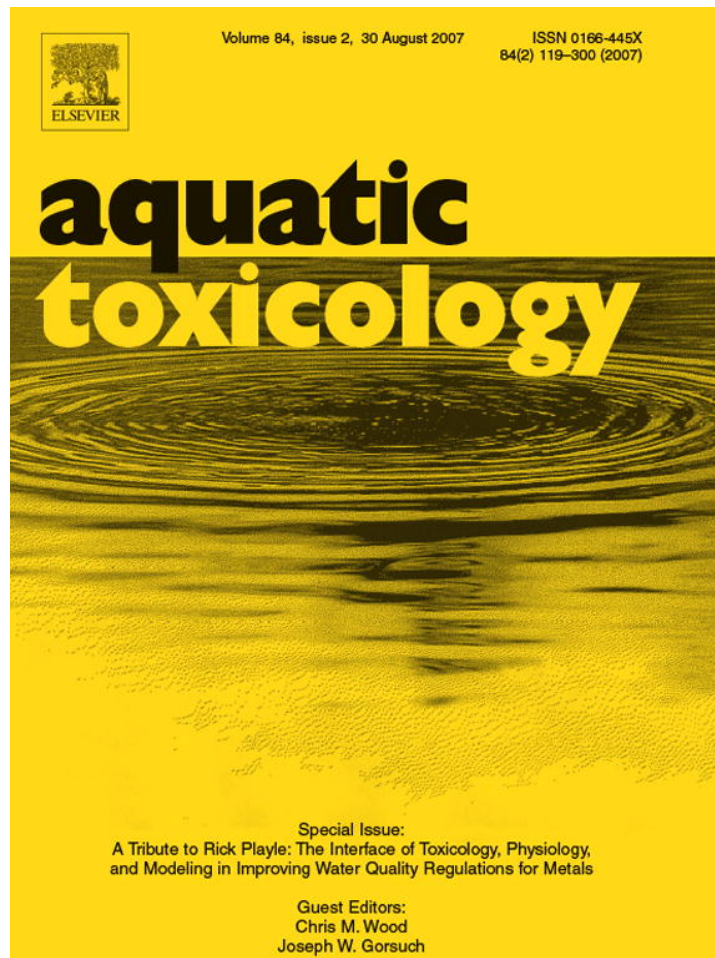


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Development and validation of a chronic copper biotic ligand model for *Ceriodaphnia dubia*

Melissa L. Schwartz^{*}, Bernard Vigneault

CANMET - Mining and Mineral Sciences Laboratories, Natural Resources Canada, 555 Booth Street, Ottawa, Ont., Canada K1A 0G1

Received 15 November 2006; received in revised form 29 January 2007; accepted 29 January 2007

Abstract

A biotic ligand model (BLM) to predict chronic Cu toxicity to *Ceriodaphnia dubia* was developed and tested. The effect of cationic competition, pH and natural organic matter complexation of Cu was examined to develop the model. There was no effect of cationic competition using increasing Ca and Na concentrations in our exposures. However, we did see a significant regression of decreasing toxicity (measured as the IC25; concentration at which there was a 25% inhibition of reproduction) as Mg concentration increased. However, taking into account the actual variability of the IC25 and since the relative increase in IC25 due to additional Mg was small (1.5-fold) Mg competition was not included in the model. Changes in pH had a significant effect on Cu IC25, which is consistent with proton competition as often suggested for acute BLMs. Finally, natural organic matter (NOM) was added to exposures resulting in significant decreases in toxicity. Therefore, our predictive model for chronic Cu toxicity to *C. dubia* includes the effect of pH and NOM complexation. The model was validated with Cu IC25 data generated in six natural surface waters collected from across Canada. Using WHAM VI, we calculated Cu speciation in each natural water and using our model, we generated “predicted” IC25 data. We successfully predicted all Cu IC25 within a factor of 3 for the six waters used for validation.

Crown Copyright © 2007 Published by Elsevier B.V. All rights reserved.

Keywords: *Ceriodaphnia dubia*; Biotic ligand model; Chronic toxicity; Copper

1. Introduction

It is widely accepted that water chemistry has a dramatic effect on the toxicity of metals such as Cu, Zn, Cd, Ag, Pb, Ni, U, Al and others (e.g. Richards and Playle, 1999; MacDonald et al., 2002; Bury et al., 1999; Janes and Playle, 1995; Hollis et al., 2000 and references therein). The influence of water hardness, which has been recognized as a moderating influence on the toxicity of a few metals within the Canadian Water Quality Guidelines, has been incorporated as a “hardness correction”, however, it is now recognized that such corrections are sometimes inappropriate (Markich et al., 2005). Although many studies have investigated the influence of water chemistry on acute metal toxicity, there are still relatively few studies on chronic metal toxicity. In recent years, jurisdictions in Canada and in the United States have taken the first steps to apply this scientific knowledge into water quality criteria, in particular using the biotic ligand approach.

In the last two decades, a better understanding of the interaction between waterborne metals and sensitive biotic receptors, such as the fish gill, has been gained through the initial work on the gill surface interaction model (Pagenkopf, 1983) and metal–gill modeling by Playle (1998) and the subsequent development of what is now termed the biotic ligand model (BLM) by Paquin et al. (2000), Di Toro et al. (2001) and Santore et al. (2001). Until recently, most work done in the BLM framework was on acute toxicity with fish and invertebrates, however a number of chronic BLMs have been developed over the last few years, including chronic Zn and Cu BLMs for *Daphnia magna* (Heijerick et al., 2005 and De Schamphelaere and Janssen, 2004, respectively). One main difference in acute versus chronic BLMs appears to be the lack of importance of competitive cations in solutions. Generally, in acute BLMs, the presence of cations such as Ca, Mg and Na can mitigate metal toxicity by competing for their own binding sites on the membrane surface. Since, Cu is taken in by the organism through the same pathway as Na, the presence of excess Na in solution can decrease Cu binding to the uptake sites by competition (Grosell et al., 2002). As De Schamphelaere and Janssen (2004) discuss, the lesser importance of this competitive interaction could be due to both

^{*} Corresponding author. Tel.: +1 613 947 7383; fax: +1 613 996 9041.
E-mail address: mschwartz@nrcan.gc.ca (M.L. Schwartz).

the increase in exposure time during a sublethal test, and the decrease in metal concentrations required to elicit the sublethal effect.

Chronic toxicity testing with *Ceriodaphnia dubia* is required under a number of United States and Canadian regulations, for example the Canadian Metal Mine Effluent Regulation – Environmental Effects Monitoring (MMER-EEM) program. The inclusion of this species in chronic BLM advancement would therefore be important especially since *C. dubia* is among the most sensitive freshwater species to Cu. Also, the current draft protocol for the derivation of Canadian water quality guidelines (CWQG) includes the use of BLM where it is applicable (CCME, 2006). Therefore, our goal of developing a chronic Cu BLM for *Ceriodaphnia dubia* validated with Canadian surface waters will aid in the derivation of site-specific CWQGs that account for the impacts of water chemistry on Cu toxicity.

2. Methods and materials

2.1. Experimental organisms

Cultures of *Ceriodaphnia dubia* were originally purchased from a commercial supplier (Aquatic Research Organisms, Hampton, NH) and cultured in our lab at CANMET-MMSL according to the recommended standard method published by Environment Canada (1992). To develop the chronic Cu BLM for *C. dubia*, organisms were cultured and tested in 75% dechlorinated Ottawa city tap water (75% tap water, 25% ultrapure water, Barnstead EASYpure LF, Fisher Scientific, Ottawa, ON) to obtain a media that was soft enough to test for competitive interactions of various cations while still being able to support healthy cultures of *C. dubia*. The pH, hardness, and alkalinity of our 75% dechlorinated tap water was 7.63, 23.3 mg/L CaCO₃ and 20.4 mg/L CaCO₃, respectively. More detailed physico-chemical data is given in Table 1.

2.2. *Ceriodaphnia dubia* chronic copper toxicity tests

Our goal was to identify the individual effects of Ca, Na, Mg, pH and natural organic matter on chronic copper toxicity to *C. dubia*. Therefore, tests of reproduction were conducted in 75% dechlorinated Ottawa city tap water where a single parameter was varied at a time in a range of concentrations. Both culture and testing of *C. dubia* was done following the Environment Canada biological test method for testing reproduction and survival. Organisms were cultured and tested under a 16 h:8 h light:dark cycle in benchtop incubators (Innova Incubator Shaker, New Brunswick Scientific) at 24.5 ± 2 °C in round-bottom polypropylene 16.5 mL test tubes (Sarstedt Inc., Montreal, QC) filled with 15 mL of culture or test media. During culturing and testing, the number of neonates per adult female daphnid was counted and the adults were transferred daily into fresh media containing 2.3 × 10⁵ cells/mL of a 3:1 mixture of *Pseudokirchneriella subcapitata* and *Chlorella kesslerii* and 100 µL of a yeast–cerophyll–trout chow mixture (YCT) purchased from Aquatic Research Organisms. Each test was started with 10 individual replicates per exposure concentra-

Table 1
Physicochemistry of 75% dechlorinated Ottawa city tap water and surface waters collected from across Canada

Exposure water	TOC (mg C/L)	TIC (mg C/L)	pH	Cu (µg/L)	Ca (mg/L)	Mg (mg/L)	K (mg/L)	Na (mg/L)	Cl (mg/L)	SO ₄ (mg/L)	IC25 _{total} Cu (µg/L)	IC25 _{freeCu} (M)
75% dechlorinated tap water	0.40	5.14	7.63	0.8	6.6	1.7	0.6	13.9	5	22	10.9 (6.2–12.5)	3.2 × 10 ⁻¹¹ (2.3 × 10 ⁻¹¹ –5.2 × 10 ⁻¹¹)
Endako mine reference water	17.41	15.88	7.95	<3	49.9	11.2	1.1	11.6	<1	120	96.5 (82.3–113.4)	2.4 × 10 ⁻¹⁰ (1.6 × 10 ⁻¹⁰ –3.2 × 10 ⁻¹⁰)
Thompson mine reference water	18.54	28.99	8.06	2.1	45.1	11.9	1.7	5.6	10	36	67.0 (25.0–95.7)	5.6 × 10 ⁻¹¹ (7.7 × 10 ⁻¹² –1.1 × 10 ⁻¹⁰)
Red Lake mine reference water	33.41	5.10	7.30	1.7	10.1	2.4	0.4	1.2	10	9	88.3 (11.6–104.0)	1.0 × 10 ⁻¹⁰ (1.8 × 10 ⁻¹² –1.5 × 10 ⁻¹⁰)
Vermillion River	5.38	7.87	7.55	2.65	21.7	3.8	1.6	8.6	12	32	20.6 (14.4–23.8)	4.1 × 10 ⁻¹¹ (1.9 × 10 ⁻¹¹ –6.1 × 10 ⁻¹¹)
Georgian Bay	1.46	18.28	8.15	<0.1	24.4	7.0	1.0	4.0	6	13	19.6 (7.7–22.4)	4.0 × 10 ⁻¹¹ (3.4 × 10 ⁻¹² –5.1 × 10 ⁻¹¹)
Lac Dawson	6.02	4.71	7.60	2.18	8.1	1.1	0.4	7.9	13	4	19.9 (16.5–35.3)	1.5 × 10 ⁻¹¹ (1.0 × 10 ⁻¹¹ –6.5 × 10 ⁻¹¹)

IC25 (lower 95% C.I. – upper 95% C.I.). See Section 2 for details on analysis.

tion, with one less-than-24 h-old neonate per replicate. The tests ended when at least 60% of control organisms had three broods (usually on the sixth or seventh day of testing at 24.5 °C). During testing, measurements of pH (Radiometer PHM290 MeterLab with a red-rod combined pH electrode, high KCl flow, London Scientific, London, ON), conductivity (Orion 1230, Fisher Scientific) and dissolved oxygen (IONcheck20, London Scientific) were done on the fresh solutions as well as the 24 h old solutions. At no point during any test did the dissolved oxygen concentration reach less than 40%. Exposure solutions were prepared and spiked with Cu (as CuSO₄; Anachemia Canada Inc., Montreal, QC) at least the day before starting a test and stored at approximately 24 °C, resulting in a pre-equilibration time of at least 20 h. This amount of time should allow for adequate equilibration of each exposure solution (Kim et al., 1999). For reference Cu toxicity testing in 75% Ottawa city tap water, Cu concentrations were 0, 5, 10, 15 and 20 µg/L, nominally. For other tests, including those with natural waters (see Section 2.6), higher Cu concentrations (up to 160 µg/L) were added as needed to achieve an IC25.

For testing the influence of pH on chronic Cu toxicity, pH of the 75% Ottawa city tap water was buffered using 750 mg/L 3-*N*-morpholinopropane sulfonic acid (MOPS, SigmaUltra min. 99.5%, Sigma–Aldrich, Oakville, ON) and adjusted as needed with HNO₃ or NaOH for pH 5.5 and 6.5, and 0.1 mM sodium tetraborate (Anachemia) adjusted with H₂SO₄ for pH 7.5 and 8.3. The use of these particular buffers is based on the fact that they show no significant Cu complexing capacity, and no effect on *C. dubia* toxicity (De Schamphelaere and Janssen, 2004). To test the effect of individual cations on chronic Cu toxicity to *C. dubia*, tests were performed with calcium (as CaCl₂; Anachemia) at added nominal concentrations of 10, 25, 50, 100 and 250 mg/L, with sodium (as NaCl; Anachemia) at added nominal concentrations of 10, 25, 50, 100 and 250 mg/L, and with magnesium (as MgCl₂; Sigma) at added nominal concentrations of 12.5, 25, 50 and 100 mg/L. Complexation tests were done using two types of natural organic matter (NOM), Suwannee River NOM and Nordic Reservoir NOM (International Humic Substances Society, St. Paul, MN, USA). Suwannee River NOM was added at nominal concentrations of 2.5, 5, 10 and 25 mg C/L and Nordic Reservoir NOM was tested at 5 and 10 mg C/L (nominal). Both NOMs were purchased as freeze-dried solid powders that were prepared as stock solutions dissolved in 75% dechlorinated tap water. Samples taken at the end of each test were analyzed for Cu, Ca, Na, Mg, K, Fe, Al, Zn, Cl, NO₃, SO₄, PO₄ and total and dissolved organic/inorganic carbon. Dissolved organic/inorganic carbon samples were filtered using 0.45 µm Acrodisc syringe filters with a Tuffryn membrane (Pall Life Sciences, VWR International Inc., Montreal, QC). For most tests, dissolved Cu samples were measured and were filtered using 0.45 µm Acrodisc PTFE syringe filters (VWR).

2.3. Chemical analyses

Analysis of Cu was done by an inductively coupled plasma-mass spectrometer (ICP-MS; ELAN 6100 DRC with AS-91 autosampler, PerkinElmer, Woodbridge, ON) which was cali-

brated before each use with a minimum of three standards, and checked for QA/QC by including a duplicate sample, a certified reference sample and a mid-range calibration standard every 10 samples or during each sample run. Calcium, Na, K, Mg, Fe and Al were analyzed using an inductively coupled plasma atomic emission spectrometer (ICP-AES; Varian Vista RL with Varian SPS-5 autosampler, Varian, Mississauga, ON) following the same QA/QC measures stated above. For all samples using ICP-MS and ICP-AES, samples contained approximately 3% HNO₃ (trace metal grade, Fisher Scientific, Montreal, QC) as a preservative. Anions in solution (Cl, NO₃, PO₄ and SO₄) were measured using high performance liquid chromatography (HPLC; Dionex-100 advanced chromatography module with Dionex autosampler, Dionex Canada Ltd., Oakville, ON) using the same QA/QC measures stated above. Total carbon and inorganic carbon concentrations were measured with a total carbon analyzer (Shimadzu TOC-V with ASI-V autosampler, Mandel Scientific, Guelph, ON). Total organic carbon concentration of each sample was calculated automatically by subtracting inorganic carbon from total carbon. The instrument was calibrated using total carbon and inorganic carbon standard solutions purchased from Pharmaceutical Resource Associates (Arvada, Colorado, USA).

2.4. Free Cu²⁺ activity measurements using a copper ion-selective electrode

Measurements of free Cu²⁺ activity were done on Cu-spiked samples in 75% dechlorinated tap water, and the natural water samples (see Section 2.6) using an Orion 5-star Series Meter with an Orion Ionplus Sure Flow Cupric Ion Electrode (Orion 96–29 Ionplus, Thermo Electron Corporation, Burlington, ON). Before use, the electrode was washed with soap and rinsed with ultrapure water and new filling solution (D Optimum results Thermo) was added. An electrode response's slope check was performed each day before calibration and the electrode was polished if required. For all Cu ion-selective electrode (ISE) measurements, a five point linear calibration curve was generated using the diluted Cu standard with concentrations ranging from 1×10^{-7} to 1×10^{-5} M Cu. To minimize the effect of ionic strength, NaNO₃ was added to all standards and solutions at a concentration of 0.01 M. The slopes of the calibration curves, on average 29.0 ± 0.5 mV per decade of Cu²⁺, were close to the theoretical value of the electrode slope at 20 °C, i.e. 29.1.

2.5. Data interpretation

The mean number of *C. dubia* neonates produced during a test in each exposure was calculated and the copper concentrations causing a 25% reproductive inhibition (IC25 ± 95% confidence intervals) were calculated using CETIS, Comprehensive Environmental Toxicology Information System software version 1.025b (Tidepool Scientific Software, McKinleyville, CA, USA) by linear interpolation based on measured initial total Cu concentrations. We chose the IC25 as our endpoint in this study, since it is currently favoured as a primary criterion of a sublethal effect (Environment Canada, 1992). As recommended

in the current Environment Canada method, in exposures where mortality occurred, any offspring produced up until that time was included in the calculated mean number of offspring. In cases where an organism died before producing offspring, a value of zero neonates was used for calculating a mean. When complete mortality occurred in a given Cu exposure, the data was not included.

2.6. Model validation

To check the robustness of our model (see Section 3 for model development), we collected six natural surface waters from across Canada. Water was collected from three mine site reference waters; Endako Mine (Thompson Creek Mining, Fraser Lake, British Columbia, 54.069N, 124.832W); Birchtree Mine (Inco Thompson, Thompson, Manitoba, 55.699N, 97.951W); and Upper Balmer Creek (Goldcorp Inc. Red Lake Mine, Red Lake, Ontario, 51.069N, 93.669W). Also, Vermillion River water near Sudbury, Ontario (46.402N, 81.312W) and lake water from Georgian Bay, Ontario (45.131N, 81.324W) and Lac Dawson, Quebec (45.748, 74.261W) were collected. The chemistry of these waters is summarized in Table 1. For testing, the unfiltered surface water was spiked with Cu and allowed to equilibrate for at least 24 h before starting the test. We used total copper concentrations because not all samples were measured for dissolved Cu. Of the samples we measured for dissolved Cu, the difference between total and dissolved concentrations was minimal.

3. Results and discussion

3.1. Effects of calcium, sodium and magnesium on chronic copper toxicity

In contrast to acute BLMs and hardness normalization, we found no significant protective effect of Ca, Na and Mg to Cu chronic toxicity to *C. dubia*. Several reference chronic Cu toxicity tests were run in 75% tap water throughout the duration of this study and the mean Cu IC25 (95% LCL – 95% UCL) was 10.9 (6.2–12.5) $\mu\text{g/L}$, $n=7$. When Ca concentrations were increased, up to 249 mg/L, no linear relationship was found between added Ca and Cu toxicity (linear regression, $r^2=0.01$, $P=0.89$; Fig. 1A). When Na concentrations were increased, up to 270 mg/L, there was also no significant effect on Cu toxicity ($r^2=0.64$, $P=0.06$), although there appears to be a slight trend towards a decrease in Cu toxicity as Na is increased (Fig. 1B). Considering the possibility of an elevated ion-induced stress, we removed the 250 mg/L Na treatment, and the 100 and 250 mg/L Na treatments from the linear regression calculation, and although the relationship improved slightly, it was still not significant ($r^2=0.70$, $P=0.08$, $r^2=0.86$ $P=0.08$, respectively). Finally, we increased the concentration of Mg in the exposures up to 54 mg/L and found there was a significant linear regression of Cu toxicity and added Mg ($r^2=0.98$, $P=0.01$), however the actual increase in IC25 is only a factor of 1.52 (Fig. 1C). At added Mg concentrations of 100 mg/L and higher we saw a Mg-induced toxicity in *C. dubia* that was

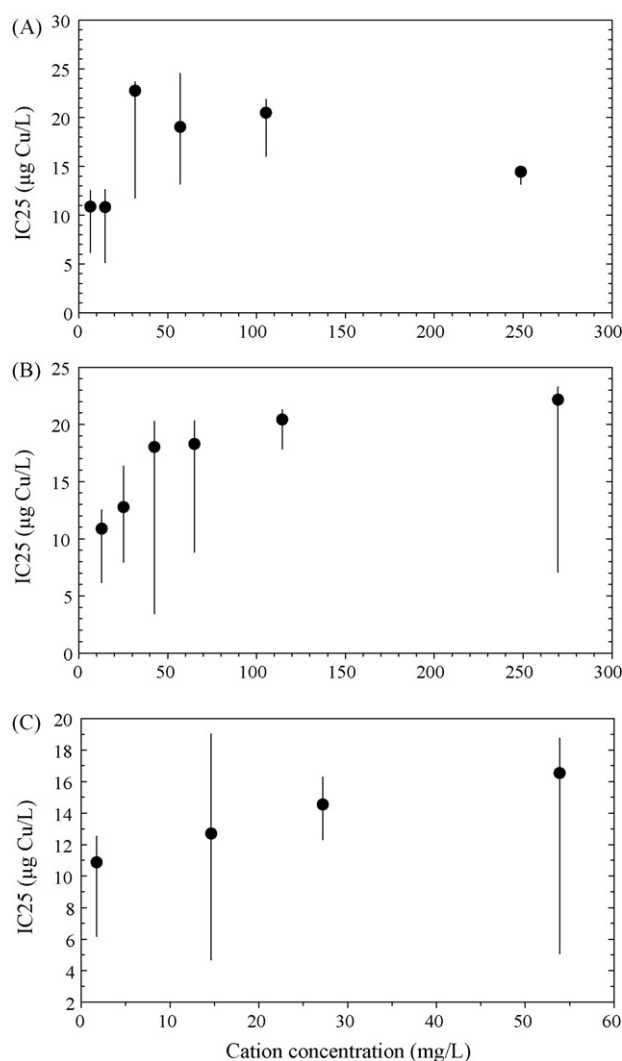


Fig. 1. The effect of calcium (A), sodium (B) and magnesium (C) on chronic Cu toxicity to *Ceriodaphnia dubia*. No significant increase in IC25 was seen with Ca concentrations up to 249 mg/L, with Na concentrations up to 270 mg/L, or with Mg concentrations up to 54 mg/L compared with the reference Cu toxicity tests in 75% dechlorinated tap water. Concentrations of Ca, Na and Mg on the x-axis refer to total amounts, including what was already present in the 75% dechlorinated tap water. The reference Cu IC25 value in 75% decl. tap water was the mean \pm 95% C.I. for 7 tests. All other data are presented as IC25 \pm 95% C.I. for single tests. In some cases an upper or lower confidence limit could not be determined. The average pH \pm standard deviation for all tests was 7.63 \pm 0.23 ($n=288$).

actually reduced as more Cu was added to the exposure (data not shown). That is, survival and reproduction increased with increasing Cu at Mg concentrations of 100 mg/L and higher. It would appear that, at higher Mg concentrations, the presence of Cu was ameliorating the effect of Mg. We saw the same effect whether we used MgCl_2 or MgSO_4 although with 100 mg/L Mg as MgSO_4 , there was no reproduction in the controls, and with 100 mg/L Mg as MgCl_2 there was reproduction albeit reduced. In both cases, the apparent toxicity of Mg was reduced as Cu was added.

Given the 95% confidence intervals for the IC25 and the actual range of Mg concentrations in natural waters, the observed reduction in the IC25 estimates is negligible. The fact that

chronic Cu toxicity was not substantially reduced as Ca, Na or Mg concentrations increased confirms that major cation competition is not important in the development of a chronic BLM for *C. dubia*. This is in contrast to the acute BLM developed by De Schamphelaere et al. (2002) for *D. magna*, where increases in major cations had a protective effect on acute Cu toxicity. They found that Ca and Mg decreased Cu toxicity to a similar extent. The small protective effect of Mg seen in our results is similar to the protective effect of Ca discussed in Di Toro et al. (2001). The approximate 2-fold increase in fathead minnow Cu LC50 with increasing Ca concentration is smaller than was expected based on the hardness correction in the copper water quality criteria employed by the US EPA (1984). However, they explain the difference as being related to the effect of CO_3^{2-} addition (as CaCO_3) in studies used to develop the hardness correction rather than the true effect of Ca competition as is seen when Ca is added as CaSO_4 . Finally, results of Hyne et al. (2005) show that acute Cu toxicity to *C. dubia* is unaffected by increasing hardness, as was the case in the development of a chronic BLM for *D. magna* (De Schamphelaere and Janssen, 2004). In the latter, they found a protective effect of Na (~3.5-fold increase in 21-day EC50) but it was mainly an effect of increased survival and not an effect of reproductive improvement. Because the Na effect was not significant in our data, we decided not to include any cationic competition in our model.

3.2. Effect of pH on chronic copper toxicity

We tested the chronic toxicity of Cu to *C. dubia* at pH 5.6, 6.5, 7.4 and 8.0. At pH 5.6 there was significant mortality (40%) seen in the control organisms, indicating a toxic effect due to the low pH alone. At this low pH, the mean number of offspring produced (± 1 S.E.) was 11 ± 5 , which is considerably lower than in the reference test control organisms (mean offspring production of 23 ± 2 over 7 tests), indicating an apparent lower pH limit for this organism. Belanger and Cherry (1990) tested *C. dubia* for chronic toxicity at pH 5–10, and findings similar to ours were seen at pH 5.3. In pH 6.5 the IC25 (95% LCL – 95% UCL $\mu\text{g Cu/L}$) was 9.3 (7.5–10.8), in pH 7.4 it was 9.2 (7.8–11.2) and in pH 8.0 it was 10.9 (8.0–13.0). The similarity of these IC25s indicates that changes in pH had little effect on Cu toxicity when expressed as total Cu concentrations. However, since changes in pH also impact Cu complexation, the competitive effect of H^+ might be masked by changes in Cu speciation. We therefore calculated free Cu^{2+} activity using WHAM VI (Tipping, 1998; see Section 3.4 for details). Copper IC25s calculated based on free Cu^{2+} activity showed a decrease in Cu toxicity with decreasing pH. In effect, more free Cu^{2+} was required to achieve an IC25 as the pH is reduced (Fig. 2). When the IC25s are expressed as free Cu^{2+} activity, the linear relationship between IC25 and pH (as H^+ concentration) is $\text{Cu}^{2+} = \text{H}^+((5.3 \pm 0.2) \times 10^{-4}) + (9 \pm 4) \times 10^{-12}$ ($r^2 = 0.99$). This protective effect of H^+ competition was observed for the chronic toxicity of Cu to *D. magna* (De Schamphelaere and Janssen, 2004) and was also the case in De Schamphelaere et al. (2003) where they noted that less free Cu was required to exert the same toxic effect at a higher pH using the algae, *P.*

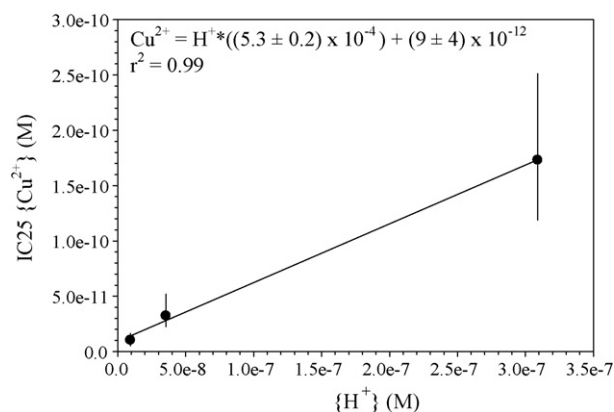


Fig. 2. The impact of H^+ competition on Cu IC25 expressed as free Cu^{2+} activity vs. H^+ concentration. As the free Cu^{2+} IC25 increased, the concentration of H^+ increased as well, resulting in a significant linear relationship ($r^2 = 0.99$). See Fig. 1 caption for details on data presentation.

subcapitata. However, this effect was non-linear which is unexpected based on the current BLM framework indicating that the assumption for proton competition at a single uptake site may not be valid for algal cells.

3.3. Complexation by natural organic matter

The most dramatic effect on chronic Cu toxicity to *C. dubia* was seen when natural organic matter was added to the exposures. Both Suwannee River NOM (SRMOM) and Nordic Reservoir NOM (NRMOM) were used as reference NOM instead of the commercially available Aldrich humic acid, based on the fact that they are naturally derived and their properties would likely be more similar to NOM found in other surface waters. Aldrich humic acid is suitable as a reference standard for NOM as well, but would represent a very optically dense, highly aromatic and highly allochthonous-type OM. The addition of both SRMOM and NRMOM reduced sublethal Cu toxicity to a large extent, however the reduction in toxicity was slightly less with NRMOM (Fig. 3). On average, IC25 data with SRMOM was 1.7 times greater than the NRMOM IC25 data at the same NOM concentration, however since the 95% confidence limits overlap and the overall linear relationship is good ($r^2 = 0.88$, $P = 0.002$), we consider this difference to be negligible. We investigated this small difference in protective effect and it does not seem to be reflected in the specific absorbance coefficients of the two NOMs (SAC₃₄₀ of both is approximately 33, see Richards et al., 2001 for SAC calculation), however SRMOM does have a slightly higher aromatic content (23% versus 19%) and lower aliphatic content (27% versus 31%) than does NRMOM (data from the International Humic Substances Society). While these differences in aromatic and aliphatic content are not large it could be one explanation for the observed differences in protective effect since more allochthonous (terrestrially derived, more aromatic) NOM binds Cu more effectively (Schwartz and Playle, 2004).

Unfortunately, these tests were not pH adjusted and when higher amounts of NOM were added, the pH in the exposures decreased to as low as 6.74 in the highest SRMOM test. However, the data can be corrected for the effect of pH differences

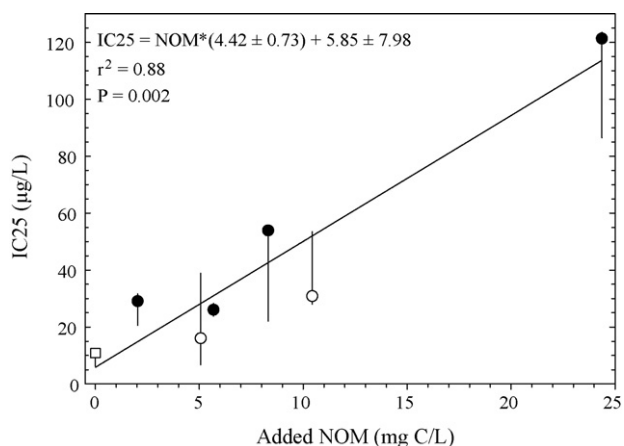


Fig. 3. Complexation of Cu by two types of natural organic matter increased the Cu IC25 significantly ($r^2 = 0.88$, $P = 0.002$). Both the SROM (filled circles) and the NROM (open circles) protected against Cu toxicity compared with 75% tap water with no NOM added (open square). Suwannee River NOM provided slightly greater protection than did NROM as seen by the higher IC25s at similar NOM concentrations. See Fig. 1 for details on data presentation.

by normalization to the 75% tap water pH using the observed relationship in the absence of NOM between IC25 (expressed as $\{Cu^{2+}\}$) and $\{H^+\}$. As expected based on the BLM framework, the IC25s based on free Cu^{2+} activities, with the inclusion of food to the media (see Section 3.4) and normalized to pH 7.63 are similar for all treatments. The average IC25 (as $\{Cu^{2+}\} \pm 95\%$ C.I.) for the 75% tap water, the SROM and NROM tests was $(9 \pm 5) \times 10^{-11}$ M, suggesting that the observed effect of NOM on Cu sublethal toxicity to *C. dubia* can be explained by the complexation effect alone.

The chronic toxicity of Cu to *C. dubia* is therefore highly influenced by the presence of NOM. Based on our data with added SROM and NROM, 88% ($P = 0.002$) of the variability in IC25 is accounted for by NOM concentration alone. This resulted in a linear correlation of *C. dubia* $IC25 = TOC * (4.4 \pm 0.7) + 6 \pm 8$, when pH was normalized to 7.63 for all tests. Other studies have found a similar strong influence of DOC on acute toxicity. For example, *Daphnia magna* 48-h EC50 in natural waters with pH ranging from 7.3 to 8.4, expressed as a function of DOC concentration resulted in a linear correlation of $EC50 = DOC * 17.2 + 30.2$, $r^2 = 0.8$; Kramer et al., 2004). The range of DOC concentrations in these waters (3–22 mg C/L) were similar to our testing with SROM. A study of acute (48-h EC50) Cu toxicity to *C. dubia* in natural waters with pH ranging from 6.8 to 7.4 also found a good linear correlation of TOC (ranging from 1.2 to 4.5 mg C/L) and EC50 ($EC50 = DOC * 7.1 + 10.6$; $r^2 = 0.99$, $P = 0.06$), when one outlier in the data was excluded (Diamond et al., 1997).

3.4. Model development

Since our testing revealed no significant protective effect of major cation competition, it was not included in the model development. Therefore, only the effect of pH and NOM complexation was included. We used WHAM VI to predict copper speciation, since Bryan et al. (2002) validated its use to predict

free Cu^{2+} in natural waters over a wide range of NOM concentrations. Inorganic carbon concentrations, major cation and anion concentrations, as well as pH were used as input parameters. The thermodynamic database used was corrected according to Martell et al. (1997). As proposed by Bryan et al. (2002) it was assumed that 68% of NOM was fulvic acid with a carbon content of 50% for all calculations. The IC25, as free Cu^{2+} activity, was first calculated based on the regression equation of our H^+ versus Cu^{2+} data (Fig. 2) to account for the effect of H^+ competition of Cu toxicity to *C. dubia*. This free Cu^{2+} IC25 was then back-calculated to obtain total copper concentrations using the WHAM VI estimations.

Because the 7-day chronic test requires the addition of food to the exposures, it was necessary to include the additional organic carbon when calculating the free Cu^{2+} activity. We found a significant improvement in the relationship between our predicted Cu^{2+} and measured Cu^{2+} activity, using the copper ISE, when this additional NOM was included (4.98 mg C/L) instead of using no TOC or our detection limit for organic carbon of 0.8 mg C/L (Fig. 4). As would be expected, the additional organic carbon content had a larger impact on Cu speciation in the low NOM exposures, and a lesser impact with the high NOM exposures. The effect of adding food to the exposure media on Cu speciation was studied by Kolts et al. (2006) where they observed a large decrease in acute Cu toxicity to *C. dubia* (9-fold increase in dissolved Cu EC50s) when organisms were fed during the test compared to unfed organisms. They attributed the increase in EC50 to a decrease in bioavailability of Cu to the organisms during testing, which is similar to the results we see in our measured free Cu^{2+} activity. The authors also looked at the timing of food addition to the exposure media, and found no difference in Cu toxicity whether food was added 30 min before the daphnid was transferred, or immediately after. In our tests, the food was always added to the exposure media approximately 2–3 h before the organisms were transferred into fresh media.

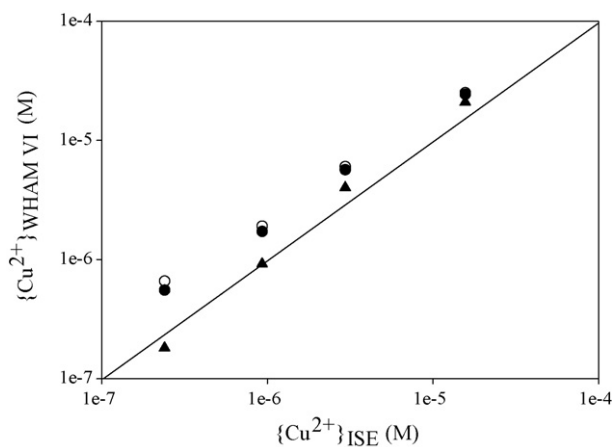


Fig. 4. Effect of food addition on free Cu^{2+} activity in 75% tap water. Measured Cu^{2+} vs. predicted free Cu^{2+} using WHAM VI assuming no fulvic acid (FA; open circles), with FA calculated using the TOC detection limit of 0.8 mg C/L (filled circles) and FA based on measured TOC in presence of food, i.e. 4.98 mg C/L (filled triangles).

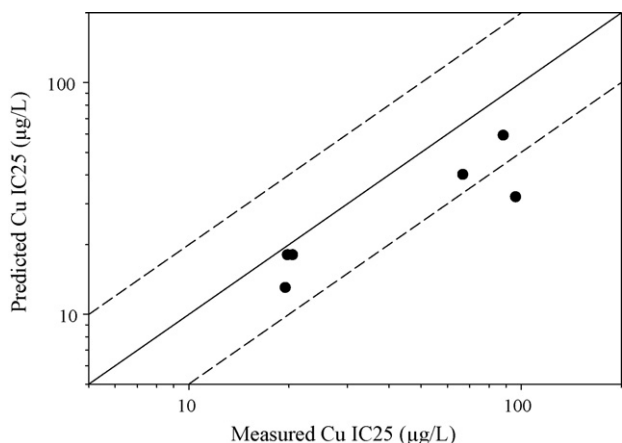


Fig. 5. The Cu IC25 values (in $\mu\text{g Cu/L}$) as predicted by our model compared with measured Cu IC25 data in six natural surface waters collected from across Canada. In all cases, the model was able to predict the IC25 within a factor of three. The solid line represents a one-to-one relationship between predicted and measured IC25 values, and the dashed lines represent a factor of 2 difference between predicted and measured IC25 values.

3.5. Model validation using natural surface waters

The IC25 based on total copper concentrations in the six natural waters collected from across Canada varied from $19.6 \mu\text{g/L Cu}$ in Georgian Bay water to $96.5 \mu\text{g/L Cu}$ in the Endako Mine reference water (Table 1). In parallel to the toxicity testing, free Cu^{2+} measurements were done on the natural waters spiked with high copper concentrations and confirmed that WHAM VI adequately predicts free Cu^{2+} concentrations. We also compared the measured free Cu^{2+} activities with the predicted free Cu^{2+} using the BLM developed by HydroQual (2002) in the chemical speciation mode. To do so we used the default assumption of DOC, i.e. 100% NOM composed of humic substances with 90% of the humic substances being fulvic acid and 10% humic acid. WHAM VI predictions were slightly closer to the measured values with a slope for the linear relationship between log measured free Cu^{2+} versus log predicted free Cu^{2+} of 1.0 ± 0.2 compared to 1.3 ± 0.3 for the BLM. It should be noted however that the actual range of free Cu^{2+} activities for the toxicity test concentrations is lower than the detection limit for our ISE measurements. Bryan et al. (2002) validated WHAM VI predictions using ISE at concentrations as low as the 10^{-10} M range, which is within the range of IC25s in our natural waters expressed as free Cu^{2+} (Table 1).

Generally, the accepted prediction error in biotic ligand modeling is a factor of two (Santore et al., 2001). Prediction error is related to both the variability associated with the IC25 and Cu speciation estimations. The inherent variability associated with the IC25 estimate for the *C. dubia* chronic test can be quite high. For example, the difference between the upper and lower limit of the 95% confidence interval can be as much as 4-fold (e.g. Fig. 1C). Bryan et al. (2002) demonstrated that WHAM VI was able to predict free Cu^{2+} in natural waters within a factor of 3.6 in 95% of cases. With our model, Cu IC25s (in $\mu\text{g/L}$) in all of our natural waters used in the validation exercise were predicted successfully by our model within a factor of three (Fig. 5) and

five of the six were predicted within a factor of 2. The largest prediction error was seen with the Endako Mine reference site water, where the measured Cu IC25 was $96.5 \mu\text{g/L}$ and the predicted Cu IC25 was $32 \mu\text{g/L}$. It appears the protective effect of this water is greater than what we would expect based on the chemistry we measured. Another possibility is that in the Endako Mine reference water, a significant proportion of Cu is not dissolved, but associated with inorganic particles. If we exclude the Endako result from our regression, the relationship of predicted Cu IC25 versus measured Cu IC25 data is significant ($r^2 = 0.84$, $P = 0.03$) indicating our model is reliable for the other five waters tested.

Given the much greater protective effect of NOM complexation compared to the effect of H^+ competition, we also tested the use of a regression approach based on NOM concentration to predict Cu toxicity. To do so, we used the NOM concentrations in our natural waters to calculate predicted IC25s based on the observed regression between SNOM and NROM concentrations and IC25 (Fig. 3). For the limited pH range of our natural waters, i.e. pH 7.3–8.2, the predicted IC25s are within a factor of 2 of the measured value with ($r^2 = 0.68$, $P = 0.04$). This confirms the predominance of the NOM protective effect compared to the competitive interactions with H^+ within that pH range. Outside that range, the BLM would be a more accurate predictor of actual toxicity. Furthermore, the developed BLM would be a more conservative approach since in no instance was the IC25 overpredicted using our model, whereas there was a maximum overprediction of 1.7-fold using the NOM regression.

4. Conclusions

To develop a chronic Cu BLM for *Ceriodaphnia dubia*, we needed to account for the effect of proton competition and complexation of Cu by natural organic matter. The competition of major cations (Ca, Na, and Mg) did not impact chronic Cu toxicity to *C. dubia*, and was therefore not included. Our model was tested with six Cu-spiked natural waters collected from across Canada, and it predicted chronic Cu toxicity (as IC25) within a factor of three. The strong influence of NOM complexation was confirmed, as we were also able to predict toxicity based on NOM concentration alone over a limited pH range within a factor of two. However, the error in the NOM-only predicted values tended to be less conservative than with our BLM. Our model adds to the increasing number of both acute and chronic BLMs that are used to predict metal toxicity to fish and other invertebrates. These models are important in advancing the development of scientifically sound environmental regulations.

Acknowledgements

We thank Barb Riordan (Thompson Creek Mining – Endako Mine), Meghan Cairns (Goldcorp Inc. – Red Lake Mine) and Rachele Trottier (Inco Thompson – Birchtree Mine) for sending us samples of their reference site water for use in the validation exercise of our model, and Carrie Rickwood (University of Saskatchewan) for the collection of water from the Vermillion River. We thank Susannah Krack for her valuable laboratory

assistance and collection of water from Lac Dawson, Quebec. Gauri Prabhakar is also thanked for her assistance in the lab.

References

- Belanger, S.E., Cherry, D.S., 1990. Interacting effects of pH acclimation, pH, and heavy metals on acute and chronic toxicity to *Ceriodaphnia dubia* (Cladocera). *J. Crustac. Biol.* 10 (2), 225–235.
- Bryan, S.E., Tipping, E., Hamilton-Taylor, J., 2002. Comparison of measured and modeled copper binding by natural organic matter in freshwaters. *Comp. Biochem. Physiol. C* 133, 37–49.
- Bury, N.R., McGeer, J.C., Wood, C.M., 1999. Effects of altering freshwater chemistry on physiological responses of rainbow trout to silver exposure. *Environ. Toxicol. Chem.* 18, 49–55.
- Canadian, 2006. Council of Ministers of the Environment (CCME), A protocol for the derivation of water quality guidelines for the protection of aquatic life. Draft 20.
- De Schampelaere, K.A.C., Janssen, C.R., 2004. Development and field validation of a biotic ligand model predicting chronic copper toxicity to *Daphnia magna*. *Environ. Toxicol. Chem.* 23 (6), 1365–1375.
- De Schampelaere, K.A.C., Heijerick, D.G., Janssen, C.R., 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. *Comp. Biochem. Physiol. C* 133, 243–258.
- De Schampelaere, K.A.C., Vasconcelos, F.M., Heijerick, D.G., Tack, F.M.G., Delbeke, K., Allen, H.E., Janssen, C.R., 2003. Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. *Environ. Toxicol. Chem.* 22 (10), 2454–2465.
- Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ. Toxicol. Chem.* 20 (10), 2383–2396.
- Diamond, J.M., Koplisch, D.E., McMahon III, J., Rost, R., 1997. Evaluation of the water-effect ratio procedure for metals in a riverine system. *Environ. Toxicol. Chem.* 16 (3), 509–520.
- Environment Canada, 1992. Test of reproduction and survival using the cladoceran *Ceriodaphnia dubia*. Report EPS 1/RM/21.
- Grosell, M., Neilson, C., Bianchini, A., 2002. Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comp. Biochem. Physiol. C* 133, 287–303.
- Heijerick, D.G., De Schampelaere, K.A.C., Van Sprang, P.A., Janssen, C.R., 2005. Development of a chronic zinc biotic ligand model for *Daphnia magna*. *Ecotoxicol. Environ. Saf.* 62, 1–10.
- Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000. Protective effects of calcium against chronic waterborn cadmium exposure to juvenile rainbow trout. *Environ. Toxicol. Chem.* 19 (11), 2725–2734.
- HydroQual, 2002. Biotic ligand model. Mahwah, New Jersey, USA.
- Hyne, R.V., Pablo, F., Julli, M., Markich, S.J., 2005. Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia cf dubia*. *Environ. Toxicol. Chem.* 24 (7), 1667–1675.
- Janes, N., Playle, R.C., 1995. Modeling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* 14, 1847–1858.
- Kim, S.D., Ma, H., Allen, H.E., Cha, D.K., 1999. Influence of dissolved organic matter on the toxicity of copper to *Ceriodaphnia dubia*: effect of complexation kinetics. *Environ. Toxicol. Chem.* 18 (11), 2433–2437.
- Kolts, J.M., Boese, C.J., Meyer, J.S., 2006. Acute toxicity of copper and silver to *Ceriodaphnia dubia* in the presence of food. *Environ. Toxicol. Chem.* 25 (7), 1831–1835.
- Kramer, K.J.M., Jak, R.G., van Hattum, B., Hooftman, R.N., Zwolsman, J.J.G., 2004. Copper toxicity in relation to surface water-dissolved organic matter: biological effects to *Daphnia magna*. *Environ. Toxicol. Chem.* 23 (12), 2971–2980.
- MacDonald, A., Silk, L., Schwartz, M.L., Playle, R.C., 2002. A lead-gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. C* 133, 227–242.
- Markich, S.J., Batley, G.E., Stauber, J.L., Rogers, N.J., Apte, S.C., Hyne, R.V., Bowles, K.C., Wilde, K.L., Creighton, N.M., 2005. Hardness corrections for copper are inappropriate for protecting sensitive freshwater biota. *Chemosphere* 60, 1–8.
- Martell, A.E., Smith, R.M., Motekaitis, R.J., 1997. Critical stability constants of metal complexes database Version 4.0. Standard Reference Database 46. National Institute of Standards and Technology, Gaithersburg, MD, USA.
- Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. *Environ. Sci. Technol.* 17, 342–347.
- Paquin, P.R., Santore, R.C., Wu, K.B., Kavvas, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. *Environ. Sci. Policy* 3, S175–S182.
- Playle, R.C., 1998. Modeling metal interactions at fish gills. *Sci. Total Environ.* 219, 147–163.
- Richards, J.G., Playle, R.C., 1999. Protective effects of calcium against the physiological effects of exposure to a combination of cadmium and copper in rainbow trout. *Can. J. Zool.* 77, 1035–1047.
- Richards, J.G., Curtis, P.J., Burnison, B.K., Playle, R.C., 2001. 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.* 20, 1159–1166.
- Santore, R.C., Di Toro, D.M., Paquin, P.R., Allen, H.E., Meyer, J.S., 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and daphnia. *Environ. Toxicol. Chem.* 20 (10), 2397–2402.
- Schwartz, M.L., Playle, R.C., 2004. Influence of natural organic matter source on acute copper, lead, and cadmium toxicity to rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* 23 (12), 2889–2899.
- Tipping, E., 1998. Humic ion-binding model WHAM VI: an improved description of the interactions of protons and metal ions with humic substances. *Aquat. Geochem.* 4, 3–38.
- United States Environmental Protection Agency, 1985. Ambient Water Quality Criteria for Copper (1984). EPA 440/5-84-031. Office of water regulations and standards criteria and standards division, Washington, DC, USA.