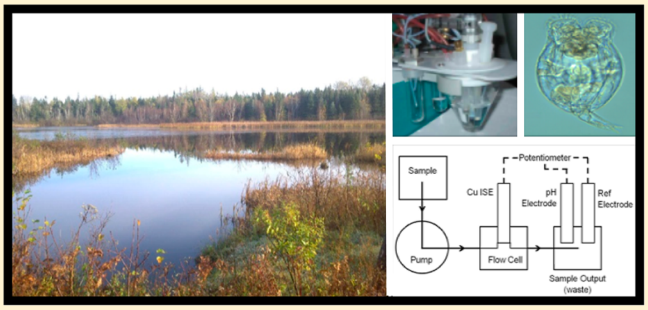
Environmental Science & Technology
Article
pubs.acs.org/est

Influence of Salinity and Dissolved Organic Carbon on Acute Cu Toxicity to the Rotifer *Brachionus plicatilis*

Christopher A. Cooper,[†] Tara Tait,[†] Holly Gray,[†] Giselle Cimprich,[†] Robert C. Santore,[‡] James C. McGeer,[†] Christopher M. Wood,[§] and D. Scott Smith*,[†]

[†]Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada
[‡]HDR|HydroQual, Incorporated, Syracuse, New York, United States
[§]McMaster University, Hamilton, Ontario L8S 4K1, Canada

Supporting Information



ABSTRACT: Acute copper (Cu) toxicity tests (48-h LC50) using the euryhaline rotifer *Brachionus plicatilis* were performed to assess the effects of salinity (3, 16, 30 ppt) and dissolved organic carbon (DOC, ~1.1, ~3.1, ~4.9, ~13.6 mg C L^{-1}) on Cu bioavailability. Total Cu was measured using anodic stripping voltammetry, and free Cu^{2+} was measured using ion-selective electrodes. There was a protective effect of salinity observed in all but the highest DOC concentrations; at all other DOC concentrations the LC50 value was significantly higher at 30 ppt than at 3 ppt. At all salinities, DOC complexation significantly reduced Cu toxicity. At higher concentrations of DOC the protective effect increased, but the increase was less than expected from a linear extrapolation of the trend observed at lower concentrations, and the deviation from linearity was greatest at the highest salinity. Light-scattering data indicated that salt induced colloid formation of DOC could be occurring under these conditions, thereby decreasing the number of available reactive sites to complex Cu. When measurements of free Cu across DOC concentrations at each individual salinity were compared, values were very similar, even though the total Cu LC50 values and DOC concentrations varied considerably. Furthermore, measured free Cu values and predicted model values were comparable, highlighting the important link between the concentration of bioavailable free Cu and Cu toxicity.

INTRODUCTION

It is vital that organisms maintain copper (Cu) homeostasis, as both deficiency and excess can be detrimental. Cu is an essential element for all aerobic organisms as the redox potential of the Cu(I)/Cu(II) transformation is utilized by a number of enzymes.[1] However, when cellular Cu levels are too high it can become very toxic. For example, excess Cu can lead to the formation of harmful reactive oxygen species (ROS);[1,2] furthermore, Cu readily binds to histidine, cysteine, and methionine sites in proteins, potentially leading to their breakdown.[3]

In the aquatic environment, Cu toxicity is not simply a function of the total concentration; the bioavailable portion of Cu must be determined to better assess potential diverse affects.[4] The most widely accepted tool for assessing metal toxicity risk to aquatic organisms is considered to be the biotic ligand model (BLM).[5] The BLM is a predictive tool that accounts for variations in metal toxicity based on chemistry information of local freshwater sources. The model incorporates a number of components, including interactions at the target site(s) on the organisms ("biotic ligand"); predicted toxicity is proportional to metal accumulation at the biotic ligand. BLMs developed for use in freshwater include protective effects of dissolved organic matter (DOM) as predicted by the Windermere Humic-Aqueous Model; WHAM v.5.[6] Competitive binding for other cations at biotic ligand sites, as well as anion−metal interactions are taken into account by key BLM water quality input parameters including calcium (Ca^{2+}) and magnesium (Mg^{2+}), dissolved organic carbon (DOC), pH, alkalinity, sodium (Na^{+}), carbonates, chloride (Cl^{-}), sulfate (SO_4^{2-}), and temperature.[4,7] The BLM has been successful in establishing ambient water quality criteria (AWQC) of copper for freshwater environments.[8]

The majority of water on this planet, however, is not fresh water. Estuaries and coastal zones receive chronic inputs of metals from both specific and nonspecific sources, such as seaports, industries, and domestic outputs.[9] According to the US EPA,[10] separate freshwater and marine numerical water quality criteria need to be derived as fresh and seawater have different chemical compositions and because species rarely inhabit both simultaneously.[4]

Received: May 15, 2013
Revised: December 20, 2013
Accepted: December 31, 2013
Published: December 31, 2013

The bioavailability and toxicity of Cu in the marine environment can differ considerably when compared to fresh water as a result of the water chemistry. Inorganic speciation of Cu in seawater is predominantly as carbonate ($CuCO_3$), with a small fraction of Cu binding to hydroxide ions ($CuOH^-$ and $Cu(OH)_2$), which can decrease Cu bioavailability.[11,12] Similar inorganic Cu speciation applies to fresh water of high alkalinity and pH,[13] while $CuOH^-$ and $Cu(OH)_2$ complexes dominate in high pH fresh water with low alkalinity.[1,14,15] In addition to inorganic speciation, Cu can also compete for uptake with certain other cations in seawater (e.g., Na^+, H^+, Ca^{2+}, and Mg^{2+}), which can decrease metal accumulation and the resultant toxic response.[16] Thus, inorganic speciation and ionic competition associated with an increase in salinity can have a protective effect against Cu toxicity, which has been shown in many different species, including copepods (*Eurytemora affinis*),[12] larval topsmelt (*Atherinops affinis*),[17] and rotifers (*Brachionus plicatilis*).[4]

Possibly the strongest effect on Cu bioavailability in both marine and fresh waters is exerted by DOC.[4,8,15] Natural DOCs may be broadly classified as ranging from allochthonous (terrigenous) to autochthonous.[18] Allochthonous DOCs are mainly humic acid based, derived from the breakdown of lignins (i.e., leaves and wood); they tend to be optically darker and are composed of larger molecules with more aromatic rings (i.e., more phenolic groups).[19] Autochthonous DOCs are mainly fulvic acid based, produced within lakes and rivers by algae or by microbial activity and/or photodegradation of allochthonous DOM. They are usually optically lighter and composed of smaller molecules with a lower content of aromatic ring structures. In general, the darker aromatic DOC is usually more protective against metal toxicity.[19] The specific absorption coefficient of the DOC in the 300−350 nm range (SAC300−350) has proven to be a simple and effective index of this protective ability.[19] Copper binds to DOC with a high affinity and this complexation can prevent or reduce Cu binding, Cu uptake, and Cu toxicity to aquatic organisms.[20−22] Interestingly, DOC has also been shown to have direct beneficial effects that would counter the toxic effects of metals (reviewed by Wood et al.),[19] including decreasing paracellular permeability,[23] hyperpolarizing branchial transepithelial potential (TEP),[24] as well as enhancing Na^+/K^+-ATPase activity,[25] and Na^+ uptake.[26] Nonetheless, the capacity of DOC to reduce Cu toxicity in freshwater organisms has been confirmed by numerous studies.[7,20−22,27,28]

Marine and estuarine DOC reactivities are influenced by the presence of high salt concentrations.[29] The typical ionic strength of seawater is around 0.7 M where fresh water ionic strength is in the range of 0.001−0.01 M, depending on local geology.[30] The several orders of magnitude larger ionic strength of seawater influences DOC reactivity in three ways: (i) activity corrections on reactivity of metal ions as well as functional groups involved in binding; (ii) competition by large cation concentrations with metals for binding sites; (iii) structural changes such as folding, unfolding[31] and potentially colloid formation.[32] In addition, marine and estuarine sourced DOM tends to be different on a molecular level from terrestrially derived DOM.[33]

Despite the established importance of DOC in decreasing Cu bioavailability, and subsequently Cu toxicity in freshwater; relatively few studies have documented the impact of DOC on Cu toxicity to marine organisms.[4,34−37] Even less is known about the combined effects of changes in both salinity and DOC concentration on Cu toxicity. In the present study, the effects of varying salinity and DOC on Cu toxicity to the euryhaline rotifer, *Brachionus plicatilis*, were examined. *B. plicatilis* is an ideal model species for a number of reasons. They can be successfully cultured in salinities ranging from 0 to 40 parts per thousand (ppt) salinity,[38] they are very sensitive to Cu,[4,39] and standard guidelines exist for toxicity testing of this species.[40] Finally, rotifers are distributed over six continents and have a major impact on several important ecological processes in coastal marine environments.[4,41] To supplement the acute Cu toxicity data (48-h LC50), total levels of Cu were measured using anodic-stripping voltammetry and free Cu concentrations using an ion-selective electrode (ISE). Data from this research will help develop the estuarine and marine BLM for Cu toxicity.

MATERIALS AND METHODS

Toxicity Method. Static acute toxicity tests (48-h duration, unfed) were performed using modified methods of those recommended by the ASTM[40] and other similar studies.[4,39] A summary of ASTM[40] recommended test methods and methodology used in this experiment are reported in the Supporting Information, Table S1. Briefly, *B. plicatilis* rotifer cysts were purchased from Florida Aqua Farms Incorporated (Dade City, Florida). Newly hatched rotifers (<6 h old) were used in each acute toxicity test, with 5−12 replicates of 10 rotifers per exposure concentration. Test rotifers were obtained by hatching resting rotifer cysts in 6-well tissue culture plates (Falcon, Becton Dickinson) at 25 °C under continuous light at 2500 lx in reconstituted artificial water at the desired salinity. Test organisms were transferred randomly to the test chamber, 24-well tissue culture plates (Falcon, Becton Dickinson), containing 2 mL of test solution via a micropipet. The test chamber was maintained at 25 ± 1 °C (continuously monitored) and in continuous darkness. Organisms were observed at the end of 48-h exposure periods using a stereoscopic microscope. Individual rotifers were scored as dead if there was no movement of body or parts (e.g., mastax) within 5 s of initiating an observation.[40]

Artificial seawater was made using the following concentrations of salts dissolved in ultrapure water (18.2 MΩ resistivity) (all in g L^{-1}): 11.31 NaCl, 0.36 KCl, 0.54 $CaCl_2$, 1.97 $MgCl_2 \cdot 6H_2O$, 2.39 $MgSO_4 \cdot 7H_2O$, and 0.17 $NaHCO_3$.[40,41] The salinity (3, 16, and 30 ± 1 ppt) was adjusted accordingly using ultrapure water and a stock solution of ∼35 ppt. The pH of the solution was adjusted to approximately pH 8.00 using 0.1 M NaOH/HCl (Orion 91-57BN pH electrode and Orion 420A+ meter, Thermo Electron Corp.). A pH of 8.00 was chosen as it was intermediate between fresh and seawater. The artificial water was made up 48-h before experimentation and mixed continuously in a 1-L glass volumetric flask. Copper ($CuSO_4 \cdot 5H_2O$; Bioshop) was spiked into the test solution prior to experimentation, mixed and allowed to equilibrate for at least 1 h. The concentration of Cu ranged from zero added to approximately 400 $\mu g\ L^{-1}$, depending on the experiment. When necessary the DOC (see below) was added to the artificial water 24-h before the addition of Cu and mixed continuously. Again, the pH of the solution was adjusted to approximately pH 8.00 prior to experimentation.

Dissolved Organic Carbon (DOC) Source and Storage. The DOC was obtained from a wetland area near Southampton, on Prince Edward Island (latitude, 46.35937; longitude, −62.59166; altitude, 7 m). The DOC sample was obtained using a portable reverse osmosis unit (for details on reverse osmosis, see Sun et al.).[42] To remove potential metal contamination, the concentrates were passed through a cation exchange resin (Amberlite IR-118H, Sigma), acidified to pH 2,

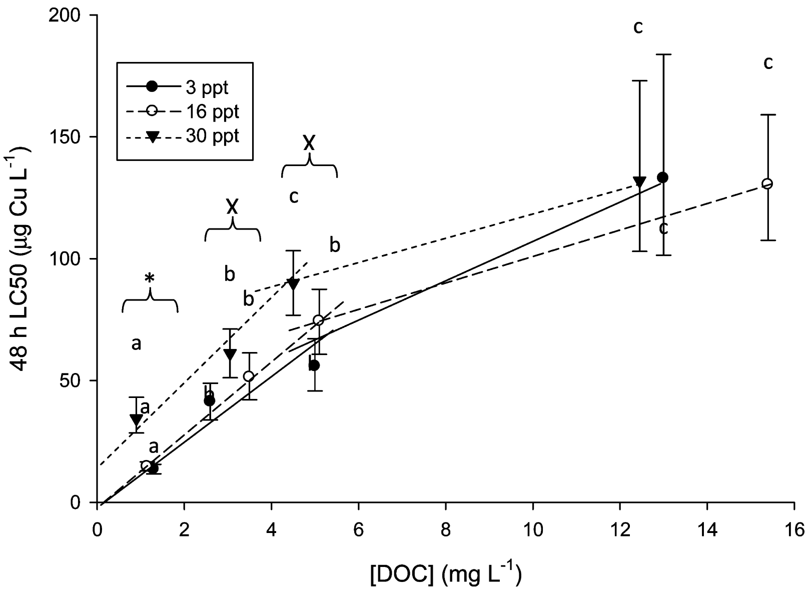


Figure 1. The 48-h LC50 values and 95% confidence limits (CL) for the rotifer, *Brachionus plicatilis*, exposed to all salinities and DOC. LC50 and CL were determined from *T*Cu measurements (voltammetry) and Probit analysis software. Different letters show significant differences in LC50 values across the DOC concentrations, for each individual salinity. An asterisk denotes a significant difference in LC50 values when comparing 3 and 16 to 30 ppt, and a "X" shows a significant difference in LC50 values when comparing 3 to 30 ppt. Significant differences in LC50 values are deemed when CL do not overlap (*N* = 5−12).

before being stored in the dark at 4 °C, in 4 L polyethylene bottles until use.[43,44]

DOC concentrations were measured using a Shimadzu TOC-$L_{CPH/CPN}$ analyzer (Shimadzu Corporation). Samples (30 mL) for storage were not acidified, but they were filtered (0.45 µm) and then kept in refrigeration until measured. Standard total carbon solutions were used as reference when TOC analysis was conducted (5, 10, and 20 mg C L^{-1}) and were prepared from potassium hydrogen phthalate (Mandel Scientific). The concentration of DOC was determined by subtracting the instrument blank area from the average peak area and dividing by the slope of the standard curve. Samples from all salinities were treated the same, except that immediately prior to measurement, 30 and 16 ppt samples were acidified with a few drops of HCl and Milli-Q water rinses were made after every seawater sample (Milli-Q water rinses every 4−5 samples in 3 ppt samples).

Dissolved Organic Carbon (DOC) Light Scattering and Fluorescence. To determine if DOC particles were aggregating at high [DOC] and salinities, light scattering measurement were made; whereas to qualitatively ascertain the relative key constituents of the Southampton DOC (i.e., humic and fulvic acid, and tryptophan) a 3D contour plot was generated from fluorescence data. (All fluorescence data are presented in the Supporting Information.) Light scattering and fluorescence measurements were conducted in a 1 cm quartz cuvette using a Varian Cary Eclipse Fluorescence Spectrometer (Varian, Mississauga, ON). For light scattering determinations, 12 solutions of variable DOC concentrations (0, 5, and 10 mg C L^{-1}) and salinities (3, 10, 16, and 30 ppt) were prepared and allowed to equilibrate for 48 h. The excitation wavelength was set for 800 nm and fluorescence emission wavelengths were measured between 750 and 850 nm. For each scan, both the excitation and emission monochromator slit widths were set to 10 nm and the photomultiplier tube was set to medium sensitivity (600 V). The 3D contour plot was generated using fluorescence emission wavelengths at 250 to 600 nm, in 1 nm increments for intervals of 10 nm excitation wavelengths between 200 and 450 nm. For each scan, the excitation and emission monochromator slit widths were set to 5 nm and the photomultiplier tube was set to high sensitivity (800 V). Using MATLAB (MathWorks, Natick, MA), 3-dimesional fluorescence excitation−emission matrices (FEEMs) were created from the fluorescence data. Scattered light at emission equal to excitation and emission equal to two times the excitation wavelength are artifacts of the fluorescence instrumentation and thus were removed from the spectra.[59]

Total Cu measurements—Voltammetry. Total Cu (*T*Cu) concentrations used in the toxicity tests were determined by differential pulse anodic stripping voltammetry (DPASV) using manufacturer recommended settings from the manual (Metrohm Application Bulletin No. 231/2e). Samples for *T*Cu measurement were taken from stock solutions used for each [Cu]. Voltammetric measurements were made with a computer controlled Autolab potentiostat/galvanostat (Eco Chemie, Metrohm) and a Metrohm 663 VA stand (Metrohm). The working electrode was a static mercury drop electrode (SMDE) and the counter electrode was made of a platinum rod (Metrohm). The analysis of peaks was done using Nova 1.7 software (Eco Chemie, Metrohm). For all the ASV measurements, the following experimental conditions were used: 1 mL of KCl-sodium acetate buffer (1.5 mol L^{-1} KCl, 0.5 mol L^{-1} CH_3COONa and 50 mL 30% w/v NaOH L^{-1}) was added to 10 mL of sample before the pH was adjusted to 6.400 (±0.005). The sample was purged with pure N_2 gas for 5 min, the deposition time was 90 s (with stirring), the equilibration time was 10 s and the differential pulse ranged from −1.250 to 0.000 V. Samples were run before and after the addition of multiple known Cu concentrations. Standard addition analysis by linear regression was used to determine the original [Cu] in the sample.

In a separate control experiment, samples from a number of wells were pooled and measured, and compared to stock values to determine if any Cu adhered to the sides of the 6-well tissue culture plates. Results indicated that 99.4% of the *T*Cu was

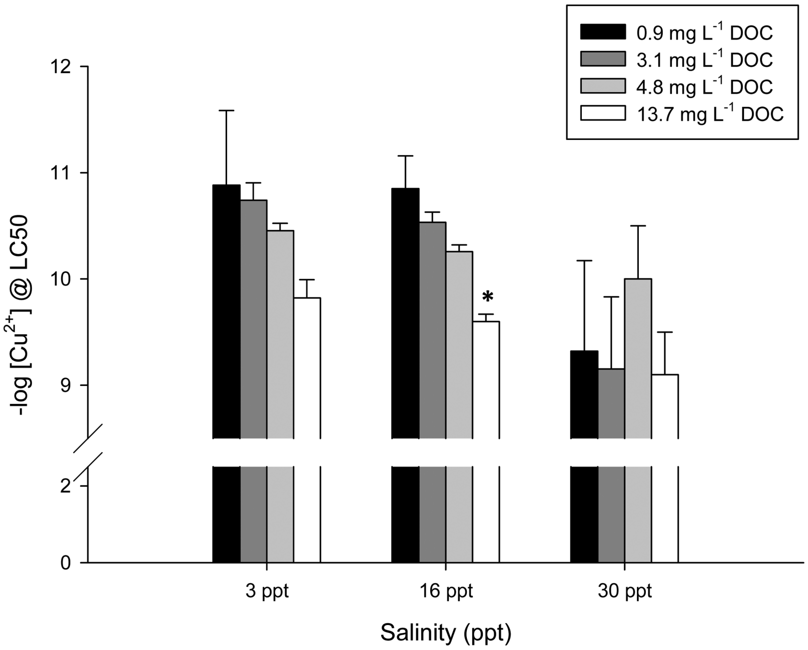


Figure 2. Concentrations of measured free Cu in samples containing total Cu concentrations equivalent to the *B. plicatilis* LC50 values obtained when using a range of DOC concentrations and salinities. The free copper activity is the negative log10 of the molar activity of Cu^{2+} ions ($-\log [Cu^{2+}]$). Shown at each salinity is the measured free Cu in the presence of different concentrations of DOC: (black) 0.9 mg L^{-1} DOC, (dark gray) 3.1 mg L^{-1} DOC, (light gray) 4.8 mg L^{-1} DOC, and (white) 13.7 mg L^{-1} DOC. Values are $-\log[Cu^{2+}]$. Mean ± S.E.M., $N = 6-3$. An asterisk denotes a significant difference in free Cu activity values between 0.9 and 13.7 mg L^{-1} DOC at 16 ppt ($P < 0.05$; ANOVA followed by Holm-Sidak post hoc test).

recovered from the culture plate wells when compared to the stock solution (data not shown).

If the sample contained Southampton DOC, the Cu−DOC complex was first UV digested before the [*T*Cu] could be measured using ASV. Briefly, 100 μL of 30% hydrogen peroxide was added to 10 mL of sample, as recommended by the manufacturer. Samples were then placed in a UV digester for 1 h at 89 ± 2 °C (Metrohm 705 UV digester).

Free Cu Measurements—Ion-Selective Electrode (ISE). Solutions containing *T*Cu concentrations equivalent to the 48-h LC50 values from all conditions (Supporting Information, Table S2) were measured using a flow-through system (from Eriksen et al.).[45] For all measurements, the Orion Cu (II) ISE (model 94-29) and an Orion double junction reference electrode (model 900200) were used, both connected to a potentiometer (Tanager, model 9501, Ancaster, ON). A Cerampump FMI "Q" Pump (GQ6) was used to deliver the sample through the flow-through system at a flow rate of 1.0 mL h^{-1}. Samples were pH adjusted to a pH of 8.00 ± 0.1 and measured until stabilization of the signal was achieved. The acceptable criterion for stability was a less than 0.1 mV change min^{-1}. The sample was then adjusted to a pH of 3.30 and delivered through the system again until the stability criterion was satisfied. This allowed for a one-point internal calibration to be performed using the lower pH measurement to determine free Cu. Checks showed that that the electrode had a Nernstian response (average slope of 28.6 ± 0.2 mV per decade of concentration) so the one point calibration allows calculation of the intercept. The free Cu concentration is determined in the low pH sample by assuming that the total Cu equals the free Cu, corrected for complexation to sulfate and chloride using NIST complexation constants.[46] Total Cu concentrations used for this determination were determined using voltammetry (see previous section).

Free Cu Modeling. The chemical equilibrium speciation component of a seawater-Biotic Ligand Model (BLM) was used to predict free Cu for all salinity/[DOC] combinations (refer to Chadwick et al.[47] SPAWAR technical report for relevant information). The model is based on fixed equilibrium binding constants for inorganic as well as organic speciation of Cu. Input parameters for the seawater-BLM include pH, salinity, DOC and total Cu. For our modeling we used the measured pH, salinity, DOC, and input total Cu at the LC50 value for each exposure condition. With these input variables, the model then outputs concentrations for all Cu species including Cu^{2+}.[47]

Statistical Analyses. The 48-h LC50 values were determined using Probit analysis as prescribed by the US EPA.[48] If the 95% confidence intervals did not overlap, the LC50 values were considered significantly different[4] (Figure 1 and Supporting Information, Table S2). A two-way analysis of variance (ANOVA) was used to compare light scattering data for the effects of salinity and DOC (Supporting Information, Figure S1). A one-way ANOVA was used to compare free Cu concentrations (Figure 2), across all DOC concentrations at each salinity. Where appropriate, an ANOVA followed by a Holm-Sidak post hoc test was used to test the data, and means were considered significantly different based on the adjusted $P < 0.05$ (SigmaStat 3.1 statistical program). All figures and calculations were performed using SigmaPlot version 11, Matlab, SigmaStat version 3.1, and Microsoft Excel.

■ RESULTS AND DISCUSSION

The rotifer, *Brachionus plicatilis*, is an excellent model species for Cu toxicity tests in different aquatic environments. Not only is it incredibly tolerant of a wide range of salinities,[37] it is also very sensitive to Cu. Mean acute Cu toxicity values (LC or EC50 values) for 44 genera range from 12 to 6448 μg Cu L^{-1}.[4] Put into this context, in the present study the lowest LC50 value observed averaged 13.5 μg Cu L^{-1} (3 ppt, zero added DOC) making *B. plicatilis* one of the most sensitive species tested to date. This may be a function of the relatively large surface area to mass ratio,[49] as it is one of the smallest organisms used for these types of toxicity tests. Increasing salinity, when no DOC was added had a protective effect against Cu toxicity to *B. plicatilis*. The addition of Southampton DOC also had a significant protective effect against Cu toxicity for all salinities. Such a relationship was particularly pronounced when going from zero added DOC to ~5 mg L^{-1} DOC (Figure 1). However, the degree of protectivity per milligram of C decreased as DOC increased at high salinities. Measurements of free Cu using an ion-selective electrode (ISE), in samples containing total Cu concentrations equivalent to the LC50, revealed that there was a threshold concentration of free Cu that accounted for the toxicity. However, it was evident that high concentrations of DOC may provide an additional protective effect, as higher concentrations of free Cu were required to elicit the same toxic effect (the LC50). Data from this research will be used in the development of a marine and estuarine biotic ligand model.

The Effects of DOC on Total Copper Toxicity. The addition of DOC provided a significant protective effect against Cu toxicity to the *B. plicatilis* in all salinities (Figure 1 and Supporting Information, Table S2). For all three salinities, as the concentration of DOC increased up to ~5 mg L^{-1}, Cu 48-h LC50 values also increased linearly (3 ppt, LC50 [μg Cu L^{-1}] = 10.76 × DOC [mg L^{-1}] + 4.98; 16 ppt, LC50 = 15.15 × DOC − 2.51; 30 ppt, LC50 = 15.22 × DOC + 19.01). This has also been shown in other studies using a variety of different species. In seawater for example, Arnold et al.[36] found a direct and significant relationship between DOC and LC/EC50 values in six different species (the mussels *Mytilus galloprovincialis* and *Mytilus edulis*, the oyster *Crassostrea virginica*, the sand dollar *Dendraster excentricus*, the sea urchin *Strongylocentrotus purpuratusi* and the copepod *Eurytemora affinis*). Cu uptake by green mussels, *Perna viridis*, was reduced when it was complexed with different DOC types in seawater.[37] Furthermore, DePalma et al.[50] showed that EC50 values in marine *Mytilus* species increased from 10 to 40 μg L^{-1} Cu when DOC concentrations were raised from 1 to 10 mg L^{-1}. Similar results in seawater were obtained in the Pacific oyster,[34] purple urchin larvae,[51] and the blue mussel.[35] In freshwater, many studies on *Daphnia magna* have also shown that the addition of DOC protects against Cu toxicity.[3,27,28,52]

Interestingly, at high concentrations of DOC (up to ~13.7 mg C L^{-1}) the relationship was still linear in 3 ppt (LC50 = 9.67 × DOC + 7.92). However, at high [DOC] the [DOC]/LC50 value relationship was no longer linear in 16 ppt (LC50 = $-0.67 \times \mathrm{DOC}^2 + 19.22 \times \mathrm{DOC} - 6.91$) or in 30 ppt (LC50 = $-0.78 \times \mathrm{DOC}^2 + 19.07 \times \mathrm{DOC} + 16.01$) (Figure 1 and Supporting Information, Table S3). Therefore, at higher salinities (30 ppt) there was a saturation point where any further increases in DOC concentrations did not significantly affect metal toxicity. This supports previous studies on other species. For example, a similar linear increase in the EC50 value for *D. magna* was observed in fresh water, with EC50 values increasing from 25 μg L^{-1} Cu (zero added DOC), up to 100 μg L^{-1} Cu (5 mg DOC L^{-1}) and 300 μg L^{-1} Cu (15 DOC mg L^{-1}).[27] Whereas in marine blue mussels (*Mytilus trossolus*), an increase in [DOC] from 0 to 10 mg L^{-1} increased the 48-h EC50 values in a linear fashion from 9.4 to 37.0 μg Cu L^{-1}. Yet, doubling the [DOC] (nominal 20 mg L^{-1} DOC) did not double the EC50 value; instead this relationship had plateaued out, with an EC50 value of only 39.0 μg Cu L^{-1}.[35]

We hypothesize that the decrease in protectivity per milligram of C L^{-1} at high salinities could be due to salt induced colloid formation of DOC particles. Continuum theory predicts that the force between two surfaces in a liquid is given by the sum of an attractive van der Waals force and a repulsive electrostatic force. Repulsive forces decrease at high ionic strength due to collapse of the electrical double layer. This is the essence of the Derjanguin–Landau–Verwey–Overbeek (DLVO) theory.[53] In certain situations (e.g., in high salt concentrations), there is a possibility of a "secondary minimum", which is when an interparticle attractive force (van der Waals force) causes colloidal particles in liquid medium to form persistent aggregates at high enough electrolyte concentrations.[32] Light scattering data, which are presented in Supporting Information, Figure S1, supported this hypothesis. Although the units are arbitrary, an increase in light intensity is directly proportional to the particle size, that is, a direct correlation between colloid formation and light intensity. When no DOC was added, the light intensity was approximately equal across all salinities. With the addition of 5 mg L^{-1} DOC, light intensity values significantly increased only at the highest salinity (30 ppt). The addition of 10 mg L^{-1} DOC resulted in light intensity values significantly increasing at a much lower salinity (16 ppt). Thus, the trend of light intensity suggests that colloid formation was more prevalent at elevated salinities and DOC concentrations, which would explain the linear increases in EC50 values and DOC in freshwater studies (where salt induced colloid formation does not occur)[27,3,28,52] and the less than expected protective effect of DOC in salt-rich marine environments (Figure 1;[35]). However, we cannot eliminate other possible explanations, such as changes in the Cu/DOC ratio, and the dependence of complexation constants on ionic strength at higher salinities.

It was evident that DOC had a protective effect against Cu toxicity; however, the source of DOC can also be a major factor determining the level of protectivity. Using the same species (*B. plicatilis*) as in the present study, Arnold et al.[4] found that in seawater (29 ppt) the 48-h LC50 value increased from 34 to 145 μg of Cu L^{-1} when the DOC concentration was increased from 1 to 4 mg L^{-1}. A very similar LC50 value was observed in the present study for 30 ppt *B. plicatilis* with zero added DOC (~1 mg L^{-1} DOC, LC50 value of 34.4 μg of Cu L^{-1}). However, at ~4 mg L^{-1} DOC the LC50 value was approximately 90 μg of Cu L^{-1}, almost half that of the previous study by Arnold et al.[4] This is also reflected in the respective slope equations (LC50 [μg Cu L^{-1}] = 15.23 × DOC [mg L^{-1}] + 19.01 versus LC50 = 37.1 × DOC − 3.2; Supporting Information, Table S3 versus Arnold et al.,[4] respectively). The cause of this difference is most likely attributed to the type DOC used. On the basis of the fluorescent spectrophotometry data (Supporting Information, Figure S2), high emission levels in the 460 to 520 nm range indicate the presence of humic acid, with only low levels of fulvic acid and tryptophan in the Southampton DOC used in this study, which is typical of allochthonous/terriginous origin.[19] In addition, as Southampton DOC was very dark this presumably means that phenolic groups were also present.[19] DOC of this nature is generally perceived to be very protective against metal toxicity.[19] However, it must be noted, the DOC used in the study by Arnold et al.[4] was that contained in natural saltwater (from Little Egg Harbor, NJ, USA), it was not subject to fluorescent spectrophotometry, so this explanation remains speculative. Nonetheless, these differences in LC50 values, for the same species, highlight the potential importance of the DOC source. Several studies have concluded that biologically meaningful

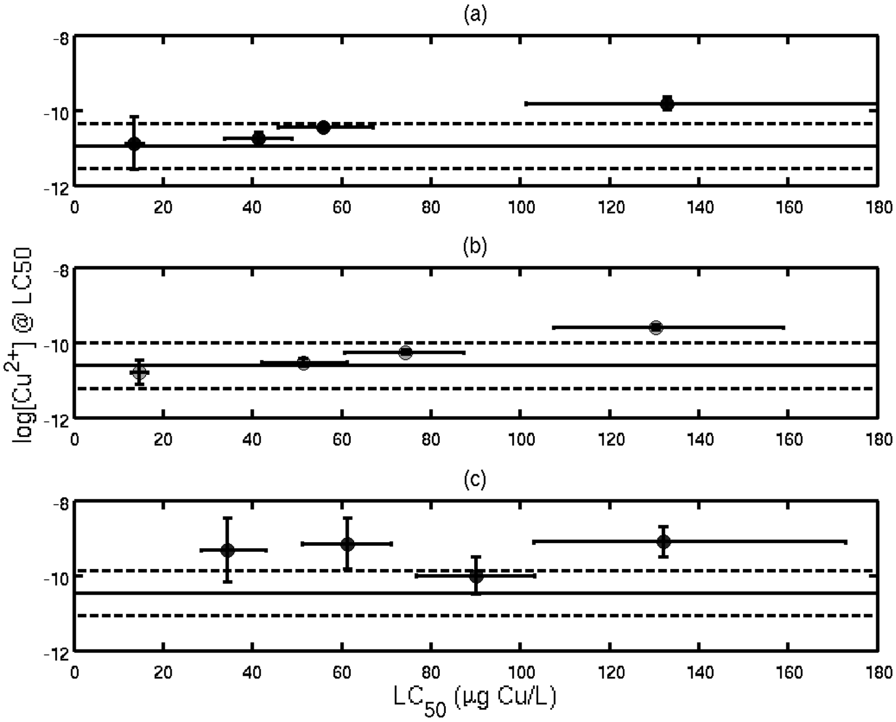


Figure 3. A plot comparing the 48-h LC50 *T*Cu values (μg Cu L^{-1}; 95% CL) and the measured free Cu values ($-\log[Cu^{2+}]$) for 3 (a), 16 (b), and 30 ppt (c). Also included for each salinity are the calculated free Cu values using a seawater-BLM[47] ($-\log[Cu^{2+}]$; horizontal solid lines) and the respective error bars (±0.6 $-\log[Cu^{2+}]$; dashed lines). Calculated free Cu error bars of ±0.6 $-\log[Cu^{2+}]$ were selected as a reasonable confidence envelope (refer to Supporting Information for an explanation of how the 0.6 value was obtained). Error bars for $-\log[Cu^{2+}]$ correspond to SEM.

differences exist in DOC isolated from different sources, leading to differential binding abilities to the free metal ion and to different subsequent toxicity or protection to organisms.[4,22,28,43,54] The importance of DOC source and/or optical qualities is something that should be incorporated into toxicity models in the future.

The Effects of Salinity on Total Copper Toxicity. Increases in salinity from 3 to 30 ppt, which approximates full strength seawater had a protective effect against Cu toxicity when no DOC was added, with LC50 values being significantly higher at 30 ppt when compared to 16 and 3 ppt (Figure 1 denoted by an asterisk, and Supporting Information, Table S2). In addition, at DOC concentrations of ~3.1 and ~4.5 mg L^{-1}, LC50 values for 30 ppt were significantly higher than for 3 ppt (Figure 1 denoted by a "X", and Supporting Information, Table S2), whereas LC50 values for 16 ppt were intermediate. This has been found in other studies as well. Using the same species, *B. plicatilis*, the 24-h Cu LC50 values were a factor of 1.5 higher at elevated salinities (20−29 ppt) when compared to lower salinities (6−15 ppt).[4] Hall et al.[12] and Lauer and Bianchini[55] both demonstrated that elevated salinity increased LC/EC50 values in estuarine copepods (*Eurytemora affinis* and *Acartia tonsa*, respectively). Similarly in vertebrates, a higher salinity also correlated with higher LC50 values in larval topsmelt, *Atherinops affinis*,[17] and 96-h LC50 values in juvenile mummichog fish, *Fundulus heteroclitus*.[49] However, the protective effect of salinity is not always evident. For example, changes in salinity had no effect on acute Cu toxicity in brine shrimp, *Artemia salinus*;[56] and in the presence of high DOC concentrations, increased salinity had no protective effect in the present study (Figure 1).

Cu is generally more toxic in fresh water because of its ability to competitively interact with major cations for sensitive sites on the biological target.[49,57] Organisms that are osmoregulators have to take up salts in fresh water and excrete salts in a marine environment to maintain osmoregulatory homeostasis. Therefore, in fresh water Cu may compete with ions such as Na^+ for uptake, which in turn would lead to increased metal toxicity. In both larval topsmelt[17] and mummichog[49] Cu LC50 values were lower in fresh water when compared to seawater. However, Cu uptakes rates were similar in both fresh and seawater, which suggests that the increased sensitivity to Cu was due to the increased physiological challenge of osmoregulation.[17]

Environmental Effects on Free Copper Concentrations, and Implications for the BLM. Bioavailability and toxicity of trace metals depend on the physical and chemical speciation of metals in the matrix.[58] One of the most bioavailable species of Cu is divalent free Cu and it is the general consensus that it is the main toxic form of Cu, although $CuOH^{-/+}$ and $Cu(OH)_2$ complexes have also been demonstrated to be bioavailable and exert toxicity.[1,5,14,15] The present study confirmed that free Cu is a toxic form of Cu to *B. plicatilis*. Comparing measurements of free Cu (the negative log10 of the molar concentration of Cu^{2+} ions ($-\log[Cu^{2+}]$)) across all DOC concentrations, for each separate salinity, revealed that, in general, free Cu concentrations were not significantly different, even though the effective *T*Cu concentrations (i.e., the LC50) varied considerably (Figure 2). There was one exception, a significant difference in free Cu measurements at 0.9 versus 13.7 mg L^{-1} DOC at 16 ppt (Figure 2), which indicates that more free Cu was required to elicit the same toxic effect (i.e., the LC50) at the highest DOC concentration. This highlights a general trend that was observed for the lower salinities, where there was a tendency toward higher DOC having higher free $[Cu^{2+}]$ concentrations (Figure 2, Figure 3a and 3b). It is feasible that with a larger data set more significant differences might be found. A possible explanation for this greater than predicted Cu protectivity, is that at high DOC concentrations, rotifers may be ingesting the DOC and using it as a nutrient; or it provides physical protection against uptake within the digestive tract. These effects would be less at high salinity if salt induced colloid formation reduces the net bioavailability of DOC (by creating fewer but larger molecules). The potential effects of suspended particulates providing additional protectivity against metal toxicity warrants future investigation, but it is not within the scope of the present study.

The reason why greater concentrations of *T*Cu were required to elicit the same toxic effect (LC50) at higher salinities and [DOC] was because a greater proportion was being bound by inorganic and organic ligands, such that more *T*Cu was required to yield the same concentration of free Cu in solution to be taken up by the animal. Kramer et al.[27] found a very similar relationship when using freshwater *Daphnia magna* and a range of DOC concentrations from 0.1 to 22 mg L^{-1}. The authors performed speciation calculations for free Cu concentrations at the effective *T*Cu concentration levels and found that the free Cu concentrations ranged from 10^{-11} to 10^{-10}, which compares well with the present study.[27] It should be noted that the measurement of free Cu using ISE is prone to large variability. The variation in some literature data sets are as much as 4 orders of magnitude (Chadwick et al.[47]) so within that context our measurements here are fairly reproducible.

One of the primary aims of this study is for the data from this research to be used in the development of a marine and estuarine (BLM). Therefore, it is critical that our measured values of free Cu are comparable to the predicted values calculated by the BLM. To ascertain if the measured values of free Cu were comparable to predicted free Cu values at each effective concentration, for each salinity/[DOC] combination, a model first presented by Chadwick et al. was used.[47] Free Cu calculations using this model were based on [DOC], salinity, and pH. In brief, the modeling presented in Chadwick et al., is based on solving simultaneous equilibrium utilized binding constants (logK values) for inorganic ligands as well as DOC. The DOC logK and binding site concentrations (mol site/mg C) are reported in Chadwick et al.[47] and were determined therein from detailed cupric ISE titrations of seawater samples. These values are presented in Figure 3 (horizontal solid lines), along with measured free Cu values (also on the *y*-axis) and the effective *T*Cu values (with 95% CL; *x*-axis). Of note, the calculated free Cu concentrations were the same at each [DOC], but gradually increased as salinity increased (due to an activity correction, where the reactivity of Cu becomes less as the salt levels increase, therefore, more Cu is required to elicit the same toxic effect) (Figure 3). Error estimates on modeled free Cu are also presented for each salinity ($\pm 0.6 -\log[\mathrm{Cu}^{2+}]$; dashed lines, refer to Supporting Information text and Figures S3 and S4 for an explanation of how the value of 0.6 was obtained) and it is evident that there is significant overlap between measured free Cu and calculated free Cu values for all low DOC data points. In fact, if 95% confidence intervals (instead of standard errors) on the measured free Cu values were plotted in Figure 3 and all but one of the data points (16 ppt, 15.4 mg DOC L^{-1}; Figure 3b) have confidence regions overlapping with model predictions. One reason for having one point as an outlier is discussed above, and is not taken into account in the BLM, and again this may lead to future research in this area. Another reason is that a very conservative approach was taken when calculating the "$\pm$ 0.6 $-\log[\mathrm{Cu}^{2+}]$" confidence envelope. Nonetheless, the measured free Cu values from the present study are in good agreement with what the BLM predicts and that these data provide further evidence that free Cu is the toxic form of Cu, and its estimates are critical for modeling and assessing metal toxicity in the aquatic environment.

Our results show that although elevated salinity did provide a protective effect against (total) Cu toxicity to *B. plicatilis* when no DOC was added, an increase in DOC provided the greatest protection against Cu toxicity. Furthermore, the combination of varying salinities and DOC concentrations resulted in similar concentrations of free Cu, even though the effective concentrations of total Cu were considerably different, thus highlighting the importance of the free ion-activity in determining metal toxicity. This study has significant environmental relevance as DOC concentrations used are comparable to those measured in marine and fresh waters and it shows how metal toxicity can be highly dependent on the environmental conditions. For example, in a typical river basin, fresh water runs through land where there could be a relatively high input of allochthonous DOC, in this environment there would be a low level of metal toxicity. As water runs through the estuary, salinity levels would increase, but the DOC may become more diluted, hence increasing relative metal toxicity. Once in the ocean the elevated salinity could offer some protection against metal toxicity, to certain organisms, but this effect will not be as significant as DOC. Metal toxicity in freshwaters is relatively well established; however, more research is required to further elucidate the effects of metal toxicity in the marine environment, particularly in estuarine environments. Hence, data from this research will be used in the development of a marine and estuarine biotic ligand model, and ultimately criteria for the protection of organisms in these environments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (+01) 519 884 0710 ext. 3046; fax: (+01) 519 745 0677; e-mail: ssmith@wlu.ca.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by an NSERC CRD grant (Scott Smith, P.I.) with cofunding from the International Zinc Association (IZA), the International Lead Zinc Research Organization (ILZRO), the Nickel Producers Environmental Research Association (NiPERA), the International Copper Association (ICA), the Copper Development Association (CDA), Teck Resources, and Vale. Also, thanks to Hassan Al-Reasi for running some of the dissolved organic carbon samples.

■ REFERENCES

(1) Grosell, M. Copper. In *Fish Physiology*; Wood, C. M., Farrell, A. P., Brauner, C. J., Eds.; Academic Press: San Diego, CA, 2012; Vol. *31A*, pp 53.

(2) Solomon, E. I.; Lowery, M. D. Electronic structure contributions to function in bioinorganic chemistry. *Science* **1993**, *259* (5101), 1575−1581.

(3) Harris, Z. L.; Gitlin, J. D. Genetic and molecular basis for copper toxicity. *Am. J. Clin. Nutr.* **1996**, *63* (5), 836S−841S.

(4) Arnold, W. R.; Diamond, R. L; Smith, D. S. The effects of salinity, pH, and dissolved organic matter on acute copper toxicity to the rotifer, *Brachionus plicatilis* ("L" strain). *Arch. Environ. Contam. Toxicol.* **2010**, *59* (2), 225–234.

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

(6) Tipping, E. WHAM—A chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. *Comput. Geosci.* **1994**, *20* (6), 973–1023.

(7) Villavicencio, G.; Urrestarazu, P.; Arbildua, J.; Rodriguez, P. H. Application of an acute-biotic-ligand model to predict chronic copper toxicity to Daphnia magna in natural waters of Chile and reconstituted synthetic waters. *Environ. Toxicol. Chem.* **2011**, *30* (10), 2319–2325.

(8) Aquatic life ambient freshwater quality criteria—copper 2007 revision. *Fed. Regis.* **2007**, 72, 3.

(9) Ferrer, L.; Andrade, S.; Asteasuain, R.; Marcovecchio, J. Acute toxicities of four metals on the early life stages of the crab *Chasmagnathus granulata* from Bahía Blanca estuary, Argentina. *Ecotoxicol. Environ. Safety* **2006**, *65* (2), 209–217.

(10) *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses*; PB85-227049; U.S. Environmental Protection Agency: Washington DC, 1985.

(11) Verslycke, T.; Vangheluwe, M.; Heijerick, D.; De Schamphelaere, K.; Van Sprang, P.; Janssen, C. R. The toxicity of metal mixtures to the estuarine mysid Neomysis integer (*Crustacea: Mysidacea*) under changing salinity. *Aquat. Toxicol.* **2003**, *64*, 307–315.

(12) Hall, L. W., Jr.; Anderson, R. D.; Lewis, B. L.; Arnold, W. R. The influence of salinity and dissolved organic carbon on the toxicity of copper to the estuarine copepod. *Eurytemora affinis. Arch. Environ. Contam. Toxicol.* **2008**, *54*, 44–56.

(13) Blanchard, J.; Grosell, M. Effects of salinity on copper accumulation in the common killifish (*Fundulus heteroclitus*). *Environ. Toxicol. Chem.* **2005**, *24* (6), 1403–1413.

(14) Chakoumakos, C.; Russo, R. C.; Thurston, R. V. The toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH, and hardness. *Environ. Sci. Technol.* **1979**, *13* (2), 213–219.

(15) Erickson, R. J.; Benoit, D. A.; Mattson, V. R.; Nelson, H. P., Jr.; Leonard, E. N. The effects of water chemistry on the toxicity of copper to fathead minnows. *Environ. Toxicol. Chem.* **1996**, *15*, 181–193.

(16) Borgmann, U.; Couillard, Y.; Doyle, P.; Dixon, D. G. Toxicity of sixty-three metals and metalloids to *Hyalella azteca* at two levels of water hardness. *Environ. Toxicol. Chem.* **2005**, *24* (3), 641–652.

(17) Anderson, B. S.; Hunt, J. W.; Piekarski, W. J.; Phillips, B. M.; Englund, M. A.; Tjeerdema, R. S.; Goetzl, J. D. Influence of Salinity on Copper and Azide Toxicity to Larval Topsmelt *Atherinops affinis (Ayres)*. *Arch. Environ. Contam. Toxicol.* **1995**, *29* (3), 366–372.

(18) Thurman, E. M. *Geochemistry of Natural Waters*; Martinus Nijhof/Dr. W. Junk Publishers: Dordrecht, Boston, USA, 1985.

(19) Wood, C. M.; Al-Reasia, H. A; Smith, D. S. The two faces of DOC. *Aquat. Toxicol.* **2011**, *105S*, 3–8.

(20) Playle, R. C.; Dixon, D. G.; Burnison, K. Copper and cadmium binding to fish gills: Modification by dissolved organic carbon and synthetic ligands. *Can. J. Fish. Aquat. Sci.* **1993**, *50* (12), 2667–2677.

(21) Richards, J. F.; Curtis, P. J.; Burnison, B. K.; Playle, R. C. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss*) and on metal binding to their gills. *Environ. Toxicol. Chem.* **2001**, *20* (6), 1159–1166.

(22) Sciera, K L.; Isely, J. J.; Tomasso, J. R., Jr.; Klaine, S. J. Influence of multiple water-quality characteristics on copper toxicity to fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2004**, *23* (12), 2900–2905.

(23) Wood, C. M.; Matsuo, A. Y. O.; Wilson, R. W.; Gonzalez, R. J.; Patrick, M. L.; Playle, R. C.; Val, A. L. Protection by natural blackwater against disturbances in ion fluxes caused by low pH exposure in freshwater stingrays endemic to the Rio Negro. *Physiol. Biochem. Zool.* **2003**, *76*, 12–27.

(24) Galvez, F.; Donini, A.; Playle, R. C.; Smith, S.; O'Donnell, M.; Wood, C. M. A matter of potential concern: Natural organic matter alters the electrical properties of fish gills. *Environ. Sci. Technol.* **2008**, *42* (24), 9385–9390.

(25) McGeer, J. C.; Szebedinsky, C.; McDonald, D. G.; Wood, C. M. The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. *Comp. Biochem. Physiol. C* **2002**, *133C*, 147–160.

(26) Glover, C. N.; Wood, C. M. The disruption of *Daphnia magna* sodium metabolism by humic substances: Mechanism of action and effect of humic substance sources. *Physiol. Biochem. Zool.* **2005**, *78*, 1005–1016.

(27) Kramer, K. J. M.; Jak, R. G.; Van Hattum, B.; Hoofman, R. N. Copper toxicity in relation to surface water-dissolved organic matter: Biological effects to *Daphnia magna*. *Environ. Toxicol. Chem.* **2004**, *23* (12), 2971–2980.

(28) Al-Reasi, H. A.; Smith, D. S.; Wood, C. M. Evaluating the ameliorative effect of natural dissolved organic matter (DOM) quality on copper toxicity to *Daphnia magna*: Improving the BLM. *Ecotoxicol.* **2012**, *21* (2), 524–537.

(29) Brunk, B. K.; Jirka, G. H.; Lion, L. W. Effects of salinity changes and the formation of dissolved organic matter coatings on the sorption of phenanthrene: implications for pollutant trapping in estuaries. *Environ. Sci. Technol.* **1997**, *31*, 119–125.

(30) Hunter, Y. R.; Kuwabara, J. S. Ionic Strength and DOC determinations from various freshwater sources to the San Francisco Bay. *Bull. Environ. Contam. Toxicol.* **1994**, *52* (2), 311–318.

(31) Minor, E. C.; Pothen, J.; Dalzell, B. J.; Abdulla, H.; Mopper, K. Effects of salinity changes on the photodegradation and ultraviolet-visible absorbance of terrestrial dissolved organic matter. *Limnol. Oceanogr.* **2006**, *51*, 2181–2186.

(32) Liang, Y.; Hilal, N.; Langston, P.; Starov, V. Interaction forces between colloidal particles in liquid: Theory and experiment. *Adv. Colloid. Interface Sci.* **2007**, *134–135*, 151–166.

(33) Coble, P. G. Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. *Mar. Chem.* **1996**, *51*, 325–346.

(34) Brooks, S. J.; Bolam, T.; Tolhurst, L.; Bassett, J.; La Roche, J.; Waldock, M.; Barry, J.; Thomas, K. V. Effects of dissolved organic carbon on the toxicity of copper to the developing embryos of the pacific oyster (*Crassostrea gigas*). *Environ. Toxicol. Chem.* **2009**, *26* (8), 1756–1763.

(35) Nadella, S. R.; Fitzpatrick, J. L.; Franklin, N.; Bucking, C.; Smith, S.; Wood, C. M. Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon. *Comp. Biochem. Physiol. C* **2009**, *149* (3), 340–348.

(36) Arnold, W. R.; Cotsifas, J. S.; Ogle, R. S.; DePalma, S. G. S.; Smith, D. S. A comparison of the copper sensitivity of six invertebrate species in ambient salt water of varying dissolved organic matter concentrations. *Environ. Toxicol. Chem.* **2010**, *29* (2), 311–319.

(37) Zhong, H.; Evans, D.; Wang, W. X. Uptake of Dissolved Organic Carbon-Complexed ^{65}Cu by the Green Mussel *Perna viridis*. *Environ. Sci. Technol.* **2012**, *46*, 2383–2390.

(38) Minkoff, G.; Lubzens, E.; Kahan, D. Environmental factors affecting hatching of rotifer (*Brachionus plicatilis*) resting eggs. *Hydrobiol.* **1983**, *104*, 61–69.

(39) Arnold, W. R.; Diamond, R. L.; Smith, D. S. Acute and chronic toxicity of copper to the euryhaline rotifer, *Brachionus plicatilis* ("L" strain). *Arch. Environ. Contam. Toxicol.* **2011**, *60* (2), 250–260.

(40) *Standard guide for acute toxicity test with the rotifer Brachionus*; E 1440-91; Annual Book of ASTM Standards, Vol. 11.05; American Society for Testing and Materials International: West Conshohocken, PA, 2004.

(41) Guillard, R. R. L. Culture of Phytoplankton for Feeding Marine Invertebrates. In *Culture of Marine Invertebrates*; Berg, C. J., Jr., Ed.; Hutchinson-Ross: Stroudsberg, PA, 1983; pp 108.

(42) Sun, L.; Perdue, E. M.; McCarthy, J. F. Using reverse osmosis to obtain organic matter from surface and ground waters. *Water Res.* **1995**, *29*, 1471−1477.

(43) Schwartz, M. L.; Curtis, P. J.; Playle, R. C. Influence of natural organic matter source on acute copper, lead, and cadmium toxicity to rainbow trout *Oncorhynchus mykiss*. *Environ. Toxicol. Chem.* **2004**, *23* (12), 2889−2899.

(44) Winter, A.; Fish, T.; Playle, R.; Smith, D. S.; Curtis, P. Photodegradation of natural organic matter from diverse freshwater sources. *Aquat. Toxicol.* **2007**, *84*, 215−222.

(45) Eriksen, R. S.; Mackey, D. J.; Alexander, P. W.; De Marco, R.; Wang, X. D. Continuous flow methods for evaluating the response of a copper ion selective electrode to total and free copper in seawater. *J. Environ. Mon.* **1999**, *1*, 483−488.

(46) *National Institute of Standards and Technology NIST Standard Reference Database 46*; National Institute of Standards and Technology: Gaithersburg, MD, 2001.

(47) Chadwick, D. B.; Rivera-Duarte, I.; Rosen, G.; Wang, P. F.; Santore, R. C.; Ryan, A. C.; Paquin, P. R.; Hafner, S. D.; Choi, W. Demonstration of an integrated compliance model for predicting copper fate and effects in DoD harbors. *Environmental Excurity Technology Certification Program (ESTCP) Project ER-0523*; SPAWAR Systems Center Pacific: San Diego, CA, 2008.

(48) *Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms*; EPA/600/R-95/136; U.S. Environmental Protection Agency: Washington DC, 1995.

(49) Grosell, M.; Blanchard, J.; Brix, K. V.; Gerdes, R. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquat. Toxicol.* **2007**, *84* (2), 162−72.

(50) DePalma, S. G. S.; Arnold, W. R.; McGeer, J. C.; Dixon, D. G.; Smith, D. S. Effects of dissolved organic matter and reduced sulphur on copper bioavailability in coastal marine environments. *Ecotoxicol. Environ. Safety* **2011**, *74*, 230−237.

(51) Sánchez-Marín, P.; Santos-Echeandía, J.; Nieto-Cid, M.; Álvarez-Salgado, X. A.; Beiras, R. Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae. *Aquat. Toxicol.* **2010**, *96*, 90−102.

(52) De Schamphelaere, K. A. C.; Janssen, C. R. Bioavailability and chronic toxicity of zinc to juvenile rainbow trout (*Oncorhynchus mykiss*): comparison with other fish species and development of a biotic ligand model. *Environ. Sci. Technol.* **2004**, *38*, 6201−6209.

(53) Christenson, H. K. DLVO (Derjaguin-Landau-Verwey-Overbeek) theory and solvation forces between mica surfaces in polar and hydrogen-bonding liquids. *J. Chem. Soc. Faraday Trans.* **1984**, *80* (1), 1933−1946.

(54) De Schamphelaere, K. A. C.; Heijerick, D. G.; Janssen, C. R. Comparison of the effect of different pH buffering techniques on the toxicity of copper and zinc to *Daphnia magna* and *Pseudokirchneriella subcapitata*. *Ecotoxicology* **2004**, *13*, 697−705.

(55) Lauer, M. M.; Bianchini, A. Chronic copper toxicity in the estuarine copepod *Acartia tonsa* at different salinities. *Environ. Toxicol. Chem.* **2010**, *29* (10), 2297−2303.

(56) Blust, R.; Bernaerts, F.; Van der Linden, A.; Thoeye, C. The influence of aqueous copper chemistry on the uptake and toxicity of copper in *Artemia*. In *Artemia Research and Its Applications: 1. Morphology, Genetics, Strain Characterization, Toxicology.* ; Proceedings of the Second International Symposium on the brine shrimp Artemia; Sorgeloos, P. et al., Eds.; 1987; pp. 311−323.

(57) McLusky, D. S.; Bryant, V.; Campbell, R. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. *Oceanogr. Mar. Biol. Ann. Rev.* **1986**, *24*, 481−520.

(58) Rachou, J.; Hendershot, W.; Sauvé, S. Soil organic matter impacts upon fluxes of cadmium in soils measured using diffusive gradients in thin films. *Commun. Soil Sci. Plant Anal.* **2007**, *38* (11−12), 1619−1636.

(59) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Plenum Publishing: New York, 1999.