Complexation and time-dependent accumulation of copper by larval fathead minnows (*Pimephales promelas*): Implications for modeling toxicity

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Abstract

Mechanistic models predicting copper (Cu) toxicity to aquatic biota in natural waters require organic and inorganic water chemistry, and quantified values for Cu binding by sensitive biological receptors. In bioaccumulation experiments using larval fathead minnows (FHM; *Pimephales promelas*), we investigated time to asymptotic accumulation of Cu and quantified the conditional stability constants (binding affinity; log $K_{Cu-FHM}$) and binding-site densities of Cu–FHM complexation. Cu bioaccumulation increased rapidly, approaching an asymptote in exposures longer than 12 h, indicating that Cu loading at 24 h is an appropriate exposure duration for modeling Cu complexation by larval FHM. Results of Langmuir and Scatchard analyses of other bioaccumulation experiments produced log $K_{Cu-FHM}$ values of 6.52, and binding-site densities of 0.39 mol g$^{-1}$ dry weight. These whole-body log $K_{Cu-FHM}$ values are approximately an order of magnitude lower than those reported for adult FHM gills. However, binding-site densities for larval and adult FHM are similar. Under similar test conditions, comparable concentrations of aqueous Cu cause 50% mortality in adult and larval FHM suggesting that binding site densities determine comparable metal–tissue loadings and have greater influence on Cu bioavailability than binding affinity.

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1. Introduction

The acute toxicity of metals such as copper (Cu) depends on the chemical activity of the bioavailable aqua ion (e.g., the cupric ion activity, $\left(\text{Cu}^{2+}\right)$) and on the associated metal complexation by sensitive biological receptors (biotic ligands) in aquatic organisms (Playle et al., 1993a,b, Azenha et al., 1995; Erickson et al., 1996; Kim et al., 1999; MacRae et al., 1999; Di Toro et al., 2001; Santore et al., 2001; Luider et al., 2004). In recognition of the importance of these processes in determining metal toxicity, a biotic ligand model (BLM) has recently been adapted to establish water quality criteria for Cu (USEPA, 2003). To adequately predict [Cu]$^{2+}$ and subsequent Cu complexation to biotic ligands such as fish gills, the BLM takes into account Cu–inorganic speciation (e.g., Cu(CO$_3$)$_2^-$, Cu(OH)$_2^-$) and Cu complexation by dissolved organic matter (DOM). Dissolved organic matter usually comprises the largest pool of organics in natural waters, is passable through a 0.45-μm filter, and originates from terrestrial vegetation and aquatic sources such as macrophytes, algae, and bacteria (McKnight and Aiken, 1998).

The conditional stability constants (i.e., binding affinity, log $K$) and binding-site densities ([L]) of biota for Cu are the crucial links between water chemistry and biologic uptake, allowing the BLM to quantify Cu-biotic complexation (Di Toro et al., 2001). To predict Cu toxicity to all aquatic species, the BLM currently uses complexation parameters developed from direct analyses of Cu complexation by the gills of adult fathead minnows (FHM; *Pimephales promelas*; Playle et al., 1993b), and then allows the LA50 (median lethal accumulation) to vary among species and their life stages to account for differences in sensitivity to Cu. However, binding affinities and binding-site densities of larval life stages of fish could vary from those of the adult fish gill because gills are only beginning to develop in larval fish, and epithelial respiratory surfaces might differ greatly in metal binding and toxic action.
2. Materials and methods

2.1. Chemistry of test waters

Unless otherwise designated, all reagents were analytical-grade except HCl (trace-metal grade; Spectrum) and HNO₃ (OPTIMA, Seastar Chemicals). The higher grade acids were used for sample preservation and pH adjustment during geochemical speciation experiments to calculate log \( K_{\text{Cu-FHM}} \) and binding-site density \([I_{\text{Cu-FHM}}]\) for a single-ligand model of Cu complexation by larval FHM. Finally, using parameters of Cu–FHM complexation entered into the geochemical speciation program MINTEQA2 (Version 3.11, Allison et al., 1991), we evaluated the effect of the biotic ligand of matrix water used in bioaccumulation experiments.

The primary goals of this study were to: (1) determine the required exposure time fore whole-body, steady-state Cu accumulation by larval FHM, (2) quantify the Cu-binding affinity \( \log K_{\text{Cu-FHM}} \) and binding-site density \([I_{\text{Cu-FHM}}]\) of larval FHM for comparison to adult FHM, and (3) determine whether Cu–FHM complexation measurably alters total aqueous Cu concentration at realistic biomass loadings in toxicity tests.

Before quantifying Cu complexation, it was essential to determine whether bioaccumulation of Cu by larval FHM reaches an asymptote. If tissue concentration does not reach a steady-state, then tissue loading depends upon the exposure time. During time-dependent bioaccumulation experiments, we determined the whole-body, steady-state concentration of Cu. In addition, we assessed whether Cu accumulation varied between 24- and 96-h-old larvae. Subsequently we conducted bioaccumulation experiments to calculate log \( K_{\text{Cu-FHM}} \) and \([I_{\text{Cu-FHM}}]\) for a single-ligand model of Cu complexation by larval FHM. Finally, using parameters of Cu–FHM complexation entered into the geochemical speciation program MINTEQA2 (Version 3.11, Allison et al., 1991), we evaluated the effect of the biotic ligand of Cu on speciation across the range of total Cu concentrations in our test solutions.

2.2. Experimental conditions for bioaccumulation tests

Larval FHM for all bioaccumulation experiments came from a resident brood population of FHM at the University of Wyoming. When fully “eyed-up” and nearing hatch (~5 days after spawn), tiles containing FHM eggs were placed in laboratory incubators containing matrix water free of added Cu or DOM (Table 1). Except for one experiment using ≤96-h-old fish, we collected larval fish for each test within 24 h after hatching and immediately started a test. Larvae were not fed during the tests.

Carboys and 150-mL test chambers were pre-conditioned for each test by filling with the appropriate test solution, allowing potential adsorption to occur for ≥24 h, and refilling with fresh test solution. We were unable to detect any loss of Cu in pre-conditioned carboys and test chambers (i.e., no systematic decrease in Cu concentrations between nominal and observed values after decanting into chambers).

All bioaccumulation experiments were conducted in a mixture of well water and reverse osmosis-treated water at pH 7.1, and alkalinity and hardness of ~0.6 meq L⁻¹ (Table 1). Copper was added as CuCl₂. Test solutions contained a low background DOC concentration of 0.1 mg L⁻¹. Although this low DOC concentration had no detectable effect on Cu toxicity, we mixed test solutions ≥24 h before the start of each test to allow sufficient time for maximal Cu–DOM complexation (Ma et al., 1999). All experiments were conducted at 25 °C. At the onset and the end of each experiment, pH, alkalinity, and hardness were measured in each exposure treatment.

To measure metal accumulation in the larval FHM exposed to Cu, we performed two types of bioaccumulation experiments without added DOM (a competing Cu ligand). We conducted time-dependent bioaccumulation experiments (BIOACC A and BIOACC B) to investigate the necessary exposure duration for steady-state Cu bioaccumulation by larval FHM. From
subsequent, concentration-dependent bioaccumulation tests (BIOACC 1 and BIOACC 2), we determined the log $K_{Cu-FHM}$ and $[L_{Cu-FHM}]$ of larval FHM for $Cu$.

In BIOACC A and BIOACC B, we exposed fish to a total $Cu$ concentration of 0.88 $\mu$mol$\cdot$L$^{-1}$, which typically would cause 100% lethality at 96 h under the conditions of this test. We chose this high concentration to maximize rapid accumulation. We measured bioaccumulation in fish removed after 0, 4, 8, 12, and 24 h. To compare potential physiological differences in $Cu$ accumulation that might occur during the rapid developmental changes taking place across the age range of fish in future 96-h, static-renewal toxicity tests, we compared accumulation by 24-h-old fish (BIOACC A) versus 96-h-old fish (BIOACC B). Thirty fish from each age group were placed in three separate exposure chambers to avoid crowding effects (10 fish per chamber) containing 100-mL solutions for the five exposure periods (i.e., 150 fish for each age group). To provide adequate biomass for Cu analysis, after each exposure period fish from the three exposure chambers were composited into a single replicate containing ~30 fish (3 chambers $\times$ 10 fish/chamber, minus mortalities). After collection, the fish were rinsed three times with 18-M$\Omega$ water to remove Cu-contaminated exposure water and weakly sorbed Cu. The fish were dried overnight at 50 °C and then digested in 70% HNO$_3$, after which the tissue digest was diluted to 5 mL with 18-M$\Omega$ water and two analytical aliquots were analyzed for Cu by GFAAS. Cu concentrations of tissue samples had coefficients of variation <1%, except for time zero (<15%) for two analytical replicates, indicating that our analyses were adequately sensitive to detect even small changes in tissue Cu concentrations.

In BIOACC 1 and BIOACC 2, we exposed the fish to six (BIOACC 1) or five (BIOACC 2) Cu concentrations (Table 2) for 24 h. To provide adequate biomass for tissue analysis, each of three replicates for each Cu concentration consisted of 30 fish, which we separated into groups of 10 fish during exposure. Thus, 90 fish in nine chambers were exposed to each Cu concentration (i.e., three replicates containing 30 fish each). At the end of the experiment, fish from the nine chambers containing the same Cu concentration were randomly composited into three final replicates that contained ~30 individuals each (3 chambers $\times$ 10 fish minus mortalities). Fish were analyzed for Cu concentration as described for BIOACC A and BIOACC B. BIOACC 2 was conducted to determine whether we could replicate initial findings from BIOACC 1.

### Table 2

<table>
<thead>
<tr>
<th>Cu ($\mu$mol$\cdot$L$^{-1}$)</th>
<th>BIOACC 1</th>
<th>BIOACC 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.09</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td>0.5 ± 0.03</td>
<td>1.1 ± 0.01</td>
<td>2.7 ± 0.07</td>
</tr>
<tr>
<td>2.2 ± 0.09</td>
<td>3.8 ± 0.07</td>
<td>3.7 ± 0.53</td>
</tr>
</tbody>
</table>

2.5. Determinations of Cu bioaccumulation, Cu–FHM complexation constants, and aqueous Cu speciation

We plotted results from time-dependent experiments of Cu accumulation (BIOACC A and BIOACC B) to examine the required exposure time for whole-body, steady-state Cu bioaccumulation by larval FHM. In addition, bioaccumulation by 24-h-old versus 96-h-old fish were compared to investigate potential age effects on Cu accumulation. Nonlinear regression was performed using Sigma Plot 2002.

Whole-body burdens of Cu for FHM from BIOACC 1 and BIOACC 2 were used to calculate $K_{Cu-FHM}$ and $[L_{Cu-FHM}]$ for a single-ligand model of Cu complexation to larval FHM, similar to analyses by Playle et al. (1993b) and MacRae et al. (1999). To calculate Cu complexation constants, we compared regression analyses of Scatchard plots and Langmuir isotherms (Scatchard, 1949; Tipping, 2002), both of which are appropriate analytical methods for small ranges of metal binding, typically within one order of magnitude (Tipping, 2002). They produced results similar to nonlinear regression for a single-ligand model (MacRae et al., 1999). For each approach, we first determined the $[$Cu$^{II}$]$ in each exposure solution by entering the water chemistries and total Cu concentrations in Tables 1 and 2 into MINTEQA2. In Scatchard analyses, the whole-body Cu concentration adjusted by subtracting whole-body Cu in control FHM mg$^{-1}$ dry weight of fish [$[Cu_{observed}]]$ is plotted versus the ratio of [Cu$_{bound}$/Cu$_{total}$]. The negative of the slope is $K_{Cu-FHM}$, and the ordinate intercept is $K_{Cu-FHM} \times [L_{Cu-FHM}]$. Using a Langmuir transform, the ratio $[Cu^{II}_{observed}/Cu_{total}]$ is plotted versus $[Cu^{II}]$. The inverse of the slope is $[L_{Cu-FHM}]$, and the ordinate intercept is $K_{Cu-FHM} \times [L_{Cu-FHM}]$. To calculate the potential influence of FHM on aqueous $[$Cu$^{II}$]$ in the test waters of each bioaccumulation experiment, we entered water chemistries (Table 1) and Cu complexation parameters for FHM into MINTEQA2.

3. Results

3.1. Time-dependent Cu bioaccumulation

In BIOACC A and BIOACC B, the whole-body accumulation of Cu by larval FHM increased rapidly until approaching an asymptote in exposure durations greater than 12 h (Fig. 1). Thus, 24 h is a reasonable exposure period for bioaccumulation experiments with larval FHM. Because Cu accumulation was nearly identical after 12 h between 24- and 96-h-old fish (the full age range of fish used in standard static-renewal toxicity tests), we concluded that the slight difference in age had no effect in Cu complexation, and described the combined data from both experiments using single-ligand nonlinear regression.

3.2. Binding affinities and binding-site densities of Cu–FHM complexation

Untransformed Cu bioaccumulation as a function of total Cu concentration was not different between the two concentration-
dependent experiments (BIOACC 1 and BIOACC 2, based on the overlap of 95% confidence intervals, Fig. 2A). Plotting Cu bioaccumulation versus \( \{\text{Cu}^{2+}\} \) (instead of total Cu) simply phase-shifted the plot but the data relationships were virtually identical (data not shown). \( \log K_{\text{Cu–FHM}} \) values calculated from Scatchard analysis were 6.65 (BIOACC 1) and 6.63 (BIOACC 2), in good agreement with values calculated by Langmuir analysis (6.48 in BIOACC 1 and 6.64 in BIOACC 2; Fig. 2A and B; Table 3). Binding-site densities estimated by the two methods were also similar (Table 3). The variation in these regressions are comparable to the variation in Cu binding on adult FHM gills using Langmuir isotherms to calculate Cu-gill stability constants (Playle et al., 1993b). Between the two experiments, tests for equality of slopes and intercepts (Zar, 1996) for either Langmuir analyses or Scatchard plots demonstrated that results were not different (all \( t_{\text{calc}} < 1.8, t_{\text{crit}} = 2.015 \)). Thus, we combined data from the two experiments (BIOACC 1 and BIOACC 2) and report values from the Langmuir analysis because of the strong fit of that regression analysis (Table 3). We entered these values into MINTEQA2 calculations of Cu speciation in the presence of fish.

### Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Slope ((\text{L} \text{mol}^{-1}))</th>
<th>Ordinate ((\text{L g}^{-1} \text{dw}))</th>
<th>(\log K_{\text{Cu–FHM}})</th>
<th>Regression</th>
<th>Binding-site concentrations ((\text{g dw mol}^{-1}))</th>
<th>(\log K_{\text{Cu–FHM}})</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOACC 1</td>
<td>3.52</td>
<td>1.27</td>
<td>6.55</td>
<td>0.75</td>
<td>0.025</td>
<td>0.361</td>
<td>4.3 × 10^{-5}</td>
</tr>
<tr>
<td>BIOACC 2</td>
<td>4.25</td>
<td>1.82</td>
<td>6.63</td>
<td>0.96</td>
<td>0.004</td>
<td>0.427</td>
<td>5.1 × 10^{-5}</td>
</tr>
<tr>
<td>Combined</td>
<td>3.70</td>
<td>1.49</td>
<td>6.57</td>
<td>0.66</td>
<td>0.002</td>
<td>0.402</td>
<td>4.8 × 10^{-5}</td>
</tr>
</tbody>
</table>

*a g dw refers to g dry weight of fish.

**3.3. Effect of Cu–FHM complexation on aqueous \( \{\text{Ca}^{2+}\} \)**

Accumulation of Cu by larval FHM had little impact on MINTEQA2-calculated Cu speciation in test waters because the small mass of larval fish in the exposure chambers (0.00012 g dry weight fish\(^{-1}\) with 10 fish chamber\(^{-1}\)) contributed very few binding sites compared to the total amount of Cu in the system. Regardless of the presence or absence of larval FHM, proportions of Cu-inorganic species were constant across the range of total Cu concentrations [([Cu\(_{\text{total}}\)]) in BIOACC 1 and BIOACC 2.
Fig. 2. Data from bioaccumulation experiments 1 (BIOACC 1) and 2 (BIOACC 2) shown as (A) whole-body Cu concentration adjusted by subtracting whole-body Cu in control FHM (\([\text{Cu}_\text{bound}]\)) as a function of total Cu (\([\text{Cu}_\text{Tot}]\)), (B) Scatchard plots, and (C) Langmuir plots for the binding of Cu to larval fathead minnows. Regressions in (B) and (C) were calculated from the combined data from BIOACC 1 and BIOACC 2 experiments. Each datum represents the value from three replicates (30 fish per replicate, 90 fish per treatment). Fish were exposed for 24 h to varied Cu concentrations (Table 2) in matrix water (Table 1); dw: dry weight.

4. Discussion

4.1. Biological considerations of Cu bioaccumulation

In addition to aqueous geochemistry, it is important to consider biological affinities and complexation capacities for metals when predicting metal accumulation in aquatic organisms. In determining the role of the biotic ligand to Cu bioavailability, it is important to consider rates of biological uptake and depuration in addition to aqueous geochemistry. If uptake kinetics vary as they do for silver on the gills of rainbow trout (Oncorhynchus mykiss) and European eel (Anguilla anguilla), then bioaccumulation depends on the sampling interval. In those situations, gill loading is not an appropriate endpoint for modeling metal complexation to biotic ligands (Wood et al., 2002). The rate of Cu removal from the gill is determined by physiological mechanisms such as transport across the basolateral membrane into the circulatory system (Bury and Wood, 1999; Bury et al., 1999) or mucous sloughing from the gill surface (McDonald et al., 1991). If removal rates are much slower than binding onto the gill surface, Cu concentrations on the gill reach a dynamic steady-state with the exposure water. The biotic ligand then exchanges Cu\(^{2+}\) in exposure waters without additional net gain, essentially acting as an ion-exchange resin for Cu. In those instances, uptake kinetics of Cu at the gill are relatively linear until eventually reaching an asymptote (Laurén and McDonald, 1986; Giles, 1988; Playle et al., 1993a,b; Hollis et al., 1997; Grosell et al., 1999). Because accumulation kinetics in BIOACC A and BIOACC B followed this pattern (Fig. 1), biotic loading was not dependent on sampling interval in exposures exceeding 12 h. In addition, a single-ligand nonlinear regression described binding trends in BIOACC A and BIOACC B (Fig. 1). Thus, although we did not directly measure depuration, we were able to calculate the Cu-fish conditional stability constants and binding site densities for larval FHM from bioaccumulation measured during the succeeding BIOACC 1 and BIOACC 2 experiments (Table 3).

4.2. Cu complexation by larval FHM and effects on aqueous Cu concentration

Cu complexation parameters have been directly measured for the gills of several adult and juvenile fish species (Table 4). Binding affinities (log \(K\)) reported here and in the literature vary from 6.5 to 8.4 (Playle et al., 1993b; MacRae et al., 1999; Taylor et al., 2003). Some of this variability may stem from pH-dependent differences among studies. However, at pH \(\sim 7\) the binding affinities for Cu on the gills of juvenile trout (O. mykiss) and yellow perch (Perca flavescens) are \(\sim 100\) fold higher (i.e., \(\sim 2\) log units) than the whole-body \(K_{\text{Cu-FHM}}\) of larval fathead minnows. Some apparent differences between larval FHM and trout or perch may arise because Taylor et al. (2003) collected samples from 3 h exposures without demonstrating that accumulation was independent of exposure duration (Taylor et al., 2000), but rather based their exposure time on that required for asymptotic concentrations in FHM (Playle et al., 1992). Nonetheless, such variation in binding affinity suggests that species-specific
or developmental differences may be maintained regardless of the geochemistry of their aquatic ecosystem. Disregarding potential pH effects, the whole-body Cu-binding affinity of larval FHM is approximately 10-fold lower than that of the adult fathead gill (log $K_{\text{Cu-FHM}} = 7.4$; Playle et al., 1993b). Assuming larval FHM are 90% water, their ~10-fold greater whole-body binding-site densities $g^{-1}$ dry weight are similar to the literature values for binding-site densities $g^{-1}$ wet weight on gills of older fish. Given dissimilar log $K_{\text{Cu-FHM}}$ values for larval and adult FHM, we expected that the lethal concentrations of Cu causing 50% mortality (LC50) would differ between larvae and adults. However, reported LC50s (median lethal concentrations) of Cu for larval FHM in waters with ≤1.8 mg DOC L$^{-1}$ are 0.11 μmol Cu L$^{-1}$ (pH 6.5, hardness = 0.08 meq L$^{-1}$; Erickson et al., 1996) and 0.08 μmol Cu L$^{-1}$ (pH 7.06, hardness = 0.40 meq L$^{-1}$; Welsh, 1996). These values are comparable to the LC50 value of 0.16 μmol Cu L$^{-1}$ for adult FHM at pH 6.6 and hardness of 0.27 meq L$^{-1}$ (Playle et al., 1993b). Despite their lower log $K_{\text{Cu-FHM}}$, which suggests that Cu complexation by larvae is less thermodynamically favorable than for adults, it appears that larval fish are equally susceptible to aqueous Cu. Therefore, larval FHM might have higher sensitivity to Cu accumulated in or on their bodies than adult FHM. That is, they have lower LA50s because the LA50 is directly proportional to $K$ (Di Toro et al., 2001).

Several mechanisms might explain this apparent discrepancy between radically different conditional stability constants and virtually identical LC50 values. Respiratory tissues are the most sensitive biologic receptors. In larval life stages, the epithelium is the primary respiratory organ (Potter et al., 1996; von Herbing et al., 1996). If high-affinity sites on the developing gill were diluted by low-affinity sites on non-keratinized epithelium, then similar Cu toxicity might result despite the low, net binding affinity of larval FHM compared to the fully developed adult gill. Aside from this affinity dilution, the two different life stages might respond differently to cations such as Ca$^{2+}$ and Mg$^{2+}$ that compete with Cu for complexation on sensitive biological surfaces. Such cation competition for binding sites can substantially alter metal toxicity (Playle et al., 1993b; McGee et al., 2000; Macdonald et al., 2002). Furthermore, as with smaller animals, larval fish might have a higher sodium turnover rate, which leads to greater sensitivity from faster depletion of internal sodium at the same relative inhibition of sodium uptake (Grosell et al., 2002). Finally, several studies show that Cu-inorganic complexes such as CuCO$_3$ and Cu(OH)$_2$ contribute to toxicity (Shaw and Brown, 1974; Howarth and Sprague, 1978; Chakoumakos et al., 1979; Laurén and McDonald, 1986). It is unknown how susceptibility to Cu-inorganic complexes changes as respiratory physiology develops. Any of these potential differences in toxic mechanism could be less thermodynamically favorable, but require the same binding site density to cause mortality. Based on our current data, we cannot exclude any of these possible mechanisms. However, our findings are consistent with studies showing that metal toxicity depends on metal–gill concentrations (Playle et al., 1993b; McGee et al., 2000; Macdonald et al., 2002).

Apparently, the log $K_{\text{Cu-FHM}}$ values of the biotic ligand for Cu have less influence on Cu bioavailability to larval FHM so long as their binding-site densities that determine metal–tissue loadings are comparable, and there is an excess of [Cu$^{2+}$] in the water column. An excess of [Cu$^{2+}$] was clearly the case in our experiments because the presence or absence of larval FHM made no substantive difference in the proportions of [Cu$^{2+}$] or other Cu species at any Cu concentration. In the exposure solutions, larval FHM complexed ≤0.2% of the total Cu. At this low percentage of the total Cu, the larval densities would have to increase at least 50-fold to 5000 fish L$^{-1}$ to complex 10% of the total Cu (Table 3). This is consistent with the apparent discrepancy of Meyer (1999) that bioaccumulation of Cu by rainbow trout gills does not considerably alter the bulk-water metal speciation of Cu at realistic biomass densities in exposure waters containing Cu concentrations near the LC50. Thus, bioaccumulation may differ greatly without apparent change in bulk water chemistry, meaning that binding site densities (and possibly binding affinities in other species or life stages of aquatic biota) are a necessary link between water chemistry and biologic uptake in biotic ligand models.

4.3. Implications for future research

Our data provide a reasonable fit to a one-ligand, bioaccumulation model. However, our tests did not determine depuration kinetics, which require analyses of decreases in Cu burdens over time.
time in Cu-free waters. Such accumulation-depletion experiments with different [Cu] and [DOC] in natural waters are needed with larval fish and adults of several aquatic species to determine whether Cu complexation is subject to competition from cations such as H\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\).

Possibly because total loading on biotic ligands appears to differ from that in Cu-free waters. Such accumulation-depletion experiments with different [Cu\(^{2+}\)] and [DOC\(_\text{aq}\)] hold constant could elucidate whether Cu complexation is subject to competition from cations such as H\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\).

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