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Determination of Copper Binding Affinity of *Ceriodaphnia dubia* Using Competition Bioassay Tests: Environmental Significance

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The speciation of trace metals in natural waters is important to their bioavailability and therefore to toxicity and water quality criteria (WQC) (Allen et al. 1980; Luoma 1983). Many researchers demonstrated that bioavailability or toxicity of trace metals is directly correlated to concentration of free metal ions rather than to total or complexed metal concentrations (Campbell 1995). Furthermore, binding of copper to biotic surfaces and subsequent toxicity to aquatic biota is influenced by copper complexation with naturally occurring inorganic and organic ligands. The gill surfaces are composed of several functional groups with negative charge (Pagenkopf 1983) and metals form complexes with these negative charged materials on gill surfaces. Copper complexation to the gill surface is especially fast, typically occurring in about 1 hr (Playle et al. 1992). In natural aquatic environments, aquatic organisms are placed in the situation where natural ligands and biotic ligands on gill surfaces compete for cations including metal ions. The presence of strong ligands in the water prevents the metal from interacting with receptor sites on the organism and the complexed copper with organic ligands is not affected by the binding affinity of the biological surface. Besides, the strong ligands facilitate the excretion of metals from tissues (Muramoto 1980). However, if the metal is only weakly complexed with ligands in the water, the metal will be transferred to the stronger biotic ligands having greater copper binding affinity on the gill surfaces (MacRae et al. 1999) and toxicity may ensue.

It is important to understand that the toxicity of metals depends on the binding affinity of metals to aquatic organisms and to know the stability constant of metals to predict metal toxicity of aquatic organisms, especially primary indicator species. However, most biotic ligand models apply uniformly the same biological stability constant for indicator species though they may have different strength of binding affinity. The objective of this study is to determine the copper binding affinity of *Ceriodaphnia dubia* using the competitive bioassay test with different synthetic organic ligands ranging from those with high stability constant to those with low stability constant. In addition, the results obtained from biotic ligand model (Di Toro et al. 2000) were compared with the results by Scatchard plot method (MacRae et al. 1999) for *C. dubia*.

MATERIALS AND METHODS

All reagents were analytical grade and were used without further purification. All the glassware, as well as test cups, was soaked in 10% HNO_3 (v/v) for at least 48 hr before use. Deionized water from a Barnstead NANOpure ultrapure water system was used throughout the study. Six organic ligands having various copper binding affinities were selected for this study. The list of ligands and their stability constants for copper are tabulated in Table 1. All ligands (Sigma, NJ) were used without further purification.

Table 1. Organic ligands used in competition bioassay and $logK_{Cu}$ (Martell and Smith, 1977).

Organic acid	Formula	MW (g)	$LogK_{Cu}$*
Ethylenediamine	$C_2H_8N_2$	60.1	8.5
Picolinic	$C_6H_5NO_2$	23.1	8.39
Citric	$C_6H_8O_7$	192.1	7.18
Oxalic	$C2H2O_4 \cdot H_2O$	108.1	6.22
Malonic	$C_3H_4O_4$	104.1	5.69
Tartaric	$C_4H_6O_2$	150.1	4.29

* The $logK_{Cu}$ of the organic ligand is at the experimental pH (pH = 8.0).

The test organism used in this study, *Ceriodaphnia dubia* and the food, *Selenastrum capricornutum* and Yeast, trout chow, and Cerophyll® (YTC) mixture were purchased from Aquatic BioSystem Inc. (Fort Collins, CO). Organisms were cultured and handled following the procedures outlined in the U.S. EPA (1993) manual. The detailed culturing and toxicity testing conditions are summarized in Ma et al. (1999). Static toxicity tests were performed to determine the copper toxicity to *C. dubia* in the dilution water according to EPA manual. Free copper was determined using ion selective electrode (ISE), and pH and DO were determined before and after the 24 hr exposure period. Mortality was determined for seven copper concentrations and one control.

In order to evaluate the binding capacity of added synthetic organic ligands with free copper ions in test waters, the titration of copper was first performed by adding synthetic organic ligands to a 5×10^{-7} M copper solution. The total copper concentration of 5×10^{-7} M was chosen to achieve at least 10% survival of test organisms in the bioassay cups. The static toxicity test in the absence of any organic ligands indicated that approximately 5×10^{-7} M of total copper is needed to achieve 10% survival of test organisms (Kim 1999). Copper was added as $CuSO_4 \cdot 5H_2O$ to test waters containing ligands and allowed to reach equilibrium before placing the copper-ligand solutions in the bioassay chambers. Competitive bioassay tests were carried out using 30-mL plastic cups with a liquid volume of 15 ml as the static exposure chamber. Based on the results of Cu titration curves with ligands, two ligand concentrations per each ligand were selected to obtain a range of percent survival (i.e. high percent survival and medium percent survival) at a fixed total copper concentration of 5×10^{-7} M. For each ligand, a control cup

without copper was also set-up to assess the toxicity of ligands to *C. dubia* neonates. The pH of the test solution was adjusted to pH 8.0 using 0.1 N HNO_3 and 0.1 N NaOH prior to the introduction of test organisms. Twenty *C. dubia* neonates were placed into four replicated test solutions (15 ml) and after a 24-h exposure period, percent survival of *C. dubia* neonates was measured by counting the number of living organisms. Free copper and total copper concentrations in the bioassay cups were measured after the 24-hr exposure period.

A Perkin-Elmer (Norwalk, CN) Model 5000 atomic absorption spectrophotometer with a graphite furnace accessory was used for the analysis of total copper concentration. The free Cu^{2+} activity was determined by a cupric ion selective electrode (Cu-ISE, model 94-29, Orion Research, Boston, MA). Detailed descriptions of Cu-ISE apparatus and calibration procedure were presented in Ma et al. (1999). The total concentrations of Cu and EN and the measured pH were put into the MINEQL+ speciation model (Schecher and McAvoy 1992) to calculate the activity of free Cu^{2+} at each of the points in the calibration.

RESULTS AND DISCUSSION

The copper titration curves for various ligands are shown in Figure 1. The results show that the degree of complexation between organic ligands and copper ions increases with increasing stability constant. The curves allowed us to determine the appropriate ligand concentration for obtaining the desired free copper concentration in the test solution containing the fixed total copper concentration of 5×10^{-7} M. In order to normalize the bioassay results by the free copper ion concentrations in the test cups rather than total Cu concentrations, the Cu^{2+} concentrations were measured after 24-hr using a ion selective electrode (ISE). The normalization of the bioassay results from static tests in the absence of organic ligands by the free copper ion concentrations resulted in a single toxicity curve (Figure 2). From the linear regression analysis, the relationship between survival percentage and Cu^{2+} concentrations was obtained:

$$\text{Survival (\%)} = 138 - 2.8 \times 10^{10} [Cu^{2+}] \qquad (1)$$

with a correlation coefficient of 0.86 (n = 21). The results demonstrate that the bioavailability or toxicity of copper is directly correlated to the free copper ion activity. In addition, the average toxic range of free copper ions to *Ceriodaphnia dubia* was 1.7×10^{-9} to 5×10^{-9} M and the free copper LC_{50} value to *C. dubia* was calculated as 2.84×10^{-9} M by the probit analysis method (Finney 1971).

To estimate the Cu-binding constant (conditional stability constant) of the biological surface of *Ceriodaphnia dubia*, the bioavailability of copper bound to the ligands was examined with known stability constants. Figure 3 shows the response of *C. dubia* neonates to the measured free Cu^{2+} concentrations in the bioassay cups containing various organic ligands with known stability constants. All test organisms survived in the control cups containing no copper, indicating that the selected ligand concentrations did not have any toxic effects on test organisms. The free copper normalized bioassay data were also compared to the free copper toxicity curve obtained from the static tests in the absence of organic

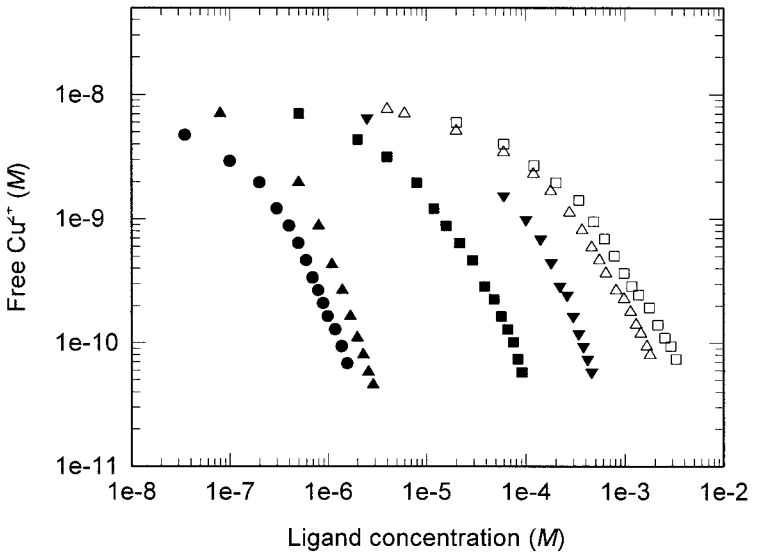

Figure 1. Copper titration using different synthetic organic ligands at pH 8.0 in dilution water. Free Cu^{2+} was measured by Cu-ISE. ●, Ethylenediamine; ▲, Picolinic acid; ■, Citric acid; ▼, Oxalic acid; △, Malonic acid; □, Tartaric acid.

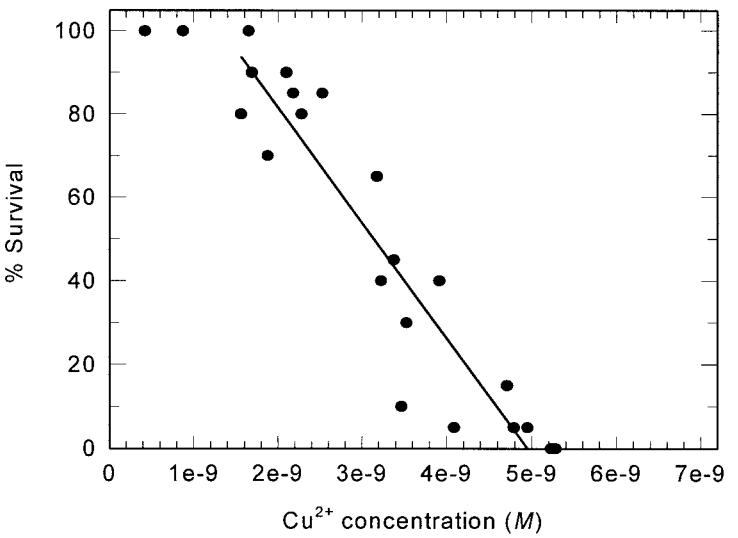

Figure 2. Effect of free Cu^{2+} on *C. dubia* survival in 24-hr static test. The linear regression result was: $\%[S] = 138 - 2.8\times10^{10}[Cu^{2+}]$.

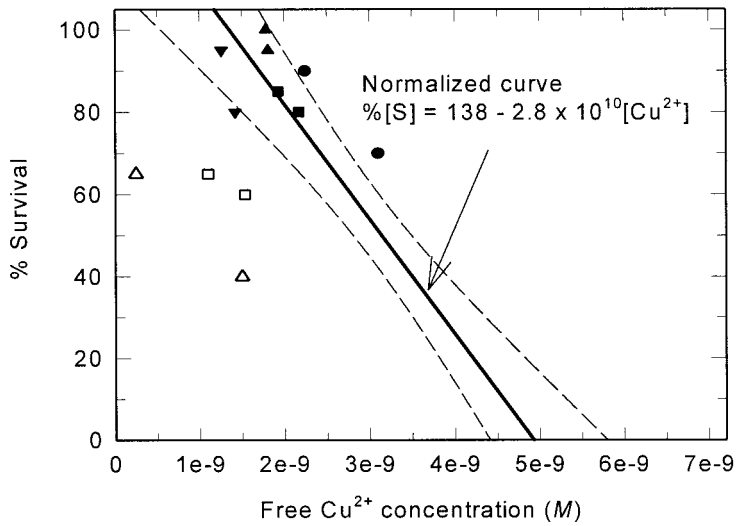

Figure 3. Effect of synthetic organic on copper toxicity of *C. dubia* in 24-hr static bioassay tests. The broken line represents the 95% confidence interval. ●, Ethylenediamine; ▲, Picolinic acid; ■, Citric acid; ▼, Oxalic acid; △, Malonic acid; □, Tartaric acid.

ligands (Figure 3). The survival data in the presence of ethylenediamine, picolinic acid, citric acid, and oxalic acid closely fitted the free copper toxicity curve while the survival data from the toxicity test with malonic acid and tartaric acid significantly deviated from the free ion activity model (FIAM) curve from Figure 2. For a malonic acid concentration of 1.2×10^{-4} M, the observed percent survival was 65% even though the percent survival of *C. dubia* should be 100% at the 2.4×10^{-10} M of free copper concentration present according to the FIAM from equation (1). This deviation from the free copper toxicity curve may be attributed to the exchange of free Cu^{2+} from the low affinity binding site (malonic acid) to the high affinity binding site (biotic surface). A statistical test revealed that these deviation of malonic acid and tartaric acid from the free ion activity model (FIAM) curve was significant (at 95% confidence interval) when the results from the same free copper concentration were compared. This result suggests that the stability constant of the copper binding sites on *C. dubia* is greater than the stability constant value for malonic acid ($\log K_{Cu} = 5.69$). On the other hand, percent survival of *C. dubia* in the presence of organic ligands having higher Cu stability constants than the stability constant of the binding site on *C. dubia* would not deviate from the FIAM curve because the free Cu^{2+} complexed with such organic ligands would remain in their sites rather than being exchanged to the biotic surface sites. Since the bioassay data for oxalic acid and the other ligands having higher stability constants was closely predicted by the FIAM, the stability constant of *C. dubia* is probably less than that of oxalic acid ($\log K_{Cu} =$

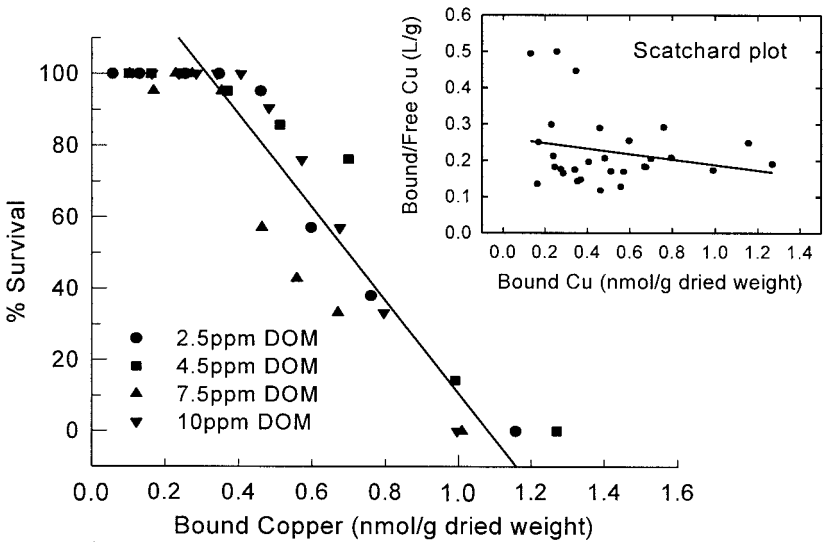

Figure 4. Bound copper concentration to *Ceriodaphnia dubia* in 24-hr acute test. Copper-DOM solution was equilibrated for 24 hr before exposing *C. dubia* to solution. Bound copper concentration was calculated using Biotic Ligand Model (BLM).

6.22). As a result, the apparent binding affinity of copper to *C. dubia* ($\log K_{Cu}$) is estimated between 5.69 and 6.22.

Using competitive bioassay, MacRae et al. (1999) determined that the copper binding affinity for the rainbow trout gill was between 6.4-7.2 (log K). Other studies showed wide range of copper binding affinities; the apparent binding affinity constant (log ABA) for fathead minnows gills was 7.4 (Playle et al. 1993) and the conditional complexation constants of Cu(II) to algae, *Chlamydomonas rheinhardii,* ranged from 8.4-10.0 at pH 5.0-6.5 (Xue and Sigg 1990). Reid and McDonald (1991), however, reported the extremely lower copper gill binding constant ($\log K = 2.7$) for rainbow trout.

By using Biotic Ligand Model (Di Toro et al. 2001), the bound copper concentration was calculated in order to compare our stability constant results with the values by Scatchard plots method (MacRae et al. 1999). It was assumed that the 10 % of humic acid exist in the dissolved organic matter because of treatment DOM with H^+-saturated resin. Figure 4 shows that % survival of *C. dubia* has a good relationship with bound copper concentration to *C. dubia* in the presence of natural dissolved organic matter that collected from Suwanee River in George (Kim et al. 1999). The bound copper LC_{50} is approximately 0.7 nM/g_w, which is much lower than that fixed in Biotic Ligand Model (BLM) for the calculation of LC_{50}. Moreover, considerably low regression value was obtained

from the Scatchard plot compared the calculated bound copper from BLM versus the measured free copper concentrations, and the *C. dubia* binding affinity to copper was not found from the Scatchard plot. This result indicates that the BLM method does not support the results from Scatchard plot method for *C. dubia* because the same value of stability constant applies equally to all test species in BLM program. Therefore, it is essential that the biological stability constant of copper should be considered to predict the bioavailability of copper for *C. dubia* bioassay test and not be applied uniformly to model approaches for all test species.

Natural waters contain many kinds of organic (humic acid, fulvic acid and particulate, etc.) and inorganic ligands that affect the toxicity of metal to aquatic organisms. Considering the gill-copper binding affinity, dissolved organic carbon (DOC) having high binding affinity constants in natural environments is an effective source for protecting aquatic organisms from metal toxicity. According to Kim et al. (1999), the natural DOM has a significant effect to decrease the toxicity on aquatic organisms. Owing to natural organic ligands having stronger binding affinity to copper than biological surfaces, copper accumulation to aquatic organisms significantly decreases and thereby the lethal impacts of copper on aquatic organisms decrease. In addition, the results of this study suggest that some of copper bound to dissolved organic matter (DOM) may be bioavailable to aquatic organisms if the DOM contains some organic ligands that have lower Cu stability constant than aquatic organisms. It is important to know the stability constant of the test organism in order to correctly predict its response to the metals in the natural environments containing a mixture of ligands.

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