a known volume and analyzed for $[Ni_{gill}]$ by flame atomic absorption spectroscopy (AAS). Aqueous $[Ni_{total}]$ also was analyzed by flame AAS. Quality control for Ni analyses included repeated injections of samples (typically ≤5% relative standard deviation among Ni concentrations determined in replicate injections) and periodic analysis of certified reference standards (a prior set of samples was rerun if analyzed value differed from the reference standard's certified value by >20%). We sampled gills at 24 h because results of preliminary studies indicated accumulation of Ni by FHM gills was relatively rapid and consistent with a one-compartment uptake-depuration model in which the amount of Ni accumulated at 24 h was ~85% of its predicted asymptotic value. Thus, sufficient Ni associated with (i.e., accumulated on or in) the gills before the majority of FHM began dying in exposure concentrations that bracketed the LC50. Median lethal accumulations (LA50s, analogous to the term LC50) of Ni on FHM gills were calculated by regressing logit(mortality) on ln($[Ni_{gill}]$).

Based on (a) the measured exposure-water quality and (b) published stability constants (but with log K values adjusted to zero ionic strength using the Davies equation (*11*)) for complexes of the major anions with the major cations and Ni (*12*), we calculated $[Ni^{2+}]$ and $\{Ni^{2+}\}$ at the $[Ni_{total}]$ LC50 for each toxicity test using the geochemical speciation program MINTEQA2 (*13*).

Cu Toxicity Calculations. To further test our hypothesis, we analyzed published acute-toxicity data for FHM exposed to $CuSO_4$ at various water hardnesses. Erickson et al. (*14*) conducted 12 96-h static-nonrenewal toxicity tests in which larval FHM were exposed to $CuSO_4$, and $[Ca^{2+}]$ and $[Mg^{2+}]$ were adjusted to vary water hardness among the tests (tests "none" and "add 2 mN $CaSO_4$" in set S2 and all 10 tests in set S3 in their Table 2, all of which were conducted at pH 7.8). We calculated $\{Cu^{2+}\}$ and $[Cu_{gill}]$ at the $[Cu_{dissolved}]$ LC50 in each test by entering $[Cu_{dissolved}]$, other inorganic water-quality parameters (*15*), and [DOC] (0.8 mg·L^{-1}) into the geochemical speciation program CHESS (*16*). For these calculations, the CHESS model was used to simulate (a) Cu-organic matter interactions described in the humic-substances model WHAM (*17*), assuming the dissolved organic matter (DOM) was composed of 10% humic acid and 90% fulvic acid, and (b) binding of Cu^{2+}, Ca^{2+} and H^+ to FHM gills using published conditional stability constants (*8*). Least-squares linear regression equations were computed for $[Cu_{dissolved}]$, $\{Cu^{2+}\}$, and $[Cu_{gill}]_{calc}$ vs [Ca], and two-tailed significance of each regression slope (H_o: slope = 0) was tested at $\alpha = 0.05$.

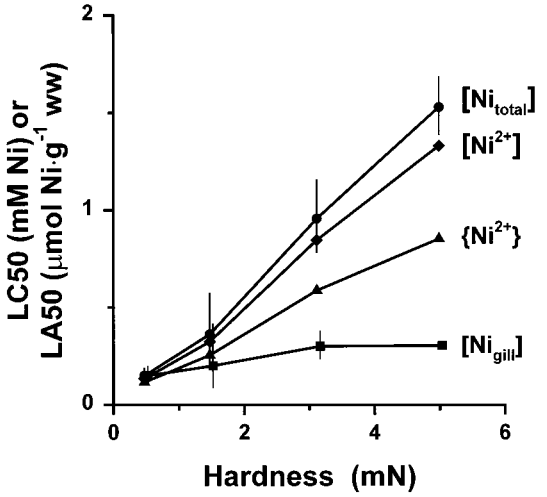

FIGURE 1. Measured 96-h LC50s (median lethal concentrations) for $[Ni_{total}]$, $[Ni^{2+}]$, and $\{Ni^{2+}\}$ in exposure waters and LA50s (median lethal accumulations, expressed on a wet weight (ww) of tissue basis) for $[Ni_{gill}]$, for fathead minnows (FHM) exposed to $NiSO_4$ at various water hardnesses. LC50s are Ni concentration ([]) or chemical activity ({}) averaged over the 96-h exposure; LA50s are measured tissue burden of Ni associated with FHM gills at 24 h (but corresponding to 50% mortality at 96 h). Error bars represent 95% confidence intervals, and, for clarity, they are shown only for $[Ni_{total}]$ and $[Ni_{gill}]$. Error bars for $[Ni^{2+}]$ and $\{Ni^{2+}\}$ at a specified hardness are proportional in size to the error bar for $[Ni_{total}]$.

Results and Discussion

The 96-h $[Ni_{total}]$ LC50 increased 10-fold as [Ca] increased 10-fold (Figure 1), as expected from the hardness correction equation in the USEPA's Ni criteria document (*2*). Moreover, the $[Ni^{2+}]$ LC50 also increased 10-fold over the same range of water hardness, and the $\{Ni^{2+}\}$ LC50 increased 7-fold (Figure 1). Thus, $[Ni^{2+}]$ and $\{Ni^{2+}\}$ were not constant predictors of Ni toxicity (i.e., one would have to know water hardness in addition to $[Ni^{2+}]$ or $\{Ni^{2+}\}$ to be able to accurately predict the LC50). But $[Ni_{gill}]$ was approximately a constant predictor of toxicity, because the LA50 increased only 2-fold over the 10-fold increase in water hardness and appeared to approach a plateau at high hardness (Figure 1).

The 96-h $[Cu_{dissolved}]$ LC50 increased significantly by 3.1-fold as [Ca] increased 5-fold (Figure 2a), as expected from the hardness correction equation in the USEPA's Cu criteria document (*3*). Moreover, the $\{Cu^{2+}\}$ LC50 increased significantly by 4.3-fold (Figure 2b). Thus, $\{Cu^{2+}\}$ was not a constant predictor of Cu toxicity. But $[Cu_{gill}]_{calc}$ was approximately a constant predictor of toxicity, because the LA50 did not increase significantly over the 5-fold increase in water hardness and appeared to approach a plateau at high hardness (Figure 2c). Tabulations of the data plotted in Figures 1 and 2 are available from the lead author.

As noted by Erickson et al. (*14*) for their plot of Cu_{total} LC50 vs hardness, the slope of our plot of $Cu_{dissolved}$ LC50 vs [Ca] (Figure 2a) appears to decrease slightly as [Ca] increases—even if both axes are ln-transformed. This curvilinear trend is counter to predictions of BLM-type models but can be

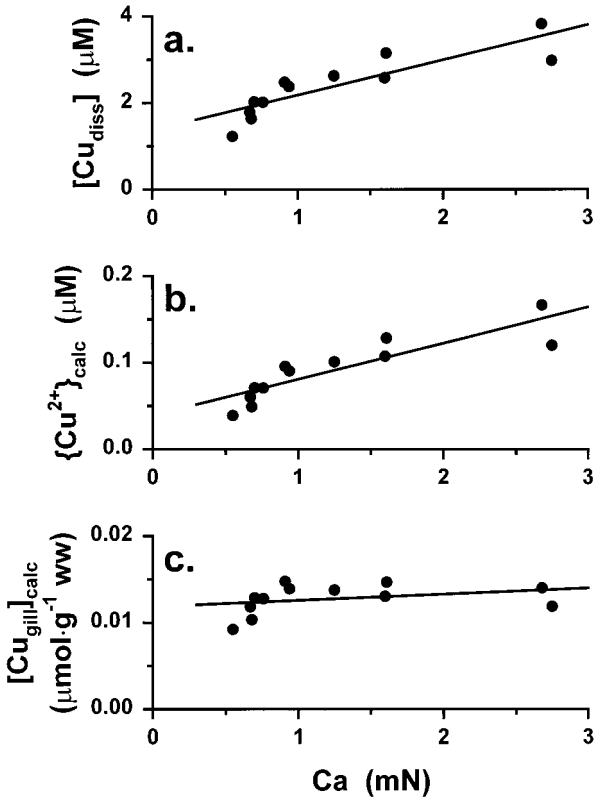

FIGURE 2. (a) Measured 96-h LC50s (median lethal concentrations) for [$Cu_{dissolved}$] in exposure waters, (b) calculated 96-h LC50s for {Cu^{2+}}, and (c) calculated LA50s (median lethal accumulations, expressed on a wet weight (ww) of tissue basis) for [Cu_{gill}], for fathead minnows (FHM) exposed to $CuSO_4$ at various water hardnesses (*14*). LC50s are Cu concentration ([]) or chemical activity ({}) averaged over the 96-h exposure; LA50s are calculated tissue burden of Cu associated with FHM gills at 2−3 h (but corresponding to 50% mortality at 96 h). Least-squares linear regression slopes are (a) 0.808 ($P < 0.001$), (b) 0.0417 ($P < 0.001$), and (c) 0.000710 ($P = 0.308$).

explained as follows. First, the decreasing slope at higher [Ca] might result from competition between Ca^{2+} and Cu^{2+} binding to the ~1 mg·L^{-1} of DOC that was present in the Lake Superior water in their toxicity tests (*14*). As a result of this cation competition, {Cu^{2+}} would have increased as [Ca] increased at a given [$Cu_{dissolved}$]—thus, tending to increase FHM mortality (i.e., decreasing the LC50 below the value expected in the absence of DOM). Winner (*18*) reported such a hardness-mediated increase in toxicity of Cu in the presence of humic acid, and Penttinen et al. (*19*) reported a loss of the protective effect of DOM as water hardness increased when *Daphnia magna* were exposed to Cd. Second, Cu disrupts ionoregulation in fish (*20*). Because gills may be more permeable to body ions at low [Ca] than they are at higher [Ca] (*20*), the lower than expected $Cu_{dissolved}$ LC50s at low [Ca] might result from diminished, direct physiological protection by gill-bound Ca—a process that is not accounted for by our BLM. This might also explain why the LA50s of [Ni_{gill}] (Figure 1) and [Cu_{gill}]$_{calc}$ (Figure 2c) appear to increase slightly at low hardness and approach plateaus at high hardness. Third, Erickson et al. (*14*) only plotted results from their toxicity test set S2 in their Figure 3, and the nonlinearity of their Figure 3 was influenced strongly by the two extreme LC50 values. However, when we included two additional data points from their toxicity test set S2 with their set S3, the trend appears to be more linear at the high [Ca] end of our curve (Figure 2a) than it does at the high hardness end of their curve. Finally, combined with the uncertainty about the LC50 estimates (see 95% confidence intervals in Table 2 and Figure 3 in (*14*)), the apparent curvilinear relationship at low [Ca] in Figure 2a might even be an artifact.

We conclude that accumulations of Ni and Cu on FHM gills are approximately constant predictors of toxicity when the concentration of Ca (the major competitor with Ni and Cu for binding to the gill) in exposure waters increases, whereas the free-ion activities of Ni and Cu in exposure waters are not constant predictors of toxicity. This also appears to be valid when pH is varied among Cu toxicity tests, although {Cu^{2+}} is just as good a predictor of toxicity as [Cu_{gill}]$_{calc}$ is when [DOC] is varied (*21*). And, emphasizing the importance of cations other than Ca^{2+} as competitors with some metals, K^+ (but not Ca^{2+}) ameliorates acute and chronic toxicity of thallium (Tl) to the amphipod *Hyalella azteca*, and body burden of Tl is an approximately constant predictor of chronic lethality and growth effects across a range of aqueous K^+ concentrations (*22*). Therefore, the BLM, which simultaneously accounts for (a) metal speciation in the exposure water and (b) competitive binding of transition-metal ions and other cations to biotic ligands predicts acute toxicity better than does free-ion activity of Cu, Ni, and Tl.

Adopting a biotic-ligand modeling approach could help advance the regulation of aqueous discharges of metals beyond the current phenomenological approach and establish a more defensible, mechanistic basis. In the future, regulatory limits might be based on accumulation of metals on biotic ligands (e.g., fish gills, soft tissues of invertebrates, algal cells) measured in the field or predicted in dynamic simulation models that would estimate the number of daily exceedences of a regulatory limit at a site downstream from a metal discharge (*21*). However, H^+ (*10*) and Ca^{2+} (*20*) bound to fish gills perform important, direct physiological functions (e.g., altering membrane permeability and ion transport) beyond just competing with transition-metal cations for binding sites. Such beneficial functions have not yet been incorporated into a BLM. Thus, biotic-ligand modeling could be used to complement (but not totally replace) toxicity testing, by providing much greater temporal coverage than is currently feasible within the financial constraints and time limitations of standard fish, invertebrate, and algal toxicity tests.

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