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DEVELOPMENT OF A REGRESSION MODEL TO PREDICT COPPER TOXICITY TO *DAPHNIA MAGNA* AND SITE-SPECIFIC COPPER CRITERIA ACROSS MULTIPLE SURFACE-WATER DRAINAGES IN AN ARID LANDSCAPE

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Abstract: The water effect ratio (WER) procedure developed by the US Environmental Protection Agency is commonly used to derive site-specific criteria for point-source metal discharges into perennial waters. However, experience is limited with this method in the ephemeral and intermittent systems typical of arid climates. The present study presents a regression model to develop WER-based site-specific criteria for a network of ephemeral and intermittent streams influenced by nonpoint sources of Cu in the southwestern United States. Acute (48-h) Cu toxicity tests were performed concurrently with *Daphnia magna* in site water samples and hardness-matched laboratory waters. Median effect concentrations (EC50s) for Cu in site water samples ($n = 17$) varied by more than 12-fold, and the range of calculated WER values was similar. Statistically significant ($\alpha = 0.05$) univariate predictors of site-specific Cu toxicity included (in sequence of decreasing significance) dissolved organic carbon (DOC), hardness/alkalinity ratio, alkalinity, K, and total dissolved solids. A multiple-regression model developed from a combination of DOC and alkalinity explained 85% of the toxicity variability in site water samples, providing a strong predictive tool that can be used in the WER framework when site-specific criteria values are derived. The biotic ligand model (BLM) underpredicted toxicity in site waters by more than 2-fold. Adjustments to the default BLM parameters improved the model's performance but did not provide a better predictive tool compared with the regression model developed from DOC and alkalinity. *Environ Toxicol Chem* 2014;33:1865–1873. © 2014 SETAC

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INTRODUCTION

The ambient water quality criteria for Cu currently applied by all states in the United States are based on a relationship between water hardness and metal toxicity to aquatic organisms, as determined by toxicity tests conducted in mostly synthetic laboratory waters. However, the federal water quality standards regulation provides states with the option to calculate site-specific criteria for Cu based on the well-established principle that the exposure-water chemistry (e.g., pH, alkalinity, hardness, and dissolved organic carbon [DOC] concentration) modifies the toxicity of cationic metals to aquatic organisms [1]. The water effect ratio (WER) procedure (see explanation of WER calculations below) is a federally approved site-specific criteria method [2,3] used to account for the toxicity-modifying properties of ambient surface waters. In addition, some states allow determination of site-specific criteria using the biotic ligand model (BLM; see explanation of the model in the *Biotic ligand model* section). Despite relatively widespread use for point-source discharges of Cu into perennial streams and rivers, the use of WER studies and BLM calculations to derive site-specific criteria for nonpoint-source discharges of Cu into intermittent and ephemeral surface waters, which are common in arid climates, is limited. Therefore, reliable approaches are needed to address such nonstandard situations.

The BLM is a computerized model that predicts the toxicity of Cu to several freshwater species of aquatic invertebrates and fish and thus is convenient to use because of time and cost savings, compared with the extensive toxicity testing required for WER-based site-specific criteria. The BLM is the US Environmental Protection Agency's (USEPA's) current recommended method for deriving national water quality criteria for Cu [4], because it incorporates a wider range of water chemistry parameters (pH, alkalinity, and concentrations of major inorganic ions and DOC) than only water hardness and thus predicts toxicity more accurately. However, none of the states in the United States have yet adopted the BLM as the primary method to calculate ambient water quality criteria for Cu. Instead, hardness-based equations are still used to derive Cu criteria, although many of the states have incorporated the BLM into their administrative code in some form as an option for deriving site-specific criteria.

Despite the ease of use and the time and cost savings the BLM provides, the default calibration of the BLM can leave uncertainty about the accuracy of its predictions of Cu toxicity for a given strain of invertebrate or fish and/or for a given type of DOC. For example, it is unknown whether the affinity and binding-site density for Cu by DOC in surface waters in arid climates differ from DOC in more mesic climates. Therefore, theoretically, a WER test can more accurately determine site-specific criteria for a given water sample than can the BLM. However, WER testing is most amenable for determining site-specific criteria for point-source discharges instead of for all combinations of spatial and temporal variability in a large number of water bodies. Furthermore, current WER guidelines [2,3] are based on experience in perennial rather than ephemeral systems.

The WER procedure compares empirical toxicity endpoints, such as median lethal effect concentrations (EC50s) measured in

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Exhibit C

site waters, with the same toxicity endpoint measured in hardness-matched laboratory waters [2,3]:

$$\text{WER} = \frac{\text{site-water EC50}}{\text{laboratory-water EC50}} \quad (1)$$

Because synthetic laboratory waters used in the WER procedure are assumed to represent water chemistry and toxicity conditions from which the hardness-based ambient water quality criteria were calculated (and this assumption is verified in the WER procedure by comparisons with other studies [2]), WER values greater than 1.0 indicate protective effects of site-specific water chemistry on metal toxicity (beyond the protective effect of hardness that is already accounted for in the hardness-matched laboratory water). Using the WER procedure, a site-specific criterion is the product of a measured WER value and the corresponding hardness-matched ambient water quality criterion [2,3].

Despite the assumed accuracy of a WER value determined for a given water sample, spatial and temporal variability in water chemistry can lead to spatial and temporal variability in WER values. This presents a challenge for site-specific criteria development because of the possible over- or underprotection that can result from a WER-adjusted site-specific criteria value, particularly when no specific mechanistic or statistical underpinning for the variability can be identified based on the WER toxicity tests. Many arid landscapes, such as those in parts of the western United States, contain intermittent and ephemeral water bodies impacted by naturally occurring or anthropogenic nonpoint sources. As a consequence, current WER guidance might need to be modified for application to arid landscapes and ephemeral aquatic systems, because spatial and temporal characteristics of water persistence and water chemistry in such landscapes can differ from perennial systems.

In the present study, the WER framework was used to develop a regression model to predict site-specific Cu toxicity in multiple ephemeral and intermittent surface waters that receive nonpoint sources of Cu in an arid landscape in the southwestern United States. Empirical measures of Cu toxicity were determined in *Daphnia magna* acute lethality tests and evaluated through correlation and step-wise multiple-regression analyses to identify the primary chemical predictors of Cu toxicity in the site waters. The regression model was then incorporated into an equation that can be used to predict the WER value in a variety of water chemistries that might occur in the multiple-watershed study area, thereby allowing derivation of site-specific criteria for any of those receiving waters. Performance of the BLM was also evaluated as an additional tool for developing site-specific criteria for Cu.

MATERIALS AND METHODS

Site description

The study area extends across an approximately 60-km^2 arid, mountainous region of the southwestern United States. Elevations range from approximately 1500 m above sea level in the lowlands (comprising desert grasslands and shrub lands) to 2300 m above sea level in the mountainous terrain. There is diffuse Cu contamination to the adjacent landscape as a result of historical industrial emissions and natural mineralized soil. The study area was categorized into a total of 9 sub-watersheds that encompass12 drainages. The surface waters are mostly ephemeral, flowing only in direct response to high-intensity precipitation that occurs over short durations, primarily during mid to late summer (i.e., southwestern monsoonal precipitation events). Following these precipitation events, surface water can persist in isolated pools for short periods of up to several weeks. These short-term pools are generally located in headwaters, where drainage channels are comprised primarily of bedrock and stable substrate. Water chemistry varies among the sub-watersheds because of localized differences in geology, geomorphology, hydrology, and surrounding upland landscapes.

Sample collection

Surface-water samples were collected from as many sub-watersheds as possible, limited by the presence of rainfall pools and seeps. A total of 18 surface-water samples were collected for chemical analyses and toxicity tests twice during summer 2011 (12 locations in August and 6 repeat samples in September from locations also sampled in August). No measurable flow was observed in these drainages during the 2 rounds of sampling, and most of the drainage areas surveyed during each field sampling round were dry. All surface-water samples were collected from pool habitats that were relatively isolated in terms of their connectivity to up-gradient or down-gradient drainage areas.

Samples were collected and processed in accordance with USEPA guidelines [2], including the use of clean techniques for all phases of field sampling, such as equipment preparation, water collection, handling, and storage. Each water sample was collected as a grab sample taken at approximately the center or the deepest section of the pool, and at mid-depth. Before collecting a water sample, the acid-rinsed, low-density polyethylene sample container was filled with water and shaken to rinse the container; this process was repeated for a total of 3 rinses for each sample container. The screw caps on the sample containers were sealed so no air space remained inside the container. Immediately after sample collection, the containers were chilled and stored in coolers for transport to the testing laboratory. Samples were shipped the same day as collected, and all samples arrived at the laboratories within 24 h of collection. Samples were maintained at <4 °C in the dark until test initiation.

Laboratory dilution water

Reconstituted laboratory dilution water was prepared by adding reagent-grade salts to 18-MΩ deionized water according to USEPA guidelines [5]. Hardness of laboratory dilution waters was matched in concentrations to water hardness of site samples according to WER testing requirements [2]. This included equal or lower water hardness in matched laboratory water, unless the hardness of a site water sample was less than 50 mg/L as $CaCO_3$. All laboratory water tests were performed at water hardness concentrations within the WER guideline-required range of 40 mg/L to 220 mg/L as $CaCO_3$ [2]. In total, 11 laboratory dilution waters were used for the WER toxicity tests, with hardness ranging from 42 mg/L to 168 mg/L as $CaCO_3$ and alkalinity concentrations ranging from 30 mg/L to 112 mg/L as $CaCO_3$. The laboratory dilution water used for all toxicity tests was comparable to waters used to develop the hardness-based Cu ambient water quality criteria, including having alkalinity similar to hardness, approximately circumneutral pH, and low concentrations of total suspended solids (<5 mg/L) and total organic carbon (TOC; <1 mg C/L; Supplemental Data, Tables S1 and S2).

Toxicity testing

To investigate the influence of site water chemistry on Cu toxicity, standard WER tests were conducted [2]. Laboratory water toxicity tests were performed concurrent with site water

toxicity tests under identical exposure conditions (except tested Cu concentrations). Acute toxicity tests were performed with neonates (<24 h old) of the freshwater cladoceran *D. magna* obtained from Aquatic Biosystems, using the USEPA-recommended protocol [5]. *Daphnia magna* was chosen for these toxicity tests because they are a USEPA-recommended species for WER testing [2,3] and a substantial database is available to compare Cu toxicity in the laboratory waters used in the present study with results in other laboratories, as required in the WER guidelines [2,3]. All cultures and toxicity tests were performed in a temperature-controlled growth chamber at 20 ± 1 °C with a 16:8-h light:dark cycle. Cultures were maintained in moderately hard reconstituted water [5] and fed a combination of yeast–trout chow (YTC; Cerophyll™) and the green alga *Pseudokirchneriella subcapitata*.

Neither the *D. magna* neonates nor their mothers were acclimated to the hardness of the water in which the neonates were tested, because the water hardness of any given site water (and thus of its required hardness-matched laboratory water) was not known a priori. The streamlined Cu guidance for WER tests specifies that site water holding time should not exceed 96 h before a WER test is started [3], thus making it difficult to acclimate organisms to an unknown water hardness and still start the WER toxicity test in time. Therefore, the WER guidance states that "Acclimation to site water is desirable but optional" (p 7 in USEPA [3]) and that "The least objectionable approach is to acclimate the organisms to a laboratory dilution water with a hardness in the range of 50 to 150 mg/L and then use this water as the laboratory dilution water when the WER is determined" [2], which was done in the present study. The potential influence of this lack of acclimation on the toxicity of Cu to *D. magna* (or other aquatic invertebrates) is unknown; and even for fish, the influence of acclimation depends on the species and metal tested [6]. However, because the hardness of the laboratory water was matched to the hardness of the site water for each site water and the WER value is the ratio of the EC50 values in the site water and the hardness-matched laboratory water, the relative effect on the WER of not acclimating the test organisms to the site water hardness might have been minimized.

Test solutions were not renewed and organisms were not fed during the 48-h exposure period. Each test treatment consisted of 4 replicate polyethylene chambers, each containing 25 mL of test solution and 5 organisms. Including controls (unspiked site or laboratory water), 6 Cu concentrations were tested for each site and laboratory water sample. Copper stock solutions used to spike test treatments were prepared by dissolving $CuCl_2 \cdot 2H_20$ in deionized water. A separate stock solution was prepared for each round of WER testing, but the same stock solution was used to spike all laboratory and site waters in each round of testing. Copper concentrations were selected for each site water sample based on the results of 24-h static range-finding toxicity tests performed on receipt of each sample and were a series of nominal 0.7× dilutions of a high concentration that was expected to produce approximately 100% mortality in 48 h. Exposure waters were mixed thoroughly and allowed to stand for a minimum of 20 h at 4 °C before test initiation to allow the metal chemistry to equilibrate, as recommended by the USEPA [3] and Ma et al. [7]. Aliquots of exposure water were collected from a replicate chamber in each treatment for Cu analyses at the beginning and end of each test. The observation endpoint was immobilization; thus, toxicity results are reported herein as EC50 values instead of median lethal concentrations (LC50s). A toxicity test was acceptable if mortality in the hardness-matched laboratory water control was $\leq$10%.

Chemical analyses

Water chemistry parameters measured in all tests included dissolved oxygen, pH, conductivity, temperature, hardness, alkalinity, DOC, Cu, and major inorganic ions (Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}). All parameters were measured on samples collected at test initiation. Physicochemical parameters (dissolved oxygen, pH, conductivity, and temperature) also were measured at 24 h in a replicate chamber in each treatment.

Concentrations of Al, Cd, Cu, Fe, Mn, Pb, and Zn in the site waters and total recoverable and dissolved Cu concentrations at the beginning and end of each site water and laboratory water toxicity test were determined by inductively coupled plasma mass spectroscopy (ICP–MS), according to USEPA method 200.8 (Agilent Technologies 7500ce series, with an Octopole Reaction System). For dissolved-metal concentrations, samples were filtered through a 0.45-μm nylon membrane filter (EMD Millipore) following prerinses with ultrapure deionized water and then sample water. Samples collected for metals were preserved with trace metal–grade nitric acid immediately after collection (total recoverable concentrations) or after filtration (dissolved concentrations). Internal laboratory blank samples and certified standards were analyzed at a rate of 1 per 10 samples, with acceptance criteria of $\pm$10% of the known concentration in the continuing calibration verifications. Concentrations of major cations (Ca^{2+}, Mg^{2+}, Na^+, and K^+) were determined by ICP–MS, according to USEPA method 200.7. Concentrations of the anions Cl^- and SO_4^{2-}were determined by low-level amperometric titration (Standard Methods 4500-E) and the turbidimetric method (D516-07), respectively.

All site waters were analyzed for DOC and TOC concentrations, but laboratory water samples inadvertently were analyzed only for TOC concentration. Samples for DOC analyses were filtered through a 0.45-μm nylon membrane filter after a 20-mL rinse with sample water, acidified with nitric acid, and stored in amber glass bottles at 4 °C. Dissolved organic carbon and TOC concentrations were determined by direct combustion/infrared detection using a Leco SC632 sulfur/carbon analyzer calibrated with a certified potassium hydrogen phthalate standard (Fisher Scientific); the method detection limit was 1 mg C/L.

Data treatment

The 48-h EC50s for immobilization/death and their 95% confidence limits were computed by maximum likelihood probit analysis using ToxCalc™ statistical software (Ver 5.0; Tidepool Scientific). Copper concentrations used to calculate EC50 values were the averages of Cu determined at the beginning and end of each test. To evaluate the influence of individual water chemistry parameters on Cu toxicity, univariate linear-regression analyses ($\alpha = 0.05$) were performed using measured dissolved Cu EC50 values and measured water chemistry parameters. With the exception of pH, all data were log-transformed for univariate and multivariate regression analyses. Step-wise, multiple linear regression analyses were performed to determine the best combination of water chemistry parameters for predicting measured Cu toxicity. Models were evaluated for predictive ability (based on the adjusted R^2 [i.e., the percentage of variance in the EC50s that is explained by the regression] and p values) and by limiting the colinearity of water chemistry parameters (evaluated by inspection of the variance inflation factor). All regression analyses were performed using SigmaPlot™ software (Ver 12.1; SYSTAT Software).

Water effect ratios

Water effect ratios were calculated as the Cu EC50 measured in site water divided by an appropriate denominator (e.g., Equation 1). For each sample, a set of 4 WER values was calculated based on 4 different denominators that potentially could be used, including individual hardness-matched laboratory water EC50 values, the USEPA-recommended species mean acute value (SMAV) for *D. magna* [3], a recalculated *D. magna* SMAV based on excluding nominal Cu concentrations from the toxicity dataset listed in the USEPA [3], and the geometric mean of the concurrent hardness-matched laboratory water EC50 values. Based on USEPA guidance [2], laboratory water Cu EC50 values were compared with results from other laboratories to evaluate the sensitivity range of the laboratory water EC50 values. In the more recent Cu WER guidance [3] designed for point-source discharge of Cu, the WER denominator is the greater of the laboratory water EC50 or the SMAV for the test species. The USEPA-recommended *D. magna* SMAV for dissolved Cu at a hardness of 100 mg/L as $CaCO_3$ is 19.31 μg Cu/L (Appendix B in USEPA [3]). Although the present study site does not receive point-source Cu discharges, the *D. magna* SMAV was used as the WER denominator if concurrent laboratory water EC50 values normalized to a hardness of 100 mg/L were less than 19.31 μg/L dissolved Cu. All EC50–hardness normalizations were performed using the hardness-based Cu criteria slope of 0.9422 [8].

Biotic ligand model

The influence of site-specific water chemistry on acute Cu toxicity was also evaluated using the Cu BLM (Ver 2.2.3; http://hydroqual.com/wr_blm.html). Concentrations of pH, alkalinity, DOC, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, and SO_4^{2-} measured in toxicity-test exposure solutions were used as the BLM input parameters. Recommended default values for the percentage of humic acid (10%) and sulfide (0.01 μM) were also used as BLM input parameters. The BLM was run in toxicity mode to produce BLM-predicted EC50 values, for comparison with measured and regression model–predicted EC50 values. Because TOC concentrations in the laboratory waters were below the method detection limit (1 mg C/L), a DOC concentration of 0.5 mg C/L was assumed for BLM calculations with the laboratory waters; however, that assumption has uncertainty associated with it because the DOC concentration in deionized laboratory waters can vary. Therefore, we performed a sensitivity analysis by also conducting the BLM calculations for the laboratory waters using lower and upper bounds on the assumed DOC concentration of 0.3 mg C/L and 0.7 mg C/L.

RESULTS AND DISCUSSION

Chemical analyses of the hardness-matched laboratory waters and the site waters (except for metals other than Cu) are listed in Supplemental Data, Tables S1 and S2. Measured dissolved Cu concentrations ranged from 40% to >99% of nominal, and measured total Cu concentrations ranged from 56% to >99% of nominal. Concentrations of other metals in the site waters are listed in Supplemental Data, Table S3.

With the exception of site water sample 1-5, at least 90% of organisms survived in control treatments of all laboratory and site water toxicity tests. Only 20% of organisms survived in the unspiked control for sample 1-5, which precluded calculation of EC50 and WER values for this sample. Therefore, that sample was excluded from the subsequent regression analyses that are discussed below.

Laboratory water tests

To verify the sensitivity of tested organisms, the Cu EC50 values from laboratory water tests were compared with the 55 *D. magna* EC50 values listed in Appendix B of the streamlined WER guidance document [3], which were used to compute the SMAV of 19.31 μg/L dissolved Cu (i.e., the recommended WER denominator). Of the 55 *D. magna* EC50 values listed in by the USEPA [3], 45 were based on measured Cu concentrations and 10 were based on nominal Cu concentrations. Laboratory water EC50 values determined in the present study were comparable to all EC50s used to calculate the *D. magna* SMAV but were more similar to the measured EC50s (Supplemental Data, Figure S1). In addition, the geometric mean of hardness-normalized laboratory water toxicity tests (13.95 μg/L at a hardness of 100 mg/L as $CaCO_3$) determined in the present study was within a factor of 1.4 of the recommended *D. magna* SMAV and within a factor of 1.2 of the geometric mean of only the measured EC50 values from the streamlined WER guidance document (16.50 μg/L [3]). The slope between *ln*[dissolved Cu EC50] and *ln*[hardness] for *D. magna* in the present study (1.189 for hardness values ranging from 42 mg/L to 168 mg/L as $CaCO_3$) was slightly greater than the pooled slope used in the hardness-based acute Cu criteria (0.9422), but it is within 13% of the slope calculated using only the *D. magna* values listed in the historic USEPA hardness-based Cu criteria documents (1.044 for hardness values ranging from 45 mg/L to 226 mg/L as $CaCO_3$ [8]). Overall, these results suggest that the sensitivity of test organisms used in the present study was comparable to previously reported laboratory water results and therefore was acceptable for determining WER values.

Water effect ratios

All but 1 of the 17 calculated WER values were greater than 1.0, using the SMAV as the WER denominator (range of WER values = 0.989–14.41; Table 1). The preponderance of WER values greater than 1.0 indicates that site water chemistry decreased Cu toxicity relative to standard laboratory dilution waters (i.e., waters used to develop the hardness-based Cu criteria). The lowest WER values were computed using the SMAV as the WER denominator, because the dissolved Cu EC50 values in the hardness-matched laboratory water were mostly less than the SMAV (the range of WER values using the hardness-matched laboratory water EC50 value as the WER denominator was 1.48–24.8; Supplemental Data, Table S4).

Because of the range of site water chemistries tested and because of the known influence of DOC and inorganic parameters (major cations, alkalinity) on aqueous Cu bioavailability [5,9], the wide variability in measured WER values was not surprising. However, the variability in WER values presents a challenge to implement site-specific criteria, especially in arid landscapes that contain ephemeral drainages not influenced by point-source discharges of Cu. For this scenario, a final site WER could theoretically be computed as the geometric mean of the measured WER values. For example, using the hardness-matched laboratory EC50s as the WER denominator, the geometric mean WER = 7.35, whereas using the SMAV as the WER denominator, the geometric mean WER = 5.00. However, it is clear that application of geometric mean WERs to this site would result in considerable uncertainty about the level of protection of derived site-specific criteria, depending on the water chemistry of a sample to which the WER is applied. In agreement with USEPA recommendations concerning WERs determined for sites not influenced by point sources of metal

Table 1. Chemistry of site waters in which *Daphnia magna* toxicity tests were conducted, and corresponding 48-h dissolved Cu median effect concentrations (EC50s) and water effect ratio (WER) values[a]

Site[b]	pH Test start	pH Test average	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	DOC (mg C/L)	Dissolved Cu (μg/L)	Dissolved (Cu EC50 μg/L)[c]	Dissolved Cu WER[d]
1-1	8.00	8.19	90	74	10.7	5.9	116.3	6.651
1-2	7.47	7.88	84	60	7.8	6.5	87.39	5.334
1-5	7.54	7.66	62	28	3.5	32.3	<32.3[e]	NA
1-6	7.57	7.88	54	42	12.5	57.4	155.7	14.41
1-7	7.93	8.06	106	66	7.8	43	96.23	4.717
1-9	8.04	8.29	88	90	2.5	7.1	37.78	2.207
1-10	8.31	8.60	262	250	4.7	5.4	134.2	2.804
1-11	8.22	8.48	154	170	15.7	4.3	172.8	5.956
1-12	9.35	8.69	76	27	1.2	2.1	14.74	0.989
1-RCS1	8.67	8.44	48	32	3.2	5	31.65	3.273
1-D1-2	8.06	8.19	54	76	10.0	32.3	141.6	13.10
1-D2-1	8.16	8.02	42	28	5.8	32.8	68.45	8.027
2-1	8.19	8.27	104	96	11.0	3.4	81.06	4.046
2-6	7.14	7.60	50	40	11.4	30.2	61.82	6.151
2-9	8.44	8.49	82	102	12.3	13.7	>184.7[f]	11.53
2-11	7.99	8.24	102	106	12.3	7.9	135.5	6.889
2-12	7.40	7.70	80	34	3.1	3.6	35.23	2.251
2-D1-2	7.82	8.03	60	64	10.5	17.9	68.31	5.724

[a]Complete water chemistry for each sample is listed in Supplemental Data, Table S2.
[b]A "1-" prefix signifies Round 1 samples collected in August 2011; a "2-" prefix signifies Round 2 samples collected in September 2011.
[c]Dissolved Cu EC50 at tested hardness before normalization to a hardness of 100 mg/L as $CaCO_3$ for calculation of WER.
[d]All WERs were calculated using the *D. magna* species mean acute value of 19.31 μg/L dissolved Cu from the US Environmental Protection Agency [3] as the WER denominator, with the dissolved Cu EC50 normalized to a hardness of 100 mg/L as $CaCO_3$ in the numerator.
[e]EC50 could not be calculated because >50% mortality occurred in unspiked site water.
[f]EC50 is reported as greater than the highest tested Cu concentration because mortality was <50% in that treatment.
DOC = dissolved organic carbon; NA = not applicable because EC50 could not be calculated.

contamination [2], subsequent analyses were performed to determine whether WER variability could be attributed to the variability of measured water chemistry parameters.

Site water chemistry

Water chemistry parameters varied considerably in the tested site waters, particularly DOC, alkalinity, and major cations (Table 1)—parameters that previously have been demonstrated to modify Cu toxicity [9]. Concentrations of DOC differed among site water samples by more than 1 order of magnitude (1.2–15.7 mg/L), and 9 of the 17 samples contained DOC concentrations greater than or equal to 10 mg/L. Water hardness ranged from soft (42 mg/L as $CaCO_3$) to hard (262 mg/L as $CaCO_3$), with a >9-fold range in alkalinity concentrations (27–250 mg/L as $CaCO_3$). Although hardness and alkalinity concentrations in site samples were moderately correlated ($r = 0.82$; Supplemental Data, Table S5), the hardness-to-alkalinity ratio ranged from 0.71 to 2.8 (Supplemental Data, Table S2). In most natural waters, alkalinity and hardness covary, with alkalinity generally equal to or slightly less than corresponding hardness [9]. Total suspended solids (TSS) were low (<13 mg/L, and mostly <5 mg/L), probably because all samples were collected from isolated pools without measurable flow.

Copper toxicity in site water

Similar to the range in WER values, we observed more than a 12-fold range among *D. magna* dissolved Cu EC50 values (14.7 μg/L to >184.7 μg/L). Hardness concentrations in these lowest and highest EC50 samples differed by only 6 mg/L as $CaCO_3$ (Table 1), and the overall correlation between hardness and toxicity in site samples was low ($R^2 = 0.102$, $p = 0.211$; Table 2). This outcome suggests that Cu toxicity in the site waters was controlled more by other water chemistry parameters, and that hardness alone was a poor predictor of Cu toxicity.

Based on the univariate regression analyses (Table 2), the best predictor of Cu toxicity in site waters was DOC ($R^2 = 0.751$; $p < 0.001$). This result agrees with numerous other studies that evaluated the effects of multiple water chemistry parameters on Cu toxicity [10–12] and supports the contention that DOC should be incorporated in the derivation of water quality criteria [9]. Inorganic parameters determined to be significant predictors of Cu toxicity included (in sequence of decreasing level of statistical significance) hardness/alkalinity ratio ($R^2 = 0.539$; $p < 0.001$), alkalinity ($R^2 = 0.428$; $p = 0.004$), K ($R^2 = 0.322$; $p = 0.018$), and total dissolved solids ($R^2 = 0.245$; $p = 0.043$). With the exception of hardness/alkalinity, correlation coefficients from regressions of water chemistry parameters and EC50 values were positive, indicating that Cu toxicity decreased as concentrations of DOC, alkalinity, K, and total dissolved solids increased. This trend is consistent with results from other studies [9] and is a manifestation of the protective effects of these parameters on Cu bioavailability and toxicity. Mechanistically, DOC and alkalinity (i.e., predominantly HCO_3^- and CO_3^{2-} ions in most fresh waters) can form complexes with Cu, thereby decreasing the available fraction of Cu for biotic uptake. In contrast, cations such as K^+ (and others such as Ca^{2+}, Mg^{2+}, and Na^+) compete with Cu for binding sites on the biotic ligand [13], thus explaining the positive relationships observed between these cations and measured EC50 values. Presumably, the positive relationship between total dissolved solids and EC50 values is also at least partly reflective of this relationship because total dissolved solids, although not ion-specific, is the sum of the concentrations of dissolved inorganic and organic constituents. Therefore, the observed protective effect of total dissolved solids on Cu toxicity might reflect a combination of competition-based

Table 2. Results of univariate-regression analysis between *Daphnia magna* 48-h dissolved Cu median effect concentrations (EC50s) and measured water chemistry parameters in site-water toxicity tests

Independent variable (x)	Regression equation	R^2	p
pH	$EC50 = 10^{3.394 - 0.186 \times x}$	0.099	0.220
Alkalinity (mg/L as $CaCO_3$)	$EC50 = 10^{0.571 + 0.730 \times \log [x]}$	0.428	0.004
Hardness (mg/L as $CaCO_3$)	$EC50 = 10^{0.965 + 0.489 \times \log [x]}$	0.102	0.211
Hardness/alkalinity	$EC50 = 10^{2.026 - 1.428 \times \log [x]}$	0.539	<0.001
DOC (mg C/L)	$EC50 = 10^{1.183 + 0.848 \times \log [x]}$	0.751	<0.001
TDS (mg/L)	$EC50 = 10^{-0.591 + 1.108 \times \log [x]}$	0.245	0.043
Ca (mg/L)	$EC50 = 10^{1.111 + 0.617 \times \log [x]}$	0.159	0.112
Mg (mg/L)	$EC50 = 10^{1.495 + 0.450 \times \log [x]}$	0.117	0.179
K (mg/L)	$EC50 = 10^{1.298 + 1.085 \times \log [x]}$	0.322	0.018
Na (mg/L)	$EC50 = 10^{1.312 + 0.555 \times \log [x]}$	0.154	0.120
SO_4 (mg/L)	$EC50 = 10^{2.583 + 0.467 \times \log [x]}$	0.179	0.091
Fe (mg/L)	$EC50 = 10^{1.520 + 0.292 \times \log [x]}$	0.153	0.120
Al (mg/L)	$EC50 = 10^{1.765 + 0.194 \times \log [x]}$	0.126	0.161

DOC = dissolved organic carbon; TDS = total dissolved solids.

mechanisms from cations and of complexation mechanisms from DOC and alkalinity.

Although hardness and alkalinity were moderately correlated with each other, only alkalinity was significantly correlated with Cu EC50s. Therefore, alkalinity is a better predictor of Cu toxicity in these site waters than is hardness. Copper toxicity increased (i.e., lower EC50 values) when alkalinity was proportionally lower than hardness, as shown by the negative relationship between EC50 values and the hardness/alkalinity ratio. Although pH commonly modifies Cu bioavailability and toxicity [9,11], pH was not significantly correlated with Cu toxicity in the present study. This might be the result of the relatively narrow range of circumneutral to alkaline waters tested (average pH from all site waters ranged from 7.6 to 8.69), compared with other studies that identified significant pH-related effects over a wider range that included slightly acidic waters (i.e., pH < 7 [11,14,15]). In fact, pH should have little direct effect on Cu toxicity at pH values above approximately 6.5 because hydrogen ions do not effectively compete for binding to biotic ligands until the pH is below approximately 6.5. However, pH can have an important indirect effect on Cu bioavailability by changing the HCO_3^-/CO_3^{2-} ratio in the exposure water, thus leading to higher concentrations of CO_3^{2-} (which has a higher affinity for Cu than bicarbonate [9]) at higher pH values.

In multiple linear regressions, the combination of DOC ($p < 0.001$) and alkalinity ($p = 0.007$) was chosen as the best model, explaining 85% of the variability in observed Cu toxicity (Table 3). Models developed using a combination of DOC, hardness/alkalinity, and total dissolved solids or a combination of DOC, alkalinity, and K^+ marginally improved the fit (based on the adjusted R^2 value), but the extra parameters were not statistically significant ($p = 0.127$ for hardness/alkalinity, and $p = 0.181$ for K; Table 3). Application of the hardness/alkalinity value as a predictor of Cu toxicity is also of potential concern because it does not account for absolute concentrations of alkalinity (i.e., a similar hardness/alkalinity ratio is possible at different alkalinity concentrations). In addition, because alkalinity was significantly correlated to K^+ ($p = 0.03$, Supplemental Data, Table S5), adding K^+ to the regression model might be duplicative and might result in unstable model predictions because of colinearity between alkalinity and K^+.

Predicted EC50 values from the DOC-and-alkalinity model were strongly correlated to and generally within a factor of 1.6 of the observed EC50 values ($r = 0.92$; Figure 1). No bias was apparent between model-predicted and observed EC50 values (i.e., no systematic over- or underprediction of toxicity), and the deviations in model predictions were not related to water chemistry variability (assessed by comparing the predicted EC50/observed EC50 ratio across water chemistry ranges; Supplemental Data, Figure S2). The strong linear relationship and lack of bias in model predictions suggest that Cu toxicity in these site waters can be accurately predicted by a combination of the DOC and alkalinity concentrations, consistent with the current mechanistic understanding of Cu toxicity to aquatic organisms.

Other researchers have developed similar regression-based predictive models to describe the effects of multiple water chemistry parameters on Cu toxicity [10,11,16] and Pb toxicity [17]. In the predictive models developed previously for Cu, DOC was included as the most significant predictor of Cu toxicity, which was also the case in the present study. Through stepwise multiple regression of dissolved Cu 48-h EC50s for larval fathead minnows (*Pimephales promelas*) on water chemistry parameters, Van Genderen et al. [16] also identified DOC and alkalinity as significant variables for predicting Cu toxicity in ambient surface waters. De Schamphelaere and Janssen [11] similarly developed a regression model to predict chronic toxicity of Cu to *D. magna* based on DOC and pH, although they tested a much wider pH range than in the present study.

Table 3. Results of multiple linear regressions between *Daphnia magna* 48-h dissolved Cu median effect concentrations (EC50s) and measured water chemistry parameters in site-water toxicity tests ($n = 17$)

Independent variables (p value in parentheses)[a]	R^2	Adjusted R^2	Regression p
DOC (<0.001); H/A (0.127); TDS (0.006)	0.868	0.838	<0.001
DOC (0.002); H/A (0.179); TDS (0.023); pH (0.736)	0.869	0.826	<0.001
DOC (<0.001); alkalinity (0.199); TDS (0.448)	0.861	0.829	<0.001
DOC (<0.001); alkalinity (0.007)	0.854	0.833	<0.001
DOC (<0.001); alkalinity (0.014); pH (0.78)	0.855	0.822	<0.001
DOC (<0.001); alkalinity (0.037); K^+ (0.181)	0.874	0.844	<0.001

[a]Except for pH, all variables were $\log_{10}$-transformed for regression analysis. DOC = dissolved organic carbon; H/A = hardness/alkalinity ratio; TDS = total dissolved solids.

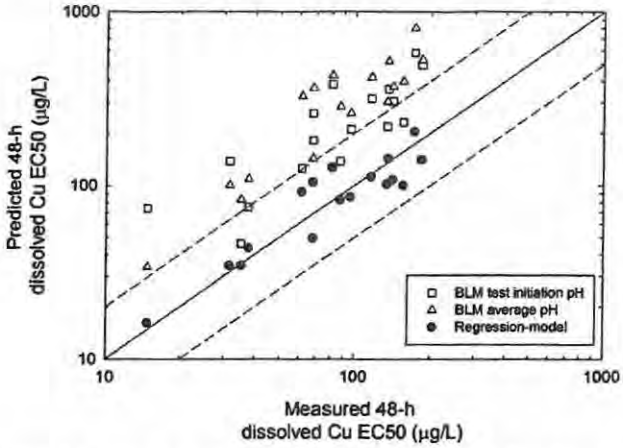

Figure 1. Observed, regression model-predicted, and biotic ligand model (BLM)-predicted 48-h dissolved Cu median effect concentrations (EC50s) in *Daphnia magna* toxicity tests conducted in site waters. Regression model included dissolved organic carbon and alkalinity as predictor variables ($R^2 = 0.854$). Biotic ligand model predictions were based on default model parameters and using pH measured at test initiation (0 h exposure; $R^2 = 0.686$) or the average pH from 0- and 24-h exposure ($R^2 = 0.855$). Solid line represents a perfect (1:1) fit; dashed lines are ± 2-fold of the perfect fit.

Model implementation

The primary source of variation in WER values is the site water toxicity endpoint (i.e., the EC50 value), which is an indicator of the toxicity-modifying properties of site water and, thus, the subsequent relationship to the current hardness-based ambient water quality criteria that are still used by individual states in the United States. The regression model developed in the present study provides a site-specific equation to accurately predict acute Cu toxicity in site waters and, therefore, an option to accurately predict WER values at a much wider variety of locations and times than would be economically reasonable using toxicity tests. Because the *D. magna* SMAV was selected as the preferred WER denominator in the present study, it can be applied uniformly to all regression model–predicted site water Cu EC50 values to calculate a WER value. This approach also eliminates the laboratory water EC50 values as a source of WER variability (e.g., laboratory water EC50 values of 4.0 µg Cu/L and 6.0 µg Cu/L at hardness concentrations of 42 mg/L and 46 mg/L as $CaCO_3$ that were determined during WER-testing Round 1 in Supplemental Data, Table S4), as suggested by others to improve interpretation of WER values [18]. A predicted WER value can thus be calculated by normalizing the regression-predicted EC50 value and the *D. magna* SMAV to the same hardness:

$$\mathrm{WER} = \frac{\text{predicted site-water EC50}_{hardness-normalized}}{D.\,magna\ \mathrm{SMAV}_{hardness-normalized}} \quad (2)$$

An advantage of applying the regression model approach to the WER procedure is that it provides an option to account for water chemistry variability when site-specific criteria are being developed. Similar to the current hardness-based ambient water quality criteria that are still used by individual states in the United States, whereby a Cu criterion is calculated based on the water hardness of the sample, a regression-based WER value can be calculated for a sample based on water chemistry values. However, the current regression model should be applied only to the surface waters from which it was developed (i.e., the present study area), because the model is calibrated to the specific chemistries of the tested waters, which includes dissolved organic matter containing site-specific types and percentages of active humic and fulvic acids. Care should also be taken when applying the model to site-specific surface waters that have DOC and alkalinity concentrations outside the range used to develop the model. For this potential scenario, the authors recommend that DOC and alkalinity be capped at the upper concentrations that were used to develop the model (i.e., a DOC concentration of 15.7 mg/L and an alkalinity concentration of 250 mg/L as $CaCO_3$). In contrast, the model can be applied to DOC and alkalinity concentrations lower than the range used to develop the model, to ensure that the predicted EC50 and WER values are sufficiently protective at low DOC and alkalinity concentrations.

BLM performance in site waters

The BLM-predicted 48-h dissolved Cu EC50 values in site waters always exceeded the corresponding measured EC50 values and in most cases were more than 2-fold greater than observed values (Figure 1). This result implies that the BLM underpredicts Cu toxicity in these site waters. Because Kolts et al. [19] concluded that most of the exposure that determines acute Cu toxicity to cladocerans occurs during the first few hours, we compared the performance of the BLM using pH measured at test initiation (0 h exposure) and the average of the 0-h and 24-h pH values. Using the average pH produced BLM-predicted EC50 values that correlated better with observed values ($r = 0.92$) than those using the initial pH values ($r = 0.83$), but they still were always at least 2-fold greater than observed values. The systematic error in BLM predictions (i.e., constant underprediction of toxicity) might suggest: (1) a sensitivity difference between tested organisms and those used to develop the BL, M and/or (2) a difference in the quality of DOC in site waters compared with those used to develop the BLM (i.e., different Cu-binding affinity or different binding-site density).

To explain the discrepancy between BLM-predicted and measured toxicity, we also evaluated the performance of the default BLM in the concurrent laboratory water toxicity tests. Most BLM-predicted EC50 values for laboratory waters were within a factor of 2 of observed values; however, a bias was still evident because predicted values consistently exceeded observed values, although the magnitude of differences for laboratory waters generally was less than for the site waters (Figure 2A). One option to optimize the BLM performance is to adjust the default sensitivity parameter (i.e., the median lethal accumulation [LA50]), because the *D. magna* used in these WER tests might have been slightly more sensitive than the composite sensitivity of the *D. magna* that were used to parameterize the BLM. This is a reasonable option in the present study because the BLM tended to overpredict the EC50 values in laboratory waters, which represent the type of water chemistries used to develop the BLM (i.e., laboratory type waters with approximately equal hardness and alkalinity, low DOC, and circumneutral pH).

To optimize the *D. magna* LA50 for the current laboratory water dataset, the default LA50 value was adjusted downward from 0.119 nmol to 0.057 nmol Cu/g wet weight so the geometric mean of the BLM-predicted EC50/observed EC50 ratio equaled 1.0 among the 11 laboratory waters, based on the approach described in Meyer and Adams [20]. This adjustment provided a reasonable fit between predicted and observed values for the laboratory water tests (Figure 2B) and was assumed to

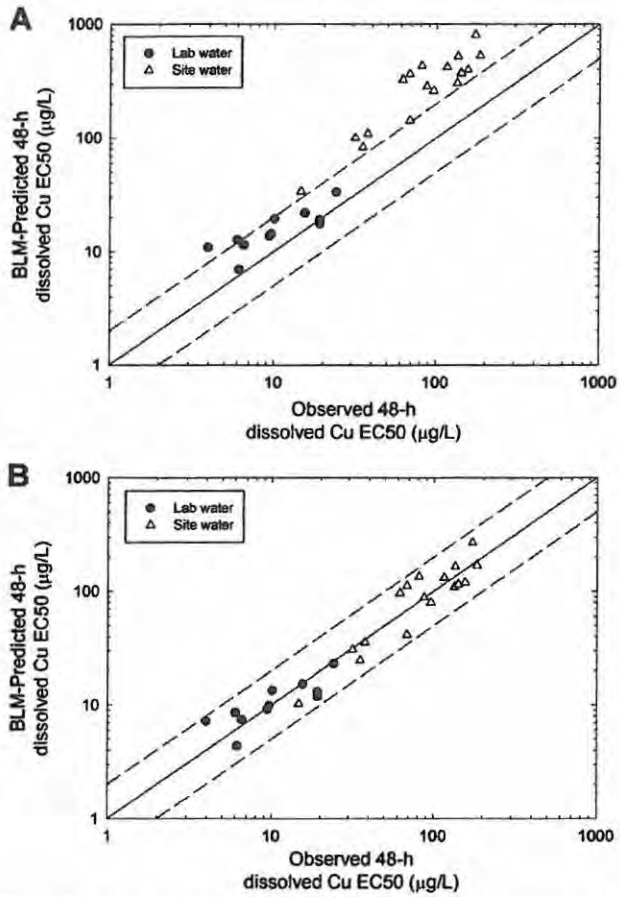

Figure 2. Biotic ligand model (BLM) predictions of *Daphnia magna* 48-h dissolved Cu median effect concentrations (EC50s) in laboratory and site waters, based on (**A**) the default BLM parameterization and (**B**) an optimized BLM. The BLM was optimized by first adjusting the sensitivity parameter (i.e., the median lethal accumulation of Cu on the biotic ligand) to improve the fit between BLM-predicted and observed laboratory water EC50 values (adjusted LA50 = 0.057 nmol Cu/g wet wt). Then the reparameterized BLM was applied to measured site water chemistries after decreasing measured site water DOC concentrations by 57%. Solid line represents a perfect (1:1) fit; dashed lines are ± 2-fold of the perfect fit.

calibrate the BLM to the sensitivity of the organisms used in the WER toxicity tests. As an indication of the sensitivity of the LA50 to the assumed DOC concentration in the laboratory waters, the LA50 increased to 0.130 nmol Cu/g wet weight when the lower-bound assumed DOC concentration was 0.3 mg C/L and decreased to 0.0333 nmol Cu/g wet weight when the upper-bound assumed DOC concentration was 0.7 mg C/L.

However, that organism-sensitivity adjustment did not completely eliminate the bias in BLM-predicted EC50 values in the site waters. One possible explanation for this difference is the quality of DOC in site waters relative to the DOC used to calibrate the default BLM. Because the BLM overpredicted the EC50 values in site waters (even after adjusting the LA50 value to optimize the laboratory water predictions), a way to decrease the predicted EC50 values is to proportionally decrease each sample's DOC concentration that is inputted into the BLM. In effect, this would increase the percentage of free Cu available to bind to the biotic ligand site(s), thereby decreasing the EC50 values. This approach has been applied previously for *D. magna* toxicity tests [21–23]. A similar approach was applied in the present study to determine the percentage of the measured DOC concentration at which the geometric mean of the BLM-predicted EC50/observed EC50 ratio of the site waters equaled 1.0.

After adjusting the LA50 downward to 0.057 nmol Cu/g wet weight for an assumed 0.5 mg DOC/L in the laboratory waters and applying that LA50 to the site water toxicity results, decreasing the measured DOC concentrations in the site waters by 57% (i.e., assuming only 43% of the DOC interacted with Cu) provided the optimized fit ($r = 0.97$) of the BLM-predicted EC50 values compared with measured values (Figure 2B). This is similar to the refined BLM model described by De Schamphelaere et al. [21,22], in which 50% of the DOC was considered to be active fulvic acid and thus the fraction of DOC that binds with Cu. As an indication of the sensitivity of that site water DOC-adjustment factor to the assumed DOC concentration in the laboratory waters, decreasing the measured DOC concentrations in the site waters by 71% (i.e., assuming only 29% of the DOC interacted with Cu) provided the optimized fit ($r = 0.97$) of the BLM-predicted EC50 values compared with measured values when the DOC concentration in the laboratory waters was 0.3 mg C/L; decreasing the measured DOC concentrations in the site waters by 42% (i.e., assuming only 58% of the DOC interacted with Cu) provided the optimized fit ($r = 0.97$) of the BLM-predicted EC50 values compared with measured values when the DOC concentration in the laboratory waters was 0.7 mg C/L. Therefore, when one is trying to determine how to parameterize the DOC in site waters for input into the BLM, it is important to determine the sensitivity (i.e., the LA50) of the toxicity-test organisms in a laboratory water that is well characterized (especially having a detectable DOC concentration) before adjusting the DOC inputs to the BLM.

CONCLUSIONS

The acute toxicity of Cu varied across site-specific water chemistries in a manner consistent with the current mechanistic understanding of Cu toxicity. Dissolved organic carbon concentration was the most significant predictor of Cu toxicity in the present study, but other water chemistry parameters were also significantly correlated with Cu toxicity, including the hardness/alkalinity ratio, alkalinity, total dissolved solids, and K^+ concentration. A multiple-regression model developed from a combination of measured concentrations of DOC and alkalinity explained 85% of the observed toxicity variability, thereby providing a strong predictive tool that can be applied to the WER procedure framework to address water chemistry and toxicity variability.

Although the multiple regression derived for this site-specific scenario predicts *D. magna* EC50 values more accurately than the BLM-predicted EC50 values, the default BLM predictions were not excessively biased and were strongly correlated to observed toxicity values. After accounting for an approximately 2-fold adjustment of the LA50 needed to compensate for the apparent difference in sensitivity between the *D. magna* used in these toxicity tests and the composite *D. magna* used to calibrate the default Cu BLM, another factor of approximately 2 was needed to adjust the DOC concentrations of the site water. Therefore, the default Cu BLM predicted *D. magna* EC50 values reasonably well in the ambient site waters, but the site-specific regression model predicted the EC50 values considerably better than did the default BLM. However, because safety margins are incorporated into the derivation of criteria concentrations for metals (i.e., criteria concentrations are derived in an environmentally conservative manner), these results do not necessarily

mean that BLM-based Cu criteria concentrations will not still be protective of aquatic life.

SUPPLEMENTAL DATA

Tables S1–S5.
Figures S1–S3. (265 KB PDF).

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