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# Determining the Effects of Major Cations ($K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$) and pH on *Scirpus mariqueter* to Assess the Heavy Metal Biotoxicity of a Tidal Flat Ecosystem

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## ABSTRACT

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The biotic ligand model (BLM) as a mechanistic bioavailability model has been used to assess the risk of metals on various ecosystems. In this study, the effects of major cations ($K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$) and pH on root elongation of the salt marsh plant *Scirpus mariqueter* were investigated by laboratory experiments, and then a tidal flat sediment–BLM was developed successfully to assess heavy metal biotoxicity on a tidal flat ecosystem for the first time. The results showed that the increase of $Na^+$ activities could decrease $Cu^{2+}$ toxicity to *S. mariqueter*. A nonlinear relationship was observed between the median effective concentration (EC50) and $H^+$ activity over the pH range (5.55–8.22). The results indicated that $H^+$ did not compete with $Cu^{2+}$ for the binding sites within the roots of *S. mariqueter* under pH $< 7.0$ (5.55–6.62), while $Cu^{2+}$ and $CuCO_{3(aq)}$, as toxic species, competed for binding sites of the biotic ligands (BL) with $Na^+$ under pH $> 7.0$ (7.03–8.22). The following conditional binding constants for the binding of $Cu^{2+}$, $CuCO_{3(aq)}$, and $Na^+$ to biotic ligands were obtained: $\log K_{CuBL} = 6.60$, $\log K_{CuCO_3BL} = 6.20$ and $\log K_{NaBL} = 1.778$. The developed Cu-BLM for *S. mariqueter* in this study was validated so that it could provide accurate predictions of Cu toxicity. This developed model could provide a scientific and reasonable basis for an environmental quality standard to assess risk in an estuarine tidal flat wetland.

**ADDITIONAL INDEX WORDS:** *Tidal wetland,* Scirpus mariqueter*, biotic ligand model, copper, bioavailability.*

## INTRODUCTION

Estuarine tidal flat wetlands represent a transition zone between sea and land. They also serve as a distribution center of material and energy between the atmosphere, hydrosphere, lithosphere, and biosphere and are highly sensitive to changes in natural processes caused by large-scale environmental change and anthropogenic activities (Xu *et al.*, 1997). In recent years, the development of tidal flats combined with the discharge of large amounts of industrial wastewater and sewage have led tidal flats to act as pools or sinks for basin pollutant (Williams, Bubb, and Lester, 1994). The net result is that tidal flat ecosystems are facing significant pressure (Feng *et al.*, 2004; Zhang *et al.*, 2001). The potential effect of pollutants, especially heavy metals, on the regional ecosystem has become a particularly hot topic. Heavy metals are of particular importance because of their widespread use and their nonbiodegradability (Morillo, Usero, and Gracia, 2002), both of which allow them to accumulate in the environment. Heavy metals pose serious risks to organisms, estuaries, and the marine environment as well as human health. Heavy metals may also be accumulated in organisms by means of organo-metallic compounds. These compounds may do more harm to tidal flat organisms and human health as they are passed through the food chain (Altındağ and Yiğit, 2005). Heavy metals can also be accumulated in tidal flat sediments and can be released back into the water column in response to environment changes. In this case, they can be viewed as "secondary pollutants" that may cause additional, direct harm to the offshore environment (Hill, Simpson, and Johnston, 2013). In light of the above, it is of vital significance to establish a rational evaluation standard of trace metal toxicity for tidal flat wetlands.

The biotic ligand model (BLM) is a mechanistic bioavailability model. It is based on the free-ion activity model (Pagenkopf, 1983) and the gill site interaction model (Morel, 1983). The BLM approach is regarded as a milestone approach that considers bioavailability in the risk assessment of metals (Rüdel *et al.*, 2015). There is considerable international interest in the use of BLMs for the assessment and management of ecological risks in the environment (Santore *et al.*, 2002; Van Sprang *et al.*, 2009; Verschoor *et al.*, 2011). For example, BLM-based water-quality criteria for Cu have been incorporated into risk assessment reports in the United States (U.S. EPA, 2007). BLMs were also incorporated in the risk assessment of Cu, Ni, and Zn in the European Union (Erickson 2013; Van Sprang *et al.*, 2009). In Australia, New Zealand, and Japan, BLMs have been applied to assess freshwater quality but are not specifically mandated (ANZECC and ARMCANZ Staff, 2000; Hayashi, 2013). BLMs have also supported site-specific risk analyses (Niyogi and Wood, 2004; Van Sprang *et al.*, 2009) and have been developed to predict metal toxicity for both

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Table 1. *Composition of the test media used in the various toxicity experiments and the measured EC50 for* S. mariqueter *root elongation with 95% confidence intervals (EC50 expressed as free* $Cu^{2+}$ *activity* ($EC50_{[Cu^{2+}]}$)).

| Set | pH | $Ca^{2+}$ (mM) | $Mg^{2+}$ (mM) | $Na^{+}$ (mM) | $K^{+}$ (mM) | $Cl^{-}$ (mM) | $SO_4^{2-}$ (mM) | EC50 (95% CI) ($Cu^{2+}$ μM) |
|---|---|---|---|---|---|---|---|---|
| Ca | | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.25 (0.17–0.38) |
| | | 1.750 | 0.333 | 2.609 | 0.059 | 6.168 | 0.333 | 0.27 (0.21–0.37) |
| | | 2.265 | 0.333 | 2.609 | 0.059 | 7.198 | 0.333 | 0.32 (0.25–0.40) |
| | | 3.500 | 0.333 | 2.609 | 0.059 | 9.668 | 0.333 | 0.38 (0.36–0.40) |
| | | 4.375 | 0.333 | 2.609 | 0.059 | 11.418 | 0.333 | 0.38 (0.30–0.48) |
| Mg | | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.25 (0.17–0.38) |
| | | 0.875 | 0.800 | 2.609 | 0.059 | 4.418 | 0.8 | 0.25 (0.20–0.31) |
| | | 0.875 | 4.000 | 2.609 | 0.059 | 4.418 | 4 | 0.29 (0.22–0.37) |
| | | 0.875 | 8.000 | 2.609 | 0.059 | 4.418 | 8 | 0.25 (0.20–0.31) |
| | | 0.875 | 12.000 | 2.609 | 0.059 | 4.418 | 12 | 0.26 (0.23–0.29) |
| | | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.25 (0.18–0.33) |
| Na | | 0.875 | 0.333 | 10.40 | 0.059 | 12.209 | 0.333 | 0.29 (0.22–0.38) |
| | | 0.875 | 0.333 | 26.08 | 0.059 | 27.896 | 0.333 | 0.39 (0.31–0.49) |
| | | 0.875 | 0.333 | 52.16 | 0.059 | 53.969 | 0.333 | 0.69 (0.55–0.87) |
| | | 0.875 | 0.333 | 78.26 | 0.059 | 80.069 | 0.333 | 1.026 (0.93–1.26) |
| | | 0.875 | 0.333 | 104 | 0.059 | 105.809 | 0.333 | 1.033 (0.72–1.43) |
| K | | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.25 (0.18–0.33) |
| | | 0.875 | 0.333 | 2.609 | 0.295 | 4.654 | 0.333 | 0.33 (0.21–0.53) |
| | | 0.875 | 0.333 | 2.609 | 0.590 | 4.949 | 0.333 | 0.34 (0.28–0.43) |
| | | 0.875 | 0.333 | 2.609 | 1.18 | 5.539 | 0.333 | 0.32 (0.17–0.60) |
| | | 0.875 | 0.333 | 2.609 | 2.36 | 6.719 | 0.333 | 0.42 (0.28–0.62) |
| | | 0.875 | 0.333 | 2.609 | 3.54 | 7.899 | 0.333 | 0.43 (0.36–0.50) |
| pH | 5.55 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.17 (0.14–0.21) |
| | 6.00 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.25 (0.18–0.33) |
| | 6.62 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.22 (0.19–0.24) |
| | 7.03 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.26 (0.22–0.30) |
| | 7.53 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.17 (0.12–0.24) |
| | 7.78 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.06 (0.04–0.10) |
| | 8.22 | 0.875 | 0.333 | 2.609 | 0.059 | 4.418 | 0.333 | 0.014 (0.003–0.05) |

*Cations were expressed as concentration. The measured EC50 values were obtained based on the four parameter logistic equation using SPSS (version 16.0) software. Within a test series values were considered significantly different when the 95% confidence intervals did not overlap.*

terrestrial and freshwater ecosystems (Cheng and Allen, 2001; Le *et al.*, 2012; Maksymiec and Baszyński, 1998; Parker *et al.*, 1998; Wang, Hua, and Ma, 2012).

This study aims to extend the sediment BLM (sBLM) developed by Di Toro *et al.* (2005) to the estuarine ecosystem. More specific aims are (1) to study the toxicity of copper on a Chinese indigenous indicator plant, *Scirpus mariqueter*, under the different conditions of pH, major cations, and (2) to develop an acute Cu sBLM for indicator plant *Scirpus mariqueter* in a tidal flat wetland.

## METHODS

*Scirpus mariqueter* was chosen as a test organism to determine the independent effects of different cations on Cu toxicity to salt marsh plants. This plant is indigenous to China; it is mostly distributed in intertidal areas of both the Yangtze River estuary and Hangzhou Bay. In general, it is a typical perennial salt marsh pioneer keystone species in the family Cyperaceae and grows in depositional tidal flats. *Scirpus mariqueter* is thought to play a critical and beneficial ecological role in maintaining the integrity of ecosystem structure and function as well as salt marsh dynamics within the Yangtze estuary.

The following methodological section describes the experimental design, test preparation, acute toxicity tests, chemical measurements, data analysis and statistics, and BLM model development.

### Experimental Design

In order to develop a tidal sediment BLM, data were required regarding the effects of major cations and pH on Cu toxicity to salt marsh plants. Thus, five sets (Ca set, Mg set, Na set, K set, and pH set) (Table 1) of acute 96-hour toxicity bioassays were conducted to assess the effects of major cations (*e g*., $K^{+}$, $Na^{+}$, $Ca^{2+}$, $Mg^{2+}$) and pH on the acute 96-hour toxicity of Cu. For each set, only one studied cationic concentration was varied at a time; the other cationic concentrations were kept the same as the basal growth solutions (De Schamphelaere and Janssen, 2002; Lock *et al.*, 2007a,b,c). Each set was composed of at least six Cu concentration tests and one control test in which Cu was not added to the solution. The concentrations of added $Cu^{2+}$ in these tests ranged from 0 μM to 8.0 μM. The cationic concentrations selected for the basal growth solutions are similar to those that occur within waters of the Yangtze estuary (Zhang, Gao, and Zhang, 2003). The cationic concentration ranges produced a salinity range of 0.022% to 1.520%, typical of those that characterize sediment in the Yangtze estuary (Chen, Li, and Chen, 2005). The experiment consisted of three replicates for each treatment.

### Test Preparation

Germination of the *S. mariqueter* seeds was carried out in a climate controlled room (25°C, 80% humidity, dark) for 96 hours. Stock solutions of Cu, Ca, Mg, Na, and K were prepared by adding different amounts of $CuCl_2{\cdot}2H_2O$, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, NaCl, and KCl to deionized water. The basal growth solution had a minimum Ca, Mg, Na, and K concentration of 0.875 mM $CaCl_2$, 0.333 mM $MgSO_4$, 2.609 mM NaCl, and 0.059 mM KCl. Test media (*i.e.*, modified basal growth solution) were prepared by adding different amounts of Ca, Mg, Na, K, and Cu. The pH values of the test media were obtained by adding 2-(N-morpholino)ethanesulfonic acid ($pH < 7.0$) or 3-[N-morpholino] propanesulfonic acid buffers (3.6 mM) ($pH > 7.0$) and diluted NaOH. Neither buffer reacts with Cu (Lock *et al.*, 2007a). In order to obtain near-equilibrium conditions, all media were stored at least 24 hours at 20°C before being used in the bioassays. All reagents used in the experiment were of analytical or higher grade. Deionized water was used throughout the experiment. The test media sets are summarized in Table 1.

## Acute Toxicity Tests

Following the germination of *S. mariqueter* seeds in a climate controlled room (25°C, 80% humidity, dark) for 96 hours, seeds with radicle lengths less than 2 mm were selected for the acute 96-hour bioassay following the Organization for Economic Cooperation and Development (OECD) guideline (OECD, 2003). According to EPA and OECD, root elongation is a sensitive endpoint descriptor of metal toxicity (OECD, 2003; U.S. EPA, 1988). To each set, an acute toxicity assay was conducted consisting of at least seven treatments (control+ six Cu concentrations) with a difference of 1 log-unit or more between the lowest and highest Cu concentrations tested. Each treatment consisted of three replicates and six organisms per replicate. The treatments were conducted in polyethylene beakers filled with 300 ml of test medium. After the initial root lengths were recorded, the seedlings were fixed in nylon nets floating on the surface of the test medium in polyethylene beakers in a climate controlled room. The air temperature was maintained at 26°C during the 12-hour light and 20°C during the12-hour dark cycles for 96 hours. A constant humidity of 80% was maintained throughout the tests. Test media were renewed every other day and analyzed after 96 hours. The length of the longest seminal root of every seedling was also measured at 96-hour intervals. The relative net elongation inhibition (RNE, %) was used to determine the toxic response of *S. mariqueter*. The parameter was calculated according to the following formula (Parker *et al.*, 1998):

$$\mathrm{RNE} = \frac{(L_T - L_0)}{(L_C - L_0)} \times 100\% \tag{1}$$

where, RNE is the relative net elongation, $L_T$ refers to the final length of the seminal root in the test medium after 96 hours, $L_0$ is the initial length of the seminal root, and $L_C$ is the final length of the seminal root in the control test medium after 96 hours.

## Chemical Measurements

The concentrations of Cu, Ca, Mg, Na, and K in the test media were measured with a graphite furnace atomic absorption spectrophotometer (PE-AA800, Perkin Elmer, Fremont, California, U.S.A.). The pH in all test media was measured before and after the test using a pH meter (pH/ION 3430; WTW, Germany); a maximum difference of 0.2 pH units before and after the test was allowed. The pH meter was calibrated daily using pH 4.01, 7.0, and 10.01 buffers.

## Data Analysis and Statistics

EC50 values for free $Cu^{2+}$ activity ($EC50_{[Cu^{2+}]}$) were calculated on the basis of a four-parameter nonlinear logistic equation using SPSS 16.0 software (Luo, Li, and Zhou, 2008):

$$Y = \mathrm{Bottom} + \frac{\mathrm{Top} - \mathrm{Bottom}}{1 + 10^{(\log \mathrm{EC50} - x) \cdot \mathrm{Hillslope}}} \tag{2}$$

where, $x$ is the logarithm of Cu ion concentration and $Y$ (RNE %) is the biological response to the chemistry of the solution. Calculated $EC50_{[Cu^{2+}]}$ values were based on the measured relative net root growth to free ionic $Cu^{2+}$ concentrations in each set. Finally, linear regressions between $EC50_{[Cu^{2+}]}$ and the activities of each cation were performed using Excel 2010. The slopes and intercepts from these linear regressions were used to determine BLM parameters (De Schamphelaere and Janssen, 2002).

Cu speciation was predicted using Visual MINTEQ version 3.0 (Gustafsson, 2012). Input data included temperature, pH, and the concentrations of Cu, Ca, Mg, K, Na, $Cl^-$, and $SO_4^{2-}$. Since the experiments were carried out in an open system, a $CO_2$ partial pressure of $3.5 \times 10^{-4}$ atm (1 atm = 101.3 kPa) was assumed in the calculation (Wang, Hua, and Ma, 2012).

## The BLM Model Development

The BLM assumes that (1) toxicity is related to $Cu^{2+}$ binding with the biotic ligand (BL) sites and (2) all cations (*e.g.*, $Ca^{2+}$, $Mg^{2+}$, $Na^+$, $K^+$, or $H^+$) may compete for the total biotic ligand (BL) sites and alleviate metal toxicity. Toxicity is determined by the fraction (*f*) of the total BL bound to $Cu^{2+}$, which can be expressed as (De Schamphelaere and Janssen, 2002; Di Toro *et al.*, 2001):

$$f_{\mathrm{CuBL}} = \frac{K_{\mathrm{CuBL}}\left[\mathrm{Cu}^{2+}\right]}{1 + K_{\mathrm{CuBL}}\left[\mathrm{Cu}^{2+}\right] + \sum K_{\mathrm{XBL}}\left[\mathrm{X}^{n+}\right]} \tag{3}$$

where, the square brackets denote molar concentration, and the binding concentration of Cu and other competitive cations to BL sites is represented by $[CuBL^+]$ and $[XBL^{n+}]$($mol\ L^{-1}$). The parameters $[Cu^{2+}]$, $[X^{n+}]$, $K_{XBL}$, and $K_{CuBL}$ refer to the activity of the free copper ion ($mol\ L^{-1}$), the activity of competing cations, and the stability constants for $[X^{n+}]$ and $[Cu^{2+}]$ binding to BL sites ($L\ mol^{-1}$), respectively.

When the inhibition of *S. mariqueter* net root elongation is up to 50% of the control net root elongation, Equation (3) can be transferred to the following form (Pagenkopf, 1983):

$$\mathrm{EC50}_{[\mathrm{Cu}^{2+}]} = \frac{f_{\mathrm{CuBL}}^{50\%}}{(1 - f_{\mathrm{CuBL}}^{50\%})K_{\mathrm{CuBL}}}\left(1 + \sum K_{\mathrm{XBL}}\left[\mathrm{X}^{n+}\right]\right) \tag{4}$$

where, $EC50_{[Cu^{2+}]}$ is the free copper ion activity that results in 50% RNE (50% of *S. mariqueter* net root elongation after 96 h of exposure with respect to the control net root elongation). The parameter $f_{CuBL}^{50\%}$ is the fraction of the BLs that results in a 50% RNE when occupied by Cu. According to the BLM concept, when $f_{CuBL}^{50\%}/1 - f_{CuBL}^{50\%}$ is constant, a linear relationship should be observed between $EC50_{[Cu^{2+}]}$ and the activity of the cation of interest, provided that the other cationic activities were kept constant in Equation (4) (Di Toro *et al.*, 2001). $EC50_{[Cu^{2+}]}$ can be predicted when $[X^{n+}]$ and the values of $K_{CuBL}$, $K_{XBL}$, and $f_{CuBL}^{50\%}$,

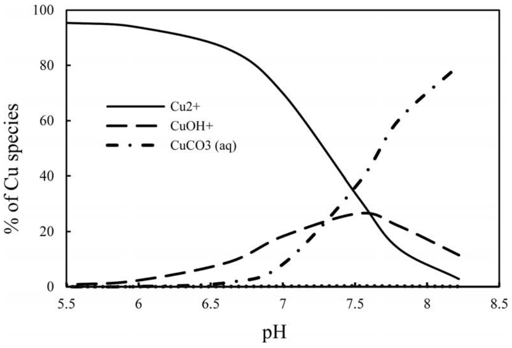

Figure 1. The distribution of Cu species in solutions with pH change from 5.55 to 8.22 simulated in Visual MINTEQ software. The solid curve, the long dotted curve and the short dotted curve represents percentages of $Cu^{2+}$, $CuOH^+$, and $CuCO_3$, respectively.

are known. Geochemical speciation modeling can be used to estimate the free ionic concentration according to the corresponding test medium concentration. Estimates of the conditional equilibrium constants for the interaction of $Ca^{2+}$, $Mg^{2+}$, $Na^+$, and $H^+$ with $Cu^{2+}$ on the biotic ligand were derived according to the method described by De Schamphelaere and Janssen (2002). The parameters $K_{CuBL}$ and $f_{CuBL}^{50\%}$ were calculated by optimizing the logit-transformed linear effect (Lock *et al.*, 2007a,b).

## RESULTS

In the following section, the distribution of copper species for different pH conditions and the effects of major cations on Cu toxicity are described. From this analysis, the parameters required for the BLM are determined and the predictive ability of the developed Cu-BLM for *S. mariqueter* is examined.

### Distribution of Copper Species for Different pH Conditions

Figure 1 shows the distribution of Cu species in pH solutions ranging from 5.55 to 8.22. Free $Cu^{2+}$ was the most dominant species at $pH < 7.53$. When pH was increased from 5.55 to 7.53, the proportions of $CuCO_{3(aq)}$ and $CuOH^+$ increased continuously as the proportion of $Cu^{2+}$ decreased. When the solution pH was above 7.53 (and within the pH range of 7.53 to 8.22), the dominant Cu species became $CuCO_{3(aq)}$; the proportions of $Cu^{2+}$ and $CuOH^+$ decreased with increasing pH. When pH was 8.22, the species distribution was 79.99% $CuCO_{3(aq)}$, 11.48% $CuOH^+$, 3.22% $Cu(OH)_{2(aq)}$, and 2.83% $Cu^{2+}$. Other Cu species, such as $Cu(OH)_{2(aq)}$ and $CuSO_{4(aq)}$ were always low in all experiments under different pH conditions. Therefore, only $Cu^{2+}$, $CuCO_{3(aq)}$, and $CuOH^+$ were considered for their toxicity to the root elongation of *S. mariqueter*.

### Effects of Major Cations on Cu Toxicity

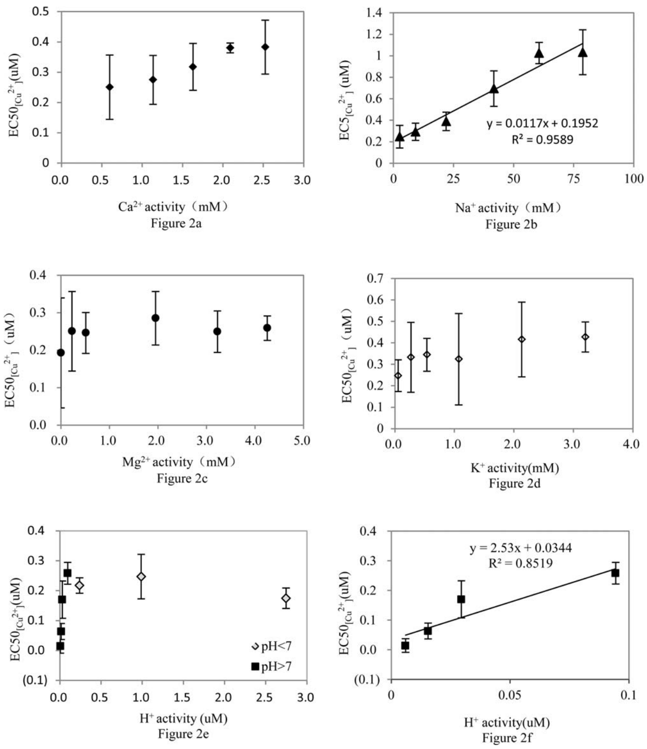

Figure 2. The EC50 values expressed as free $Cu^{2+}$ activity for *S. mariqueter* root elongation as a function of the activity of (a) $Ca^{2+}$, (b) $Na^+$, (c) $Mg^{2+}$, (d) $K^+$, (e) $H^+$, and (f) $H^+$ ($pH > 7$) in simulated media. Error bars indicate 95% confidence intervals. Significant correlations are represented by a solid line.

Figure 2 illustrates the cationic 96-hour $EC50_{[Cu^{2+}]}$ effect for net root elongation of *S. mariqueter*. The measured $EC50_{[Cu^{2+}]}$ values (Table 1) were calculated by fitting a logistic model. It was found that the measured $EC50_{[Cu^{2+}]}$ values ranged from 0.014 to 1.033 μM for all treatments. The 96-hour $EC50_{[Cu^{2+}]}$ significantly increased with increasing $Na^+$ activity when the concentrations of $Na^+$ ranged from 2.609 to 104 mM. Over this range, EC50 values increased from 0.247 to 1.033 μM $Cu^{2+}$. The slope of the linear regression line for the effect of $Na^+$ on EC $50_{[Cu^{2+}]}$ confirmed that a significant decrease in acute Cu toxicity was observed when $Na^+$ was increased (Figure 2b). Figures 2a, 2c, and 2d illustrate the relation between $Ca^{2+}$ activity, $K^+$ activity, $Mg^{2+}$ activity, and $EC50_{[Cu^{2+}]}$. $EC50_{[Cu^{2+}]}$ did not change significantly across the concentration ranges used here for $K^+$ (0.059–3.54 mM), $Mg^{2+}$(0.333–12 mM), and $Ca^{2+}$ (0.875–4.375 mM) (Table 1; Figure 2a, 2c, 2d). The slope of the regression lines describing the relationship between $K^+$ and $Cu^{2+}$, $Ca^{2+}$ and $Cu^{2+}$, and $Mg^{2+}$ and $Cu^{2+}$ toxicity did not differ significantly. Thus, they were omitted for the BLM of *S. mariqueter*, and the values of log $K_{MgBL}$, log $K_{CaBL}$, and log $K_{KBL}$ were set to zero.

Figure 2e illustrates the effect of $H^+$ activity versus $EC50_{[Cu^{2+}]}$. With an increase in pH from 5.55 to 8.22, Cu toxicity increased by a factor of 12 (Table 1). According to Equation (5), if $H^+$ can compete with $Cu^{2+}$ binding sites on the *S. mariqueter* roots, then a linear relationship between EC $50_{[Cu^{2+}]}$ and $H^+$ activity should exist in the pH set. However, there was a nonlinear relationship between $EC50_{[Cu^{2+}]}$ and $H^+$ in the culture medium over the range of pH values examined. Apparently the relationship between $EC50_{[Cu^{2+}]}$ and $H^+$ activity differs as a function of the pH (Figure 2e). When $pH < 7.0$, the $EC50_{[Cu^{2+}]}$ values were not significantly

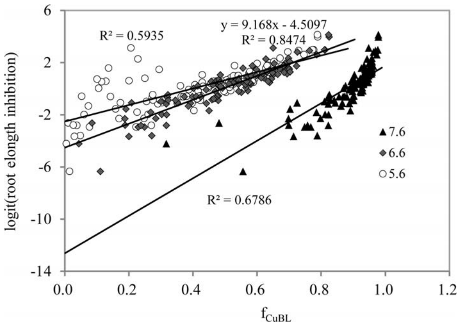

Figure 3. Relationship between the logit of the observed percent root growth inhibition of *S. mariqueter* after 96 h of exposure and the calculated fraction of the biotic ligand sites occupied by Cu ($f_{\mathrm{CuBL}}$) for log $K_{\mathrm{CuBL}} = 5.60$, 6.60, and 7.60.

different ($p < 0.05$). This suggests that there was a lack of significant competition between $H^+$ and $Cu^{2+}$ for *S. mariqueter* root binding sites for the pH range of 5.55–7.0 (the pH range studied here). When pH increased from 7.03 to 8.22, the values of $EC50_{[Cu^{2+}]}$ decreased sharply with increasing solution pH (Table 1; Figure 2e). The trend may be explained by the change of Cu species in the solutions. According to the distribution of Cu species shown in Figure 1, the percentages of $CuCO_{3(aq)}$ and $CuOH^+$ to the total Cu in solution increased with an increase in pH.

Equation (4) can be used to derive Equation (5), and Equation (5) could be used to derive the effects of $CuCO_{3(aq)}$ and $CuOH^+$ on Cu toxicity to *S. mariqueter* root elongation when pH > 7.0:

$$\frac{1}{\mathrm{EC50}_{[\mathrm{Cu}^{2+}]}} = \frac{1 - f^{50\%}_{\mathrm{CuBL}}}{f^{50\%}_{\mathrm{CuBL}}\left(1 + \mathrm{K}_{\mathrm{NaBL}}\left[\mathrm{Na}^+\right]\right)} \times \left(\mathrm{K}_{\mathrm{CuBL}} + \mathrm{K}_{\mathrm{CuCO_3BL}}\frac{[\mathrm{CuCO_3}]}{\left[\mathrm{Cu}^{2+}\right]} + \mathrm{K}_{\mathrm{CuOHBL}}\frac{\left[\mathrm{CuOH}^+\right]}{\left[\mathrm{Cu}^{2+}\right]}\right) \tag{5}$$

Equation (5) was transformed to a linear equation with $1/EC50_{[Cu^{2+}]}$ as a dependent variable, and $[CuCO_{3(aq)}]/[Cu^{2+}]$ and $[CuOH^+]/[Cu^{2+}]$ as independent variables. This transformation is consistent with prohibiting proton competition in the calculations, since proton competition would not be significant at a pH greater than 6.5. Since there was no significant relationship between $1/EC50_{[Cu^{2+}]}$ and $[CuOH^+]/[Cu^{2+}]$, $[CuOH^+]/[Cu^{2+}]$ was removed from Equation (5). Thus, the multiple linear pairwise regression between $1/EC50_{[Cu^{2+}]}$ and $[CuCO_{3(aq)}]/[Cu^{2+}]$ was calculated as follows:

$$\frac{1}{\mathrm{EC50}_{[\mathrm{Cu}^{2+}]}} = 4.156(\pm 0.976) + 1.672(\pm 0.048)\frac{[\mathrm{CuCO_3}]}{\left[\mathrm{Cu}^{2+}\right]} \tag{6}$$

Only the intercept and coefficient of $[CuCO_{3(aq)}]/[Cu^{2+}]$ are significant at $p < 0.05$ level. This demonstrates that Cu toxicity to *S. mariqueter* root elongation could be caused by $Cu^{2+}$ and $CuCO_{3(aq)}$ when $CuCO_{3(aq)}$ exists in solution. According to these data, the toxicities of $Cu^{2+}$ and $CuCO_{3(aq)}$ should be included in Cu-sBLM development.

## Determination of the Parameters in BLMs

When $CuCO_{3(aq)}$ toxicity is considered, Equation (3) can be transformed to Equation (7):

$$f_{\mathrm{CuBL}} = \frac{\mathrm{K}_{\mathrm{CuBL}}\left[\mathrm{Cu}^{2+}\right] + \mathrm{K}_{\mathrm{CuCO_3BL}}[\mathrm{CuCO_3}]}{1 + \mathrm{K}_{\mathrm{CuBL}}\left[\mathrm{Cu}^{2+}\right] + \mathrm{K}_{\mathrm{CuCO_3BL}}[\mathrm{CuCO_3}] + \mathrm{K}_{\mathrm{NaBL}}\left[\mathrm{Na}^+\right]} \tag{7}$$

*S. mariqueter* root elongation $EC50_{[Cu^{2+}]}$ can subsequently be rewritten as follows:

$$\mathrm{EC50}_{[\mathrm{Cu}^{2+}]} = \frac{f^{50\%}_{\mathrm{CuBL}}}{(1 - f^{50\%}_{\mathrm{CuBL}})\left(\mathrm{K}_{\mathrm{CuBL}} + \mathrm{K}_{\mathrm{CuCO_3BL}}\mathrm{K}_{\mathrm{CuCO_3}}\left[\mathrm{CO}_3^{2-}\right]\right) \times \left(1 + \mathrm{K}_{\mathrm{NaBL}}\left[\mathrm{Na}^+\right]\right)} \tag{8}$$

In order to determine the stability constants $K_{\mathrm{NaBL}}$ for competing ions, linear regression analysis was carried out between $EC50_{[Cu^{2+}]}$ and competing ion activity. The slope and intercept of the regression lines were then determined. In the absence of other competing ions, $K_{\mathrm{NaBL}}$ can be estimated by the ratio of the slope to the intercept:

$$\mathrm{Intercept}_{\mathrm{Na}} = \frac{f^{50\%}_{\mathrm{CuBL}}}{(1 - f^{50\%}_{\mathrm{CuBL}})\left(K_{\mathrm{CuBL}} + K_{\mathrm{CuCO_3BL}}K_{\mathrm{CuCO_3}}\left[\mathrm{CO}_3^{2-}\right]\right)} = 1.952 \times 10^{-7} \tag{9}$$

$$\mathrm{Slope} = \frac{f^{50\%}_{\mathrm{CuBL}}}{(1 - f^{50\%}_{\mathrm{CuBL}})\left(K_{\mathrm{CuBL}} + K_{\mathrm{CuCO_3BL}}K_{\mathrm{CuCO_3}}\left[\mathrm{CO}_3^{2-}\right]\right)}K_{\mathrm{NaBL}} = 1.17 \times 10^{-5} \tag{10}$$

$$\frac{\mathrm{Slope}}{\mathrm{Intercept}} = K_{\mathrm{NaBL}} = 59.939 \qquad \log K_{\mathrm{NaBL}} = 1.778 \tag{11}$$

The development of a BLM for *S. mariqueter* required the calculation of three additional parameters: $K_{\mathrm{CuBL}}$, $K_{\mathrm{CuCO_3BL}}$, and $f^{50\%}_{\mathrm{CuBL}}$. According to Equation (6), $K_{\mathrm{CuCO_3BL}}/K_{\mathrm{CuBL}} = 0.4023$; therefore, $K_{\mathrm{CuCO_3BL}}$ can be derived when $K_{\mathrm{CuBL}}$ is known. For every treatment (consisting of six Cu media concentrations), $f_{\mathrm{CuBL}}$ was calculated using Equation (7) for varying log $K_{\mathrm{CuBL}}$. The best fit ($R^2 = 0.8474$) was produced when values for log $K_{\mathrm{CuBL}} = 6.60$, log $K_{\mathrm{CuCO_3BL}} = 6.20$, and the associated $f^{50\%}_{\mathrm{CuBL}} = 0.492$. These values were retained for the BLM. For comparison, the values for log $K_{\mathrm{CuBL}}$ with 1 log-unit difference (5.60 and 7.60) from the best fit (6.60) were also presented (Figure 3).

## Relationship Between Measured and Predicted 96-Hour EC50 for *S. mariqueter* Exposure to $Cu^{2+}$

An autovalidation was performed in order to examine the predictive ability of the developed Cu-sBLM for *S. mariqueter*. The acquired BLM (Eq. 8), defined by the stability constants $K_{\mathrm{CuBL}}$, $K_{\mathrm{CuCO_3BL}}$, $K_{\mathrm{NaBL}}$, and by $f^{50\%}_{\mathrm{CuBL}}$ were used to predict the 96-hour $EC50_{[Cu^{2+}]}$ for all bioassays conducted in this study (Table 1). $Na^+$ and $[CO_3^{2-}]$ activities were used as input. The

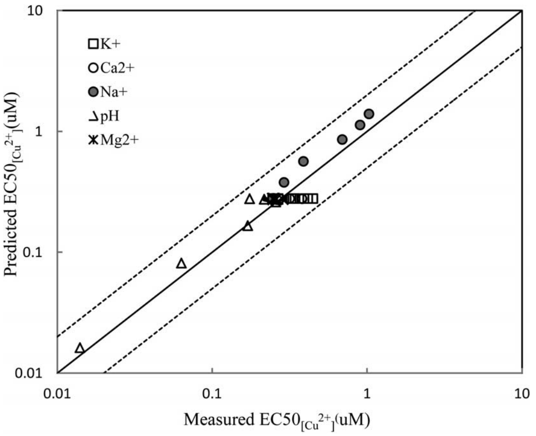

Figure 4. Comparison of predicted and measured 96-hour $EC50_{[Cu^{2+}]}$; both axes are on a logarithmic scale. The solid line (1:1 line) represents a perfect match between the measured and predicted $EC50_{[Cu^{2+}]}$ values; the dashed line (located on both sides of the 1:1 line) represents ratios that are a factor of 2.0 between measured and predicted $EC50_{[Cu^{2+}]}$.

predicted $EC50_{[Cu^{2+}]}$ values were plotted against the calculated values, together with the 1:1 line indicating a perfect match between the predicted and measured $EC50_{[Cu^{2+}]}$ (Figure 4). The dashed lines in Figure 4 represent a twofold deviation between the predicted and measured $EC50_{[Cu^{2+}]}$ values. Comparisons made in previous toxicity studies between BLM predictions and measured toxicity data have used the ±twofold values as a benchmark of predictive ability (Di Toro *et al.*, 2001; Santore *et al.*, 2001; U.S. EPA, 2002). From Figure 4, it can be deduced that the predicted EC50s differed less than twofold from the measured $EC50_{[Cu^{2+}]}$, indicating that the model can be used as the BLM-development dataset.

## DISCUSSION

In this section, the effects of salinity, $Na^+$, on Cu toxicity and pH on Cu species are listed. The binding constants for copper in terrestrial BLMs are here compared to this study.

Predicting Cu toxicity in estuarine salt marsh environments is a challenging scientific question that has vexed researchers for many years. Estuarine salt marsh environments are characterized by fluctuating salinity, and salinity can impact Cu toxicity through its influence on chemical factors (bioavailability) or biological (physiological) factors (Hall and Anderson, 1995). Some studies have indicated that Cu toxicity increased with increasing salinity, but the relationship between the two was nonlinear (Deruytter *et al.*, 2015; Grosell *et al.*, 2007). Other investigators found that salinity had an important protective effect on acute waterborne Cu toxicity to the euryhaline copepod *Acartia tonsa* (Pinho and Bianchini, 2010) and other euryhaline crustacean species (Pinho *et al.*, 2007). Salinity effects are related to the type and concentration of major cations and anions in solution. Sprague (1985) has suggested that euryhaline organisms exhibit the most resistance to toxic conditions at isosmotic salinities due to minimization of osmotic stress across a wide range. Thus, salinity will not be the cause for biological toxicity. In fact, salinity can reduce Cu toxicity as the result of anion complexation and cationic competition for binding sites. High salinity, however, may alter physiological functions that allow salinity to aggravate the toxic responses to Cu (Hall and Anderson, 1995).

During this investigation, a BLM was use to predict Cu toxicity to a sensitive salt marsh species using a procedure similar to that applied to terrestrial organisms. The 96-hour $EC50_{[Cu^{2+}]}$ value varied 73.6-fold, which clearly demonstrated the limitations of using free $Cu^{2+}$ activity alone to predict Cu toxicity. According to biotic ligand model principles, the decrease in toxicity based on $Cu^{2+}$ activity is expected with increasing salinity. When $Na^+$ concentration increased from 2.609 mM to 104.348 mM (a free $Na^+$ activity of 2.6009–78.76 mM), $EC50_{[Cu^{2+}]}$ values increased 4.13-fold (Figure 2b). The differences of the 96-hour $EC50_{[Cu^{2+}]}$ value can be explained by the positive relationship between $EC50_{[Cu^{2+}]}$ and $Na^+$ activities. This relationship demonstrates the effect of Na competition with Cu for *S. mariqueter* root binding sites, a process that decreased Cu toxicity. This is in agreement with the assumptions inherent in the development of a BLM. The competitive effect of $Na^+$ activity on $Cu^{2+}$ toxicity is probably related to similarities in the ionic radius of $Na^+$ and $Cu^{2+}$ (Lock *et al.*, 2006).

The competition for binding sites between toxic metal ions and cations is a widespread phenomenon in aquatic and terrestrial BLMs (tBLMs). However, in most Cu-BLMs, it is assumed that $Na^+$ does not affect metal toxicity. Nevertheless, the influence of $Na^+$ concentration on $Cu^{2+}$ toxicity for organisms has been shown for *Daphnia magna* (De Schamphelaere and Janssen, 2002) and fathead minnows (*Pimephales promelas*) (Erickson *et al.*, 1996) in freshwater, as well as earthworms (*Aporrectodea caliginosa*) (Koster *et al.*, 2006) in soil. In this study, $Na^+$ activity was found to affect the toxicity of Cu to *S. mariqueter*. Similar to $Na^+$, in freshwater and terrestrial BLMs, $Ca^{2+}$, $Mg^{2+}$, and $K^+$ also decreased $Cu^{2+}$ toxicity. The effect of Ca and Mg on Cu toxicity in tBLM was previously reported in the literature (Le *et al.*, 2012; Luo, Li, and Zhou, 2008; Thakali *et al.*, 2006a,b; Wang, Hua, and Ma, 2012).

The conditional binding constants of $Cu^{2+}$, $CuCO_3$(aq), and $Na^+$ (presented as log $K_{CuBL}$, log$K_{CuCO_3BL}$, and log $K_{NaBL}$) generated by this study for root growth inhibition (96 h EC50) of *S. mariqueter* were compared with those reported in Cu-tBLM for barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and lettuce (*Lactuca sativa*) root elongation, as well as tomato (*Lycopersicon esculentum*) shoot yield (Table 2) (Le *et al.*, 2012; Luo, Li, and Zhou, 2008; Thakali *et al.*, 2006a,b; Wang, Hua, and Ma, 2012). The comparison shows that the value of log $K_{CuBL}$ (6.60) in this study was lower than the reported value for barley by Thakali *et al.* (2006b) in soil solutions and lettuce by Le *et al.* (2012) in culture solutions. However, the value of log $K_{CuBL}$ is higher than the reported value for wheat (*T. aestivum*) by Luo, Li, and Zhou (2008), barley by Wang *et al.* (2012) and tomato (*L. esculentum*) by Thakali *et al.* (2006b) in culture solutions. The binding constant log $K_{CuCO_3BL}$ (6.2) in the present study was higher than the reported number for barley by Wang *et al.* (2012). These binding constant differences may be produced by differences in

Table 2. *Binding constants for copper and cations in plants species. The log* K *values are in L/mol.*

| Reference | Test Organisms | Conditional Binding Constants | | | |
|---|---|---|---|---|---|
| | | $\log K_{CuBL}$ | $\log K_{CuCO_3BL}$ | $\log K_{NaBL}$ | $f^{50\%}_{CuBL}$ |
| This study | *Scirpus mariqueter* | 6.60 | 6.2 | 1.778 | 0.49 |
| Thakali *et al.* (2006a) | *Hordeum vulgare* | 7.41 ± 0.23 | — | — | 0.05 |
| Luo, Li, and Zhou (2008) | *Triticum aestivum* | 6.28 | — | — | 0.44 |
| Wang *et al.* (2012) | *Hordeum vulgare* | 6.33 | 5.7 | — | — |
| Le *et al.* (2012) | *Lactuca sativa* | 7.40 | — | — | 0.36 |
| Thakali *et al.* (2006b) | *Lycopersicon esculentum* | 5.65 | — | — | 0.05 |

$f^{50\%}_{CuBL}$ = *the faction of biotic ligand sites occupied by metal at which 50% effect is observed.*

organisms, target tissues, exposure time, endpoints, or toxicity mechanisms (Lock *et al.*, 2006).

In an estuary tidal wetland, pH in sediment pore water generally changes with changing topographic/hydrologic environment. Such changes could affect the influence of pH on Cu speciation. The calculated results produced by the developed BLM indicate that Cu speciation was closely related to solution pH. Some studies in terrestrial environments included $CuOH^+$ and $CuCO_{3(aq)}$ in their developed BLM when pH > 6.5 (Li *et al.*, 2009). Another study showed that $CuCO_{3(aq)}$, rather than $CuOH^+$, contributed to Cu toxicity in alkaline growth medium (Wang *et al.*, 2012). In this study, where organic ligands were not considered, Cu speciation was dominated by carbonate complexation, which has a strong impact on $Cu^{2+}$ concentration. This finding is consistent with the results presented by Wang *et al.* (2012). In estuarine environments, the effect of pH on Cu speciation is relevant to carbonate complexation at relatively high salinities (De Polo and Scrimshaw, 2012). However, the mechanism of toxicity of $CuCO_{3(aq)}$ is still unclear (Wang *et al.*, 2012). The developed Cu-BLMs did not incorporate $CuOH^+$ bioavailability into the BLM framework. It should be noted that the toxicity of $CuCO_{3(aq)}$ can be incorporated into the BLM by allowing direct binding of these complexes to the BL sites (*i.e.* transport or toxic action sites). The incorporation of $CuCO_{3(aq)}$ into BLMs may be needed for specific environments. In the estuarine environment, pH and salinity are relatively high, and $CuCO_{3(aq)}$ is the main form of Cu.

## CONCLUSIONS

Toxicity assessment of heavy metals in tidal flat sediments is a complex scientific problem that needs to consider cationic competition (*e.g.*, $Ca^{2+}$, $Mg^{2+}$, $Na^+$, $K^+$, and $H^+$) for binding sites with free metal ions in the estuarine environment. In other words, the analysis must quantify metal bioavailability in site-specific tidal flat sediment solutions.

This study used a hydroponic solution system to simulate estuarine sediment pore water in salt marsh environments and to determine changes in the toxicity of Cu to *S. mariqueter* in response to alterations in pH and cationic concentration. The analysis used the effects of Cu on root elongation of the salt marsh plant *S. mariqueter* as a toxicity sensitivity index. Ultimately, a Cu-sBLM for *S. mariqueter* was developed and validated to provide accurate predictions of Cu toxicity. Experiment and simulation results show that (1) $Na^+$ can compete for binding sites with Cu on *S. mariqueter* roots, thus reducing Cu toxicity to the target plants; (2) Over the pH range examined in this study, there was a nonlinear relationship between EC50 and $H^+$ ion activity; at relatively high pH values, the increase of Cu toxicity to the target plant was associated with the change of Cu species in the medium; (3) For the simulated estuarine tidal flat environment, the results suggest that $Cu^{2+}$ and $CuCO_3$(aq) serve as toxic species that have an acute effect on *S. mariqueter*; (4) In the estuary salt marsh environment, the development of the BLMs for Cu should consider the toxicity effects of $Na^+$, $Cu^{2+}$, and $CuCO_3$(aq). Conditional binding constants were obtained for the binding of $Cu^{2+}$, $CuCO_{3(aq)}$, and $Na^+$ with biotic ligands where $\log K_{CuBL}$ is 6.60, $\log K_{CuCO_3BL}$ is 6.20, and $\log K_{NaBL}$ is 1.778.

This is the first report of a successfully developed BLM for a salt marsh plant found in a tidal wetland. The BLM method shows considerable potential for predicting Cu bioavailability in salt marsh sediments and can be widely applied to other salt marsh plants. It may even be used to develop environmental quality criteria that assess the risk associated with metal toxicity. The limitation of this model is that the developed Cu-BLM was based on plant nutrient solutions. Further validation experiments with sediment containing particulate organic carbon and other types of natural salt marsh sediments are needed before the Cu-BLM could be used for predicting Cu bioavailability in salt marsh sediments and for assessing the associated risk.

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