

Fractionation of Fulvic Acid by Iron and Aluminum Oxides—Influence on Copper Toxicity to *Ceriodaphnia dubia*

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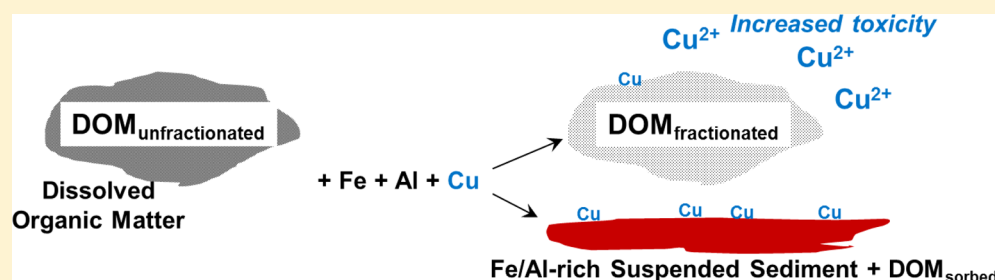
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S Supporting Information



ABSTRACT: This study examines the effect on aquatic copper toxicity of the chemical fractionation of fulvic acid (FA) that results from its association with iron and aluminum oxyhydroxide precipitates. Fractionated and unfractionated FAs obtained from streamwater and suspended sediment were utilized in acute Cu toxicity tests on *Ceriodaphnia dubia*. Toxicity test results with equal FA concentrations (6 mg FA/L) show that the fractionated dissolved FA was 3 times *less* effective at reducing Cu toxicity (EC_{50} $13 \pm 0.6 \mu\text{g Cu/L}$) than were the unfractionated dissolved FAs (EC_{50} 39 ± 0.4 and $41 \pm 1.2 \mu\text{g Cu/L}$). The fractionation is a consequence of preferential sorption of molecules having strong metal-binding (more aromatic) moieties to precipitating Fe- and Al-rich oxyhydroxides, causing the remaining dissolved FA to be depleted in these functional groups. As a result, there is more bioavailable dissolved Cu in the water and hence greater potential for Cu toxicity to aquatic organisms. In predicting Cu toxicity, biotic ligand models (BLMs) take into account dissolved organic carbon (DOC) concentration; however, unless DOC characteristics are accounted for, model predictions can underestimate acute Cu toxicity for water containing fractionated dissolved FA. This may have implications for water-quality criteria in systems containing Fe- and Al-rich sediment, and in mined and mineralized areas in particular. Optical measurements, such as specific ultraviolet absorbance at 254 nm ($SUVA_{254}$), show promise for use as spectral indicators of DOC chemical fractionation and inferred increased Cu toxicity.

INTRODUCTION

The chemical speciation of metals influences their bioavailability and toxicological effects on aquatic organisms, with free ions typically being the most bioavailable form. The toxicity of dissolved Cu to aquatic organisms has been shown to depend on Cu^{2+} concentration, dissolved organic matter (DOM) and other factors.^{1–5} DOM, which is derived from degradation of plant and animal matter, is an important constituent in many natural waters and has been demonstrated to be an important control on metal speciation, transport, and toxicity in aquatic systems.^{6–9} Some of the functional groups on DOM, such as carboxylic acid groups and amino acid residues, have a high binding affinity for metals,^{10–14} and DOM can reduce metal toxicity by complexing with metal cations and making them less bioavailable to aquatic organisms.⁹ This protective effect of

DOM against metal toxicity to aquatic organisms has been well documented by several researchers for $\text{Cu}^{1–4,15–25}$ and other metals, and the degree of protection can be related to the metal-binding affinity and capacity of the DOM.^{10,26–29}

Federal regulatory authorities recognize that DOM is an important water-quality variable that affects the acute toxicity of many metals, and the U.S. Environmental Protection Agency (U.S. EPA) has incorporated a biotic ligand model developed by HydroQual³⁰ (the “Cu BLM”) into the aquatic life ambient freshwater quality criteria for Cu.³¹ The Cu BLM is used in this

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study to evaluate aquatic Cu toxicity in test solutions containing fractionated or unfractionated FA. The Cu BLM is a predictive toxicological model that mathematically estimates the effects of water chemistry on the speciation of metals and their acute toxicity to aquatic organisms.^{32–34} It can be used to determine site-specific water-quality criteria, site-specific metal toxicity, and to assess aquatic risk for metal exposure. Given select parameters of site-specific water chemistry, a chosen metal, and a chosen organism, the Cu BLM can predict the EC₅₀ for the pairing. The EC₅₀ is the median effective concentration of the toxicant that produces an adverse response, such as lack of mobility in the case of daphnia species, for 50% of a group of test organisms within a given time frame (typically 48 to 96 h). The Cu BLM can be used to perform calculations for several combinations of a metal and an aquatic organism, one pair at a time. It applies to dissolved and freshwater systems only, assumes equilibrium, and computes speciation of the chosen metal between its various inorganic and organic complexes and with a *biotic ligand* (BL, e.g., gill surface). The required input includes pH, temperature, and concentrations of Ca, Mg, Na, K, sulfate, chloride, alkalinity, dissolved organic carbon (DOC), and the metal of interest. The Cu BLM uses the Windermere Humic Aqueous Model (WHAMV)^{10,35} to estimate metal binding with DOC. The WHAMV data set was based almost entirely on laboratory data from studies of the binding of single metals by isolated humic substances. There are several varieties of BLMs,^{36–38} and other types of BLM modeling approaches have been developed that successfully address the complexity of multiple metal mixtures, speciation and toxicity in natural systems.^{5,39–41} These new approaches are expansions of the original concepts contained in the Cu BLM.

In many mined or mineralized environments, Fe and Al are present in acidic, metal-enriched streams and precipitate as oxide and oxyhydroxide phases under increasing pH conditions. Iron oxide minerals are known to sorb dissolved metals in these environments,⁴² and to sorb or coprecipitate with DOM.^{43,44} McKnight et al.⁴⁵ demonstrated that DOC behaved non-conservatively at the confluence of an acidic, metal-enriched stream with a nonimpacted stream, and as a consequence molecules with greater contents of aromatic moieties, carboxylic acid groups, N- and S-containing groups, and amino acid residues were preferentially sorbed by Fe- and Al-rich oxyhydroxide precipitates. These interactions resulted in chemical fractionation of DOM between the suspended sediment and the dissolved phase.

The DOM source also can affect the properties of DOM that influence the protective effect of DOM against metal toxicity to aquatic organisms, and these source effects have been more widely studied than have the possible effects of DOC fractionation. Terrestrially derived DOM tends to be optically darker and to be enriched in aromatic humic and fulvic substances whereas DOM of autochthonous (microbe- or algae-derived) origin tends to be optically lighter and contain fewer aromatic compounds.⁴⁶ The influence of DOM physicochemical characteristics (quality) on its protective effect is poorly understood due to the complicated nature and heterogeneity of DOM. However, the overall findings of a number of studies indicate that plant-derived DOM of terrestrial origin tends to be more protective against Cu toxicity than does DOM of autochthonous origin. Playle et al.^{9,47} reported the importance of DOM source on protective ability, and subsequent researchers have observed that terrigenous DOM has enhanced protective effects against

metal toxicity compared to autochthonous DOM.^{20,48–51} Al-Reasi et al.⁵² and Wood et al.⁵³ provide a review of the physicochemical characteristics of DOM that have been reported to play a role in reducing metal toxicity. In the case of freshwater Cu toxicity, Pempkowiak et al.⁵⁴ proposed that reduction of Cu uptake by blue mussels (*Mytilus trossulus*) was related to a higher aromaticity (containing more aromatic ring structures or more phenolic groups) of DOM. Richards et al.⁴⁸ demonstrated that DOM optical characteristics were related to differences in toxicity from metal mixtures (including Ag, Cd, Co, Cu, Hg, and Pb) to rainbow trout (*Oncorhynchus mykiss*) in systems containing DOM from different sources. De Schampelaere et al.²⁹ reported that DOM from different sources differ in their ability to decrease acute Cu toxicity to the freshwater-water flea *Daphnia magna*, and Stoiber et al.⁵⁵ report variable Cu uptake by the alga *Chlamydomonas reinhardtii* when different humic acids were present in the exposure media. Al-Reasi et al.⁵⁶ observed up to a 4-fold difference in protection against Cu toxicity for *D. magna* due to the presence of DOM from different sources. Their results indicate that DOMs of terrigenous origin, being more aromatic, are more protective against Cu toxicity. Al-Reasi et al.⁵⁷ introduced the Proton Binding Index (PBI) to describe proton binding capacities of DOM. They examined the correlation between PBI and various spectroscopic parameters and concluded that optically dark DOM is more protective against metal toxicity and has a greater proton binding ratio. Gheorghiu et al.⁵⁸ examined Cu toxicity and gill binding to *O. mykiss* in water containing different types of DOM and found no significant gill binding differences to explain toxicity differences at low/moderate Cu concentrations. However, they did note reduced gill binding in water with more terrigenous DOM at higher Cu concentrations. They attributed the differences in their results to possible direct or indirect quality-dependent effects of DOM on the gills themselves, which is consistent with the findings of Galvez et al.⁵⁹ Direct interaction of DOM with gills is summarized by Wood et al.⁵³ Collectively, these studies support the premise that altered characteristics of fractionated DOM may influence the protective ability of the DOM against metal toxicity.

Because terrigenous DOM tends to be optically darker, and contains higher molecular weight molecules and more aromatic ring structures than autochthonous DOM, absorbance and fluorescence spectroscopy have been used to distinguish the molecular variability of DOM from various sources.⁶⁰ Al-Reasi et al.⁵² summarized and evaluated various optical techniques that have been used to explain DOM protective ability. They concluded that optical techniques are more diagnostic of protective ability than are analytical techniques such as metal-complexation capacity by titration, elemental content analysis, protein-to-carbohydrate ratio assay, or lipophilicity assay. Optical properties such as the specific absorbance coefficient (SAC)^{48,61} and the fluorescence index (FI)⁴⁶ have been reported to distinguish between DOM sources and composition. Weishaar et al.⁶² demonstrated that specific ultraviolet absorbance at 254 nm (SUVA₂₅₄) is strongly correlated with percent aromaticity for organic matter isolates obtained from a variety of aquatic environments. However, they found that SUVA₂₅₄ did not provide adequate information about reactivity of DOM derived from different types of source materials. De Schampelaere et al.²⁹ demonstrated the utility of UV absorbance at 350 nm and SAC₃₅₀ in determining differences in Cu toxicity to *D. magna* in the presence of DOM from different sources. Other studies also have observed correlations

Table 1. Composition of Artificial Test Water Consisting of Moderately Hard Reconstituted Water Amended with One of the Fulvic Acid (FA) Isolates Used in Laboratory Toxicity Tests

test solution	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	Cl (mg/L)	SO ₄ (mg/L)	DOC ^a (mg/L)	Alk ^b (mg/L)	pH
no added FA ^c	12.9	10.4	23.2	2.1	1.9	78.0	0.3 ^d	45	8.0
SRFA (IHSS) ^e	14.9	13.5	29.8	2.3	2.1	94.1	3.2	45	8.0
Deer Cr dissolved	15.0	13.4	29.4	2.4	2.1	93.4	3.1	45	7.9
Snake R dissolved	14.6	13.5	29.9	2.4	2.2	93.4	3.0	45	8.0
Snake R sediment	15.0	14.6	33.3	2.9	2.7	100.8	3.0	45	8.0

^aDOC = dissolved organic carbon. ^bAlk = alkalinity as mg/L CaCO₃, which was determined for moderately hard reconstituted water. ^cFA = fulvic acid. ^dBackground DOC is near the instrument detection limit and is unlikely to behave like FA with respect to metal binding. ^eSuwannee River Fulvic Acid from the International Humic Substances Society, St. Paul, MN.

between Cu binding affinity and spectral measurements for natural DOM.^{13,20,63,64} Hence, optical measurements might provide information about the degree of chemical fractionation of DOM and the subsequent effectiveness of DOM protection against Cu toxicity.

The focus of this work is to determine if the chemical fractionation observed by McKnight et al.⁴⁵ between FA sorbed by suspended sediment and FA remaining in streamwater is sufficient to produce observable differences in the protective ability of the FA against acute Cu toxicity to aquatic organisms. In this study, we investigated the ability of FA previously isolated by McKnight et al.⁴⁵ from the Snake River and Deer Creek confluence to reduce acute Cu toxicity to *Ceriodaphnia dubia*, a sensitive fresh-water invertebrate commonly used in toxicity testing of contaminants. We conducted toxicity tests in water amended with FAs from (1) the suspended sediment and (2) the water collected from below the confluence of an acidic, metal-enriched stream with a circumneutral stream, (3) water from the circumneutral stream, and (4) Suwannee River FA (SRFA; International Humic Substances Society (IHSS), St. Paul, MN). Furthermore, we sought to identify an optical measurement that would allow for estimation of the degree of FA chemical fractionation. This study is the first to link aquatic Cu toxicity to the in-stream process of Fe- and Al-oxyhydroxide precipitation, which alters DOM characteristics.

MATERIALS AND METHODS

Sampling Sites. In this study, we used FA samples isolated by McKnight et al.⁴⁵ from filtered streamwater and suspended sediment collected downstream of the confluence of an acidic, metal-enriched stream (Snake River) with a circumneutral stream (Deer Creek) near Montezuma, Colorado, U.S.A. (39.579 N, 105.868 W), and from Deer Creek upstream of the confluence. Downstream of the confluence, Fe- and Al-rich sediment coats the streambed as a result of the increased pH when the acidic, metal-enriched Snake River water mixes with the circumneutral Deer Creek water. Sampling sites are shown in Figures 1 and 2 of McKnight et al.⁴⁵ and sampling site designations for FAs are (1) Deer Creek dissolved corresponds to sample site DC5, (2) Snake River dissolved corresponds to sample site SN3, and (3) Snake River suspended sediment corresponds to sample site SN3_{susp}. The downstream Snake River dissolved FA (SN3) and suspended sediment FA (SN3_{susp}) are both examples of fractionated FA, whereas the upstream Deer Creek dissolved FA (DC5) is an example of an unfractionated FA. Suwannee River Fulvic Acid Standard (SRFA; International Humic Substances Society (IHSS), St. Paul, MN) also was used in this study as an example of an unfractionated FA.

The Snake River and Deer Creek are in adjacent small, headwater catchments that consist of a mixture of forest cover, willows, and grasses. The Snake River is acidic and enriched in metals due to the weathering of pyrite present in the basin.⁶⁵ The watershed area is 11.7 km² for the Snake River and 10.4 km² for Deer Creek, and the two streams have nearly equal flows at their confluence.⁴⁵

Fulvic Acid Sources and Isolation. FA samples were isolated by McKnight et al.⁴⁵ according to the methods of Thurman and Malcolm⁶⁶ and Aiken et al.⁶⁷ Stream water from the Snake River and Deer Creek was filtered through 1 and 0.3 μm glass-fiber filters in sequence. Snake River suspended sediment from below the confluence with Deer Creek that was collected on the 1 μm glass-fiber filter was dissolved in 0.1 N HCl. The extract was diluted with distilled water to pH 1.8, and insoluble material was removed by settling and centrifugation. FA was isolated using the XAD-8 resin method. The resulting H⁺-saturated FA was freeze-dried and stored in the dark at room temperature.

Toxicity Tests. Acute 48-h static nonrenewal Cu toxicity tests using *C. dubia* were carried out in artificial test water amended with the four FAs used in this study. U.S. EPA standard operating procedures were used with moderately hard, reconstituted water (MHRW),⁶⁸ and FA stock solutions were prepared by adding each freeze-dried FA to MHRW to obtain a final concentration of 6 mg FA/L. For each FA stock solution, a series of test solutions were prepared by adding Cu (as Cu(NO₃)₂) to obtain a range of five Cu concentrations and one copper-free control, and multiple test series (2–3) were performed with variable Cu concentrations to adequately bracket the EC₅₀ for each FA. Table SI-1 in the Supporting Information (SI) lists Cu concentrations and organism responses for the various toxicity tests. The FA+Cu test solutions were equilibrated for at least 24 h before *C. dubia* were added to allow time for Cu to complex with the FA. At each concentration 4 test chambers, each containing 5 organisms, were used that resulted in a total of 20 test organisms exposed to each concentration. Each of the organisms was less than 24 h old, cultured in MHRW, and fed until 2 h prior to transfer into the test solutions. After 48 h, each test solution was examined to determine immobility of the *C. dubia*. The reported toxicity measure is the 48-h EC₅₀ for the test organisms estimated using OriginPro 9.1 software. The results of the test were considered valid if ≥90% of the *C. dubia* in the control (no added Cu) survived. Copper toxicity tests in MHRW (no added DOC) also were conducted using the same procedures. Measured values of constituents in the toxicity test solutions are listed in Table 1.

Statistical analyses were performed using OriginPro 9.1 software. For these analyses, all of the test series for a given FA

were combined to obtain a global sigmoidal fit of the concentration–response curve. The concentration–response function in OriginPro 9.1 utilizes least-squares regression fits of logit-transformed data. The upper and lower parameters were fixed at 0 and 100 to represent the percentage range for mortality, and the center of the distribution is the estimated EC_{50} value.

Analytical Methods. Toxicity test solutions were analyzed for inorganic elements using inductively coupled plasma-mass spectrometry (ICP-MS).⁶⁹ DOC was analyzed by a UV-catalyzed persulfate oxidation method using a Sievers TOC 800 Turbo instrument on samples acidified to pH <4 with phosphoric acid.

After completion of the toxicity tests, copper ion-selective electrode (Cu ISE) measurements were conducted on the toxicity test solutions using a Cole-Parmer combination ion-selective electrode (model WU-27504-10). The Cu ISE was prepared according to the manufacturer's instructions and then soaked in MHRW for 24 h prior to use. Measured potentials were accepted when the drift was <0.1 mV over 3 min. Because all the test solutions were in the MHRW matrix, the ionic strength of the solutions was not adjusted. The Cu ISE measurements (mV) in the “No added FA” test solutions were converted to free Cu^{2+} concentrations using Visual MINTEQ chemical speciation software (v. 3.0, beta). Input to Visual MINTEQ included measured total Cu concentrations and the composition of the “No added FA” toxicity test solution (Table 1) with no DOC present. The Visual MINTEQ-calculated Cu^{2+} data exhibited a linear Nernstian response down to about $10^{-7.8}$ M. Because much of our Cu^{2+} data are below this concentration, we fit a plot of the Visual MINTEQ-calculated Cu^{2+} data versus measured Cu ISE (mV) to a polynomial function ($R^2 = 0.93$). This polynomial fit extended the range to about $10^{-8.6}$ M Cu^{2+} and was then used to recalculate measured Cu ISE (mV) readings to Cu^{2+} for the test solutions containing DOC from the added FAs (see SI Figure SI-1).

Spectral Characterization. Spectral characterization was conducted on the stock solutions (FA in MHRW) for the four FAs used in this study. Ultraviolet (UV) absorbance was measured on a Beckman DU 800 UV–vis spectrophotometer using a wavelength scan method. Absorbance between wavelengths 200 and 800 nm (1 nm interval) was monitored with the instrument dwelling at each wavelength for 0.05 s using a 1 cm quartz cell. The absorbance reading at 254 nm was used to calculate specific UV absorbance ($SUVA_{254}$, L/mg·m) as follows:

$$SUVA_{254} = ((A_{254}/\text{pathlength})/[\text{DOC}]) \times 100$$

where A is the measured UV absorbance at the stated nm and $[\text{DOC}]$ is the DOC concentration (mg C/L).

Specific absorbance coefficients (SAC , cm^2/mg) measured at 350 nm^{48,61} or 340 nm⁵⁸ were calculated as follows:

$$SAC_{350} = ((2.303 \times A_{350})/\text{pathlength}) / ([\text{DOC}]/1000\text{cm}^3)$$

Absorbance coefficients (ϵ , L/mg·cm) measured at 350 nm²⁹ were calculated as follows:

$$\epsilon_{350} = A_{350}/([\text{DOC}] \times \text{pathlength})$$

Spectrofluorescence of the FA samples was measured by collecting excitation–emission matrices (EEMs) on a Fluoromax 4 (HORIBA Jobin Yvon, New Jersey, U.S.A.). Excitation

wavelengths varied from 240 to 450 nm at 10 nm increments, and emission wavelength varied from 290 to 580 nm at 2 nm increments. The samples were corrected according to Cory and McKnight⁷⁰ by subtracting a Milli-Q blank EEM and then applying instrument-specific excitation and emission corrections. The samples were normalized to the area under the water Raman curve at an excitation wavelength of 350 nm. Finally, the UV absorbance scan was used to account for the inner filter effect. Absorbance at wavelength 254 nm ranged from 0.07 to 0.17 cm^{-1} . For plotting purposes, the EEMs data were interpolated to every 2.5 nm excitation wavelength and every 1 nm emission wavelength⁷¹ (see SI Figure SI-3). The fluorescence index (FI) was calculated using the corrected fluorescence measurements as the ratio between emission wavelengths 470 and 520 nm at excitation wavelength 370 nm.⁷⁰ We used a 13-component parallel factor analysis (PARAFAC)^{71–73} model developed by Cory and McKnight⁷⁰ from EEMs of 379 DOM samples from diverse aquatic environments to compare components of the FAs used in this study (see SI Table SI-2). PARAFAC is a statistical modeling approach that separates a data set of EEMs into mathematically and chemically independent components (single fluorophores or a group of strongly covarying fluorophores) multiplied by their excitation and emission spectra (representing either pure or combined spectra).⁷¹

Biotic Ligand Model (Cu BLM). The HDR/HydroQual Cu BLM v. 2.2.3 (www.hydroqual.com/wr_blm.html)³⁰ was run in “toxicity mode” to predict EC_{50} values for Cu and *C. dubia* using measured values of constituents in the toxicity test solutions as input values (Table 1). The Cu BLM default value for the adjustable parameter for the percentage of humic acid (HA) versus FA is 10% HA. Ryan et al.⁵⁰ reported that adjusting the HA parameter between 1 and 100% HA had little influence on predictions of Cu toxicity to *Pimephales promelas*. Because FAs were used in this study, a value of 0.01% HA was used, and 25 °C was input for the temperature.

RESULTS AND DISCUSSION

Toxicity—Estimated EC_{50} and Model Predictions.

Figure 1 shows concentration–response results for the various FAs, and Table 2 lists 48-h acute EC_{50} values estimated using OriginPro 9.1 software. Although the test waters had nearly identical DOC ($\sim 3 \text{ mg C/L}$) and pH (pH ~ 8) values (Table 1), there are evident differences in estimated EC_{50}

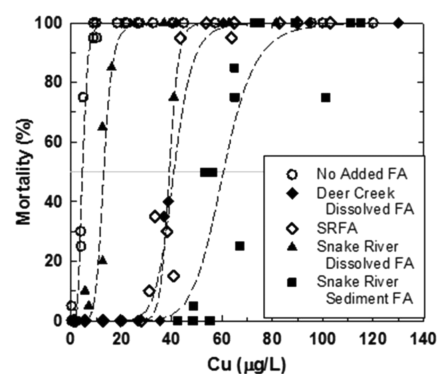


Figure 1. Concentration–response results for 48-h static nonrenewal Cu acute toxicity tests using *Ceriodaphnia dubia* in moderately hard reconstituted water amended with 6 mg/L of fulvic acid (FA) isolates. SRFA is the Suwannee River Fulvic Acid standard.

Table 2. Results from 48-h Static Nonrenewal Cu Acute Toxicity Tests Using *Ceriodaphnia dubia* (Estimated EC₅₀ with 95% confidence intervals), Cu Biotic Ligand Model (BLM) Computation of Median Effect Concentration Values (Predicted EC₅₀), and Characterization Measurements of Fulvic Acid (FA) Isolates^a

test solution	estimated EC ₅₀ ($\mu\text{g Cu/L}$)	predicted EC ₅₀ ($\mu\text{g Cu/L}$)	SUVA ₂₅₄ (L/mg·m)	ϵ_{350} (L/mg·cm)	SAC ₃₄₀ (cm ² /mg C)	SAC ₃₅₀ (cm ² /mg C)	FI
no added FA	4.3 \pm 0.04	6.6 ^b					
SRFA (IHSS)	41 \pm 1.2	66	4.0	0.008	23	18	1.4
Deer Cr dissolved	39 \pm 0.4	56	3.6	0.006	17	13	1.4
Snake R dissolved	13 \pm 0.6	62	2.3	0.003	9.9	7.5	1.5
Snake R sediment	60 \pm 2.1	63	5.1	0.012	34	29	1.4

^aPredicted EC₅₀ values were calculated using input data from Table 1 and 0.01% humic acid (HA). Suwannee River Fulvic Acid (SRFA) and Deer Creek dissolved samples are considered to be unfractionated. FI is the fluorescence index. ^bWhen Cu BLM predictions are performed for 0.1 mg C/L (background-subtracted value) instead of 0.3 mg C/L (Table 1), the predicted EC₅₀ value is 2.8 $\mu\text{g Cu/L}$.

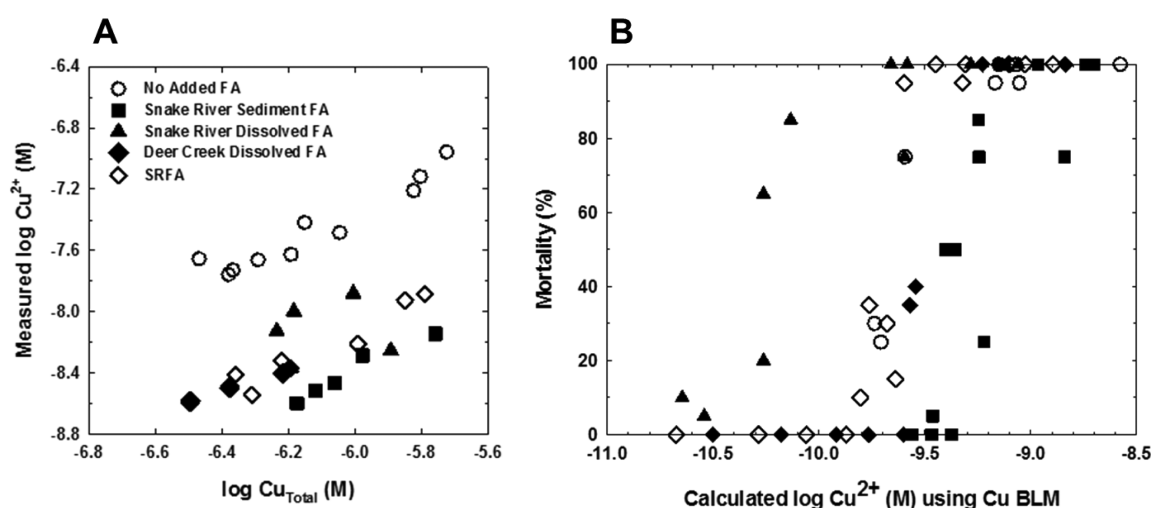


Figure 2. A. Cu-ISE measurements of free Cu²⁺ versus measured total Cu concentrations in toxicity test solutions containing moderately hard reconstituted water amended with 6 mg/L of fulvic acid (FA) isolates. B. Calculated free Cu²⁺ using the Cu Biotic Ligand Model (BLM) versus percent mortality from 48-h static nonrenewal Cu acute toxicity tests using *Ceriodaphnia dubia* in moderately hard reconstituted water amended with 6 mg/L of FA isolates.

values derived from toxicity tests for the different FAs. Estimated EC₅₀ values for test waters with added FA (13 to 60 $\mu\text{g Cu/L}$) are higher than with no added FA (4.3 $\mu\text{g Cu/L}$), which corroborates the expected protective effect of DOM on Cu toxicity. Estimated EC₅₀ values for the two waters containing FA isolated from unfractionated sources (Deer Creek dissolved and SRFA) are similar (39 \pm 0.4 and 41 \pm 1.2 $\mu\text{g Cu/L}$, respectively). In contrast, estimated EC₅₀ values for the FAs from the Snake River dissolved and suspended sediment samples are significantly different (13 \pm 0.6 $\mu\text{g Cu/L}$ and 60 \pm 2.1 $\mu\text{g Cu/L}$, respectively). This contrast between toxicity results for the two fractionated Snake River samples is apparent in the differences in their concentration–response behavior shown in Figure 1. These results demonstrate that FA isolated from streamwater that received acidic Fe- and Al-rich drainage (Snake River dissolved) is 3 times less effective at reducing aquatic Cu toxicity than is FA isolated from unfractionated sources (Deer Creek dissolved and SRFA). In comparison, FA isolated from suspended sediment in the Fe- and Al-rich stream (Snake River sediment) is 1.5 times more effective at reducing Cu toxicity than is FA isolated from the two unfractionated sources. These findings imply that FA components with greater affinity for metal binding tend to be preferentially sorbed (or coprecipitated) to the sediment phases in Fe- and Al-rich systems. This might be explained by the findings of McKnight et al.^{45,74} that Snake River sediment-

derived FA contains organic molecules with greater contents of aromatic moieties, carboxylic acid groups, and amino acid residues, whereas the DOM remaining in the streamwater is depleted in these constituents. The chemical fractionation of organic matter in streams, which occurs through contact with Fe- and Al-rich sediment, can result in DOM with reduced metal-binding capacity, which results in more bioavailable dissolved Cu and greater potential for Cu toxicity to aquatic organisms. This highlights the importance of considering the possibility of chemical fractionation of DOM when attempting to anticipate potential toxic effects of metals to aquatic organisms in mineralized and mined areas that contain streams in contact with Fe- and Al-rich sediment.

Cu BLM-predicted EC₅₀ values do not vary appreciably for the different toxicity test-water solutions containing added FA (EC₅₀ values range from 56 to 66 $\mu\text{g Cu/L}$). Currently, the WHAMV algorithm used in the Cu BLM does not incorporate DOC “quality” with respect to Cu complexation, and in this case does not account for the effects of DOM fractionation by Fe- and Al-oxyhydroxides. Bryan et al.²⁸ tested the ability of WHAMV to simulate Cu speciation measured in natural water samples containing varying amounts of DOM. In optimizing the model, they assumed that the only proton and metal-binding agents in the water samples were humic substances, represented by FA, and FA concentration was the adjustable parameter used for model calibration. They found that

WHAMV provided realistic predictions of Cu speciation in the field samples. Lofts and Tipping⁷⁵ report that uncertainties in binding predictions for Cu are largely attributable to uncertainty in the Cu binding affinity. It is likely that a modified version of the Cu BLM that allows for adjustment of metal–organic binding constants and/or site densities will be necessary to compute site-specific water-quality criteria and potential metal toxicity in many mineralized and mined areas. For example, Chappaz and Curtis⁵ demonstrated the importance of DOM quality on Cu speciation and toxicity, and they provided an approach to account for DOM characteristics on Cu^{2+} concentration by empirically adjusting binding site densities.

The test solution with no added FA has a DOC value near the limit of detection and it is unclear if the DOC measurement of 0.3 mg/L is accurate (Table 1). Comparison of Cu BLM-predicted EC_{50} values for 0.3 mg/L DOC and 0.1 mg/L DOC solutions results in values of 6.6 and 2.8 $\mu\text{g Cu/L}$, respectively (Table 2). Similar issues regarding EC_{50} variability as a function of low-DOC values were reported by Wang et al.⁷⁶

Figure 2A shows results for Cu-ISE measurements of free Cu^{2+} concentrations in toxicity test solutions containing MHRW and FAs. Although the free Cu^{2+} concentrations show some scatter because they are near the limit of detection of the electrode, the data still illustrate differences between free Cu^{2+} in test solutions containing the various FAs. For any given total Cu concentration, the free Cu^{2+} concentration measured in test solutions for the Snake River dissolved fraction is higher than in test solutions containing Snake River sediment-derived FA. The higher free Cu^{2+} concentration in the dissolved fraction likely is responsible for increased toxicity observed in the laboratory tests. The MHRW test solutions with no added FA contain higher free Cu^{2+} than do all of the test solutions with added FA, which illustrates the degree of Cu complexation by the added FAs.

Figure 2B compares free Cu^{2+} calculated by the Cu BLM (WHAMV) using compositions in Table 1 with the percent mortality determined in the laboratory toxicity tests. The Cu^{2+} data calculated for the two unfractionated samples (Deer Creek and SRFA) and the sample with no added FA (input DOC value of 0.3 mg/L) converge. In contrast, Cu^{2+} data for the dissolved Snake River sample indicate toxicity at much lower Cu^{2+} concentrations. Conversely, toxicity tests using the Snake River suspended sediment-derived FA show a higher level of Cu^{2+} is necessary to induce toxicity. The most likely explanation is that this deviation is due to an overestimate of the Cu^{2+} binding capacity of the dissolved Snake River FA and an underestimate of the Snake River suspended sediment FA binding capacity. This illustrates the merit of using model-calculated Cu^{2+} to evaluate potential toxic effects of metals. However, care must be taken in relating toxicity to model-predicted Cu speciation as different models yield different Cu^{2+} values. SI Figure SI-2 illustrates differences in Cu^{2+} concentrations calculated using the Cu BLM and Visual MINTEQ. Furthermore, it is recognized that CuOH^+ also is a likely contributor to toxicity but inclusion of this species will not change the general observed differences among FAs.

Spectral Indicators of Chemical Fractionation. Finding a simple predictor of FA metal binding characteristics would provide a powerful tool for estimating site-specific effects of DOC on aquatic toxicity of metals. Table 2 lists spectral measurements for the FAs used in this study. These FAs were previously characterized by McKnight et al.⁴⁵ who reported that

the dissolved Snake River FA was significantly less aromatic (20%) than the FA extracted from suspended sediment (35%). The various spectral measurements listed in Table 2 have been used in the literature to provide an index of color or aromaticity, or as a predictor of DOM protective ability or Cu complexing properties. The SUVA_{254} values range between 2.3 (for Snake River dissolved FA) and 5.1 $\text{L mg}^{-1} \text{m}^{-1}$ (for Snake River sediment-derived FA). The elevated SUVA_{254} for Snake River sediment-derived FA is out of the range reported for FAs and within the range reported for HAs. For example, Weishaar et al.⁶² measured SUVA_{254} for seven FAs obtained from a variety of aquatic environments, and their values ranged from 0.6 (for Pacific Ocean FA) to 3.9 $\text{L/mg}\cdot\text{m}$ (for Coal Creek FA). The UV absorbance at 350 nm and SAC values exhibit similar variability for the FAs, with end-member values exhibited by the fractionated Snake River samples. The FI is primarily a measure of DOM source and generally is correlated with aromaticity.⁴⁶ SanClements et al.⁷⁷ demonstrated the utility of FI as a monitoring tool for DOM source and quality. The FI values for our FAs exhibit little variability and are within the range generally accepted for a terrestrial source. McKnight et al.⁴⁶ reported that chemical fractionation by sorption does not change the FI even when the SUVA_{254} is lowered, and Al-Reasi⁵² reported no correlation between Cu toxicity and the FI. These observations are consistent with the FI values for the FAs in this study.

Figure 3 shows normalized optical measurements versus estimated EC_{50} values in this study. With the exception of FI,

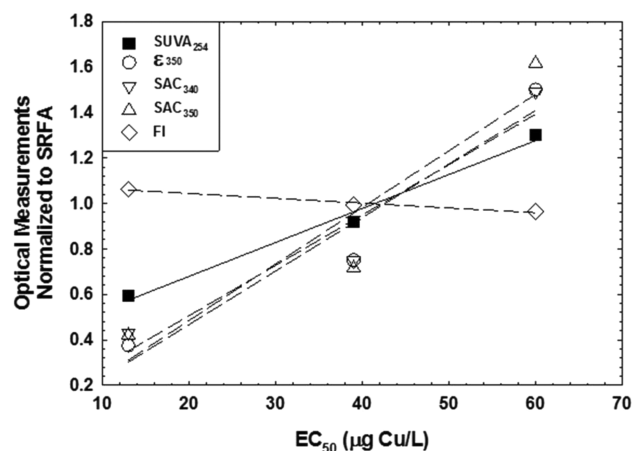


Figure 3. Normalized optical measurements versus estimated EC_{50} values from Cu acute toxicity tests using *Ceriodaphnia dubia* in moderately hard reconstituted water amended with 6 mg/L of fulvic acid (FA) isolates. Optical measurements are normalized to Suwannee River Fulvic Acid (SRFA) values listed in Table 2. Lines are best-fit regression lines, and measurement uncertainties are within the size of the symbols. The EC_{50} is the 48-h median effective concentration (mobility), SUVA_{254} is the specific ultraviolet absorbance measured at 254 nm, ϵ_{350} is the absorbance coefficient measured at 350 nm, SAC refers to specific absorbance coefficients measured at 340 and 350 nm, and FI is the fluorescence index.

there is a linear relationship among the limited data that delineates our observed chemical fractionation of DOM and its relationship to observed aquatic toxicity, with SUVA_{254} exhibiting the best regression fit. This relationship supports the findings of Weishaar et al.⁶² and Traina et al.⁷⁸ that SUVA determined at 254 nm is strongly correlated with percent aromaticity in organic matter, which has been considered to be

related to metal binding capability. Consequently, SUVA_{254} shows promise as a simple optical test for chemical fractionation of DOM, and inferred increased Cu toxicity, in areas with elevated Fe and Al sediment concentrations.

Fluorescence spectroscopy has been used to assess DOM origin, transformations, and redox state^{46,70,79,80} and was used in this study to examine various components of the FA samples. SI Figure SI-3 shows EEMs for the FAs in this study. The most intense excitation–emission pair for the unfractionated Deer Creek FA gives a fluorescence intensity of 0.74 at 270 nm excitation and 451 nm emission wavelength. The corresponding maximum value for Snake River dissolved FA is 0.53 (at 265/448 nm), and for Snake River sediment FA is 1.03 (at 265/454 nm). Snake River sediment FA displays greater fluorescence intensities at all excitation emission wavelength pairs. This is consistent with the findings of McKnight et al.⁴⁵ that DOM with a greater aromaticity preferentially sorbs to precipitating Fe and Al oxides because these moieties (such as carboxylic acids and amino acid components) tend to exhibit greater fluorescence, and that these molecules also have a greater binding affinity for metals. The relative differences between the fluorescence intensities appears to be a result of the fractionation of the DOM such that the Fe- and Al-rich suspended sediment DOM fraction has the greatest fluorescence intensity and the greatest amount of metal-binding molecules. In contrast, the Snake River dissolved fraction is depleted in metal-binding molecules and exhibits the lowest intensities. We infer that the different intensities are due to changes incurred by the fractionation process. PARAFAC enables the statistical separation of chemically independent components that have overlapping spectral components. Using PARAFAC, Cory and McKnight⁷⁰ identified 13 fluorescent components to create a generalized model for DOM. Table SI-2 in the SI lists the loadings of the 13 fluorophore components identified by Cory and McKnight⁷⁰ for our three FAs. The components most enriched in the suspended sediment FA relative to the dissolved FA are tyrosine-like, amino acid-like, hydroquinone, reduced quinone-like, and semiquinone and reduced quinone-like moieties. The relationship between these moieties and the protective effect of DOC on copper toxicity has not currently been established.

Implications for Use of BLMs in Systems with Fe- and Al-Rich Sediment. This study demonstrates that the chemical fractionation between FA sorbed by Fe- and Al-rich suspended sediment and FA remaining in streamwater reported by McKnight et al.⁴⁵ is sufficient to produce observable differences in the protective ability of the FA against acute Cu toxicity to aquatic organisms. Our results show that there is more than three times greater Cu toxicity observed in the presence of fractionated FA than for unfractionated FA. This is because the chemical fractionation produces FA with reduced metal-binding capacity, which results in more bioavailable dissolved Cu and greater potential for Cu toxicity to aquatic organisms. Because most BLMs do not currently take DOM characteristics, including Fe- and Al-mediated alterations, into account in their computations, modifications (such as adjustable binding constants and/or site densities) may be necessary to refine site-specific water-quality criteria in systems with Fe- and Al-rich sediment. Several optical measurements exhibit a linear relationship to observed aquatic toxicity, with SUVA_{254} having the best regression line fit, and show promise for use as an indicator of DOM chemical fractionation. Additional studies of other Fe- and Al-rich systems will determine the role of DOM

source material and the overall importance of this fractionation phenomenon.

■ ASSOCIATED CONTENT

Supporting Information

Concentration–response data for Cu acute toxicity tests using *C. dubia* (Table SI-1). Loadings of 6 mg/L FA suspensions for 13 fluorophore components identified by a PARAFAC general model⁷⁰ (Table SI-2). Calibration curve for Cu ISE measurements used to calculate Cu^{2+} concentrations in test solutions containing FAs (Figure SI-1). Free Cu^{2+} measurements versus calculated Cu^{2+} concentrations using the Cu BLM and Visual MINTEQ (Figure SI-2). Excitation–emission matrices (EEMs) for 6 mg/L suspensions of Deer Creek dissolved FA, Snake River dissolved FA, and Snake River sediment FA (Figure SI-3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on Oct 7, 2014 with incorrect units in the sixth paragraph of the Results and

Discussion section, and two other minor corrections. The corrected version was reposted on Oct 8, 2014.