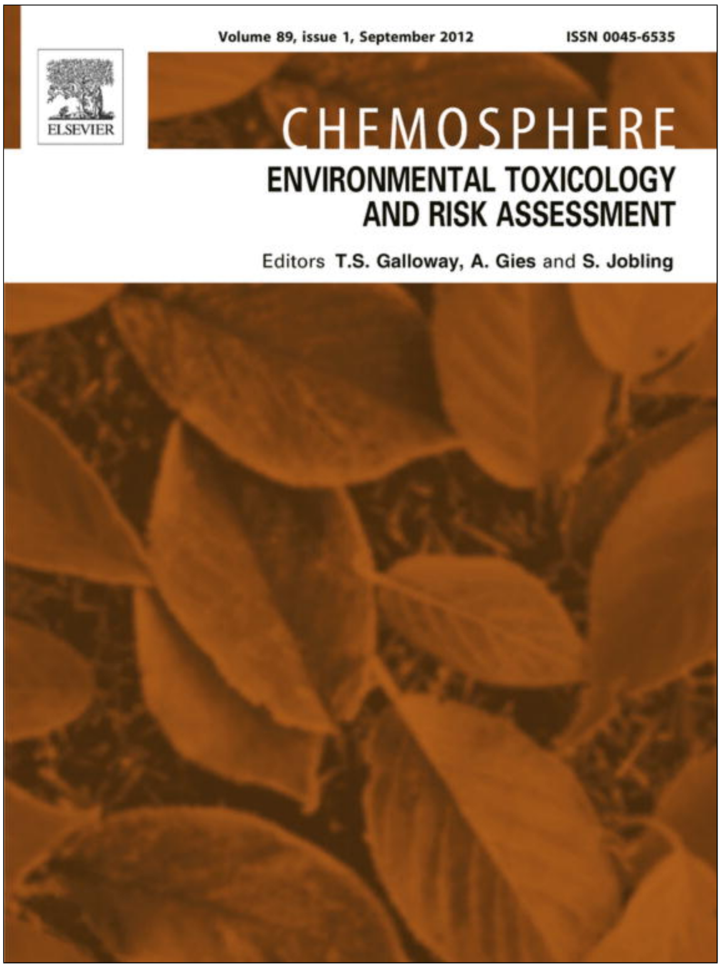
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# A biotic ligand model predicting acute copper toxicity for barley (*Hordeum vulgare*): Influence of calcium, magnesium, sodium, potassium and pH

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ABSTRACT

The effects of selected cations and pH on the acute toxicity of copper (Cu) to barley root elongation were investigated to develop an appropriate biotic ligand model (BLM). The results showed that increasing activities of $Mg^{2+}$ and $Ca^{2+}$, but not $Na^+$ and $K^+$, linearly increased the EC50 (as $Cu^{2+}$ activity). Unchanged EC50 at solution pH less than 6.5 and sharply decreased EC50 with increasing of solution pH when greater than 6.5 can be explained by toxicity of the $CuHCO_3^+$, $CuCO_3(aq)$ and $CuOH^+$ complexes. Conditional binding constants were obtained for the binding of $Cu^{2+}$, $CuHCO_3^+$, $CuCO_3(aq)$, $CuOH^+$, $Mg^{2+}$ and $Ca^{2+}$ with biotic ligand: $\log K_{CuBL}$ 6.33, $\log K_{CuHCO_3BL}$ 5.71, $\log K_{CuCO_3BL}$ 5.70, $\log K_{CuOHBL}$ 6.39, $\log K_{MgBL}$ 2.92 and $\log K_{CaBL}$ 1.96. Using the estimated constants, a BLM was successfully developed to predict Cu toxicity to barley root elongation as a function of solution characteristics.

## 1. Introduction

Copper (Cu) is an essential element for plants and animals, but when present in excess, it can exert toxic effects. Copper concentration may be elevated in soils because of (i) widespread use of pesticides, (ii) land application of sewage sludge and Cu-rich animal manures, and (iii) mining and smelting activities (Baker and Senft, 1995). Therefore, environmental quality criteria are developed for metals such as Cu. Current soil quality criteria and risk assessment of metals in soils are based on total or soluble metal concentrations. However, a large body of evidence indicates that both total and soluble metals are unrelated directly to ecotoxicity (Smolders et al., 2004). Toxicity is affected by the bioavailability of the metal in the soil and concentrations of other elements which moderate toxicity responses.

Recently, a biotic ligand model (BLM) has been proposed as a tool to evaluate quantitatively how water chemistry affects the speciation and biological availability of metals in aquatic systems (Di Toro et al., 2001). The most important assumption of the BLM is that metal toxicity is caused by free metal ions reacting with biological binding sites. The cations of $H^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$ might compete with metal ions for these binding sites and decrease the toxicity of the free metal ions. The BLM concept, developed originally for aquatic metal toxicity (Di Toro et al., 2001; De Schamphelaere and Janssen, 2002), has recently attracted increasing attention for predicting metal toxicity in terrestrial systems (Antunes et al., 2006; Thakali et al., 2006a,b; Lock et al., 2007a,b,c; Li et al., 2009; Wang et al., 2010). In most BLMs developed for metals, the linear relationship between $H^+$ and median effective concentration (EC50) suggests that competition exists between $H^+$ and free ions of the metals on binding sites of biotic ligands (Erickson et al., 1996; Meyer et al., 1999; Thakali et al., 2006a,b). However, it has been reported that $H^+$ activity had no significant effect on metal toxicity (De Schamphelaere and Janssen, 2002; Lock et al., 2007b,c), therefore, it was unjustified to incorporate $H^+$ competition into the BLM. For example, proton competition with $Cu^{2+}$ bound to biotic ligand (BL) was used to describe the effects of pH on Cu toxicity to barley in the terrestrial BLM for soils with pH $\leqslant 7$ (Thakali et al., 2006a,b), but it was unable to explain effects of pH on toxicity of Cu to barley in a wide range of pH (Lock et al., 2007a). The pH effects on toxicity of Cu to barley may be due to the change of species of Cu in solutions. In addition to $Cu^{2+}$, other inorganic species (such as $CuCO_3(aq)$ and $CuOH^+$) are probably toxic to organisms, especially in a medium with relatively high pH (Niyogi and Wood, 2004). The BLMs developed recently for both aquatic and terrestrial ecosystems assumed that the effect of pH on metal toxicity at relatively high pH was a speciation effect, but without any significant competition between protons and metal ions (De Schamphelaere and Janssen, 2002; Markich et al., 2003; Li et al., 2009; Wang et al., 2010). To our knowledge, no data are available to assess the possible toxicity of other inorganic species of Cu to plants in aquatic culture, except for $CuOH^+$ cation (Wang et al., 2009). The present study therefore aimed to investigate the

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effect of $H^+$ competition on the toxicity of $Cu^{2+}$ to barley root elongation across a wide range of pH values and to determine if other Cu species are implicated in toxicity responses. Further, the effects of $Ca^{2+}$, $Mg^{2+}$, $Na^+$ and $K^+$ on Cu toxicity to barley root elongation were determined across a wide range of ion levels in order to obtain conditional binding constants for $Cu^{2+}$ as well as other cations with BLs. Finally, a BLM was established that can be used to predict Cu toxicity to barley for a broad range of solution characteristics.

## 2. Materials and methods

### 2.1. Experimental design

To determine the effect of different cations on Cu toxicity, the concentration of one was varied, while keeping the concentration of all others as constant as possible (De Schamphelaere and Janssen, 2002; Lock et al., 2007a,b,c). Five sets of Cu toxicity tests were performed: Ca-set, Mg-set, Na-set, K-set and pH-set (Table 1). Each set consisted of a series of media in which only the concentrations of considered cation varied, while $CaCl_2$ was kept at 0.2 mM as background electrolyte. There were six Cu concentrations plus one treatment without added Cu as a control for all series. The test concentrations of Cu in solution ranged from 0 to 6.30 μM. The selected cation concentrations were based on the ranges that occur in natural pore waters (Oorts et al., 2006). All treatments were done in triplicate.

### 2.2. Preparation of the solutions

The chemicals used were all analytical reagent (AR) or higher grade, and deionized water was used throughout the experiment. Test media (the basal growth solutions) were prepared by adding different volumes of stock solutions of $CaCl_2$, $MgSO_4$, NaCl and KCl to deionized water. Buffering with 1 mM MES (2-[*N*-morpholino] ethane sulfonic acid) was used for pH < 7.0 treatments and with 3.6 mM MOPS (3-[*N*-morpholino] propane sulfonic acid) for pH ⩾ 7.0 treatments because MES and MOPS do not form complexes with Cu (De Schamphelaere et al., 2004; Lock et al., 2007a). The pH was adjusted to the desired value with 1 M NaOH or 1 M HCl. Except for the pH-sets, the pH values in the media were always adjusted using MES. The test medium prepared for each bioassay was then used to set up a Cu concentration series (added as $CuCl_2$). The chemical characteristics of the different test media are summarized in Table 1.

### 2.3. Toxicity assays

The barley root elongation test was performed according to ISO 11269-1 (ISO, 1993). Barley seeds (*H. vulgare* cv. Pinggu No. 1) were germinated at 20 °C in the dark for 24 h on filter-paper moistened with deionized water. When the radicle emerged (<2 mm in length), six seeds were transferred to nylon net fixed on the surface of plastic culture pots containing 350 mL of the test solution, which was changed every 24 h to maintain the composition. Solutions were not stirred or agitated because earlier studies of Cu toxicity to barley at pH 6.0 found no difference between unstirred and stirred systems (Wang et al., 2009). The culture pots were placed randomly in the growth chamber. The air temperature was maintained at 20 °C during the 16 h light (22 klux)/8 h dark cycles. Root length was measured after 5 d and elongation (RE, %) was calculated as percentage of the control:

$$\mathrm{RE} = \frac{\mathrm{REt}}{\mathrm{REc}} \times 100 \tag{1}$$

where REt is the root length in the test medium and REc is the root length in the control.

### 2.4. Chemical measurements

Atomic absorption spectrophotometry (Varian AA240FS/GTA120; Melbourne, Australia) was used to measure the concentration of Cu, Ca, Mg, Na and K. The pH of the solutions was determined with a pH meter (Delta 320; Mettler, Zurich, Switzerland).

### 2.5. Prediction of Cu speciation

Speciation was calculated by WHAM 6.0 (Windermere Humic Aqueous Model) (Lofts and Tipping, 2002). Input data for WHAM were pH and the concentrations of Cu, Ca, Mg, K, Na, Cl and $SO_4$. As 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 of WHAM (Lofts and Tipping, 2002).

### 2.6. Mathematical description of the BLM

Based on the BLM assumption, when the competing cations $H^+$, $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$ are considered, the fraction (*f*) of the total biotic ligand sites bound by $Cu^{2+}$ is given by following equation (De Schamphelaere and Janssen, 2002):

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

where $K_{\mathrm{CuBL}}$ and $K_{\mathrm{XBL}}$ are conditional binding constant for the binding of Cu and cation X (e.g., $Ca^{2+}$, $Mg^{2+}$, $K^+$ or $H^+$) to the BL sites (M), respectively, and curly brackets $\{\cdot\}$ indicate ion activity, such as $\{X^{n+}\}$, which is the activity of $X^{n+}$ (M). {XBL} is the concentration of the specific cation–BL complex (M).

According to the methodology described in detail by Pagenkopf (1983) and De Schamphelaere and Janssen (2002), when inhibition of barley root elongation is up to 50% of the control, Eq. (2) becomes:

$$\mathrm{EC50}\{\mathrm{Cu}^{2+}\} = \frac{f_{\mathrm{CuBL}}^{50\%}}{(1 - f_{\mathrm{CuBL}}^{50\%})K_{\mathrm{CuBL}}}\left(1 + \sum K_{\mathrm{XBL}}\{X^{n+}\}\right) \tag{3}$$

where $\mathrm{EC50}\{\mathrm{Cu}^{2+}\}$ is the free $Cu^{2+}$ that results in 50% RE (50% of barley root elongation with respect to the control) and $f_{\mathrm{CuBL}}^{50\%}$ is the fraction of the BLs that results in 50% RE when occupied by Cu.The barley root elongation is correlated to the fraction of the BLs ($f_{\mathrm{CuBL}}$) and follows the log–logistic dose–response relationship according to Thakali et al. (2006a).

**Table 1**
Composition of the test media used in the various bioassay sets and the observed $\mathrm{EC50}\{\mathrm{Cu}^{2+}\}$ for barley root elongation.

| Bioassay set | Varied concentrations and pH values | Characteristics of background solutions | Observed series of $\mathrm{EC50}\{\mathrm{Cu}^{2+}\}$ |
|---|---|---|---|
| Ca | 0.2, 1, 2, 5, 7, 10, 15 mM | 0.05 mM Mg, 2.5 mM Na, 0.08 mM K, pH 6.00 | 0.50, 0.63, 0.67, 0.60, 0.63, 0.76, 0.73 μM |
| Mg | 0.05, 0.2, 0.5, 1, 2, 3 mM | 0.2 mM Ca, 2.5 mM Na, 0.08 mM K, pH 6.00 | 0.38, 0.43, 0.55, 0.72, 0.89, 1.00 μM |
| K | 0.1, 1, 2, 3, 5, 7.5, 10 mM | 0.2 mM Ca, 0.05 mM Mg, 2.5 mM Na, pH 6.00 | 0.41, 0.38, 0.38, 0.38, 0.46, 0.43, 0.53 μM |
| Na | 2.5, 5.5, 10.5, 15.5, 20.5, 25.5 mM | 0.2 mM Ca, 0.05 mM Mg, 0.08 mM K, pH 6.00 | 0.48, 0.45, 0.50, 0.51, 0.56, 0.57 μM |
| pH | 4.5, 5, 6, 6.5, 7, 7.5, 7.8, 8 | 0.2 mM Ca, 0.05 mM Mg, 2.5 mM Na, 0.08 mM K | 0.45, 0.44, 0.41, 0.47, 0.49, 0.35, 0.17, 0.10, 0.05 μM |

$$\mathrm{RE} = \frac{100}{1 + \left(\frac{f_{\mathrm{CuBL}}}{f^{50\%}_{\mathrm{CuBL}}}\right)^{\beta}} \tag{4}$$

where $\beta$ is the shape parameter. Substituting $f$ from Eq. (2) in Eq. (4) yields:

$$\mathrm{RE} = \frac{100}{1 + \left(\frac{K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\}}{f^{50\%}_{\mathrm{CuBL}}\left(1+K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\}+K_{\mathrm{XBL}}\{X^{n+}\}\right)}\right)^{\beta}} \tag{5}$$

Eq. (5) provides the mathematical basis for the BLM that explicitly relates the biological response to the chemistry of the solution. An additional model based on the log–logistic relationship Eq. (4) was also fitted to the same dataset for comparison to the BLM. The model is the free ion activity model (FIAM) and uses the computed $\mathrm{Cu}^{2+}$ activities as the dose. The dose–response curves are plotted in terms of free $\mathrm{Cu}^{2+}$ activity (FIAM) and the fraction ($f$) of the barley root sites bound by toxic Cu species (BLM) by fitting a logistic model. The fitting parameters are conditional binding constants of all cations to BL ($K_{\mathrm{XBL}}$), $f^{50\%}_{\mathrm{CuBL}}$ and $\beta$ for BLM and EC50$\{\mathrm{Cu}^{2+}\}$ and $\beta$ for FIAM. When comparing different models, the lower value of the root-mean-square error (*RMSE*) is indicative of a better model.

The parameters of models were estimated by minimizing the *RMSE* of the predicted root elongation using Data Processing System 9.0 (DPS 9.0). DPS is a statistics and analytics software package developed by Tang and Feng (2007). It is widely used by many researchers and students in agriculture, entomology and other biological fields.

## 3. Results

### 3.1. Copper species distribution in different pHs

Fig. 1 shows the distribution of Cu species in solutions with pH increasing from 4.5 to 8.0. Free $\mathrm{Cu}^{2+}$ was the most dominant Cu species at pH $\leqslant$ 6.5; With increasing pH, the proportions of $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$ and $\mathrm{CuOH^+}$ increased continuously concomitant with the decreasing proportion of $\mathrm{Cu}^{2+}$. At pH greater than 7.5, the dominant Cu species become $\mathrm{CuHCO_3^+}$ and $\mathrm{CuCO_3}$, which were 84% of total Cu at solution pH 8.0. The fourth most abundant Cu species, $\mathrm{CuOH^+}$, increased to 7.3% of total Cu at pH 7.5. Meanwhile, other Cu species, such as $\mathrm{Cu(OH)_2(aq)}$, were always quite low (<0.2% of total Cu) and not considered for BLM development. Hence, the four main Cu species, $\mathrm{Cu}^{2+}$, $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$, and $\mathrm{CuOH^+}$ were considered for toxicity to barley root elongation.

### 3.2. Effects of cations on Cu toxicity

A summary of the effective concentration (EC50s) expressed as free $\mathrm{Cu}^{2+}$ activity is given in Table 1. It was found that the measured EC50$\{\mathrm{Cu}^{2+}\}$ values ranged from 0.05 μM to 1 μM for all treatments (20-fold difference), which clearly demonstrated the limitation of using free $\mathrm{Cu}^{2+}$ activity alone to predict Cu toxicity as done by FIAM. However, a part of the difference can be explained by the positive relationship between EC50$\{\mathrm{Cu}^{2+}\}$ and $\mathrm{Mg}^{2+}$ and $\mathrm{Ca}^{2+}$ activity. An increase of the $\mathrm{Mg}^{2+}$ activity from 0.04 mM to 1.65 mM resulted in the reduction of EC50$\{\mathrm{Cu}^{2+}\}$ by a factor of 2.6. A linear relationship ($p < 0.01$, $R^2 = 0.97$) was found between the $\mathrm{Mg}^{2+}$ activity and EC50$\{\mathrm{Cu}^{2+}\}$ (Fig. 2A and Table 1). Addition of Ca also resulted in an increase of EC50$\{\mathrm{Cu}^{2+}\}$. The values of EC50$\{\mathrm{Cu}^{2+}\}$ increased linearly up to 1.53-fold with an increase of $\mathrm{Ca}^{2+}$ activity from 0.18 mM to 7.48 mM ($p < 0.05$, $R^2 = 0.68$, Table 1).

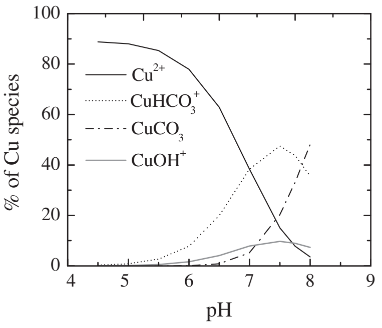

**Fig. 1.** Calculated Cu species (% distribution) in pH-set.

This result indicated that $\mathrm{Ca}^{2+}$ and $\mathrm{Mg}^{2+}$ can compete with $\mathrm{Cu}^{2+}$ for barley root binding sites and decreased Cu toxicity. However, no significant change in the EC50$\{\mathrm{Cu}^{2+}\}$ was found when the activity of K and Na varied (Table 1). Therefore, competition between $\mathrm{K}^+$, $\mathrm{Na}^+$ and $\mathrm{Cu}^{2+}$ for binding sites on barley roots could be neglected when BLM was developed, and the values of $\log K_{\mathrm{KBL}}$ and $\log K_{\mathrm{NaBL}}$ could be set to approximately zero.

Increasing pH from 4.5 to 8.0 resulted in an increase in Cu toxicity expressed based on the free $\mathrm{Cu}^{2+}$ activity by a factor of 9 (Table 1). According to Eq. (3), if $\mathrm{H}^+$ can compete with $\mathrm{Cu}^{2+}$ binding sites of barley root, then a linear relationship between EC50$\{\mathrm{Cu}^{2+}\}$ and $\mathrm{H}^+$ activity should exist in the pH-set. However, there was a non-linear relationship between EC50$\{\mathrm{Cu}^{2+}\}$ and $\mathrm{H}^+$ activity in culture solution over the whole range of pH examined. Apparently, the relationship between EC50$\{\mathrm{Cu}^{2+}\}$ and $\mathrm{H}^+$ activity could be described by two change trend (Fig. 2B). When pH $\leqslant$ 6.5, the values of EC50$\{\mathrm{Cu}^{2+}\}$ were not significantly different ($p > 0.05$). This means that there was not any significant competition effect between $\mathrm{H}^+$ and $\mathrm{Cu}^{2+}$ for barley root binding sites in the test range of pH 4.5–6.5. When the pH increased from 7.0 to 8.0, the values of EC50$\{\mathrm{Cu}^{2+}\}$ decreased sharply with increasing solution pH (Table 1 and Fig. 2), which could not be explained by $\mathrm{H}^+$ competition because of the low $\mathrm{H}^+$ activity and may be explained by the change of Cu species in the solutions. From Cu species distribution, it was known that the percentages of $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$ and $\mathrm{CuOH^+}$ to total Cu in solution increased with an increase of pH (Fig. 1). In order to determine if $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$ and $\mathrm{CuOH^+}$ could be used to explain the effects of pH on Cu toxicity to barley root elongation when pH > 6.5, Eq. (3) was transformed to Eq. (6) when $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$ and $\mathrm{CuOH^+}$ were considered as toxic species as well as $\mathrm{Cu}^{2+}$ in the pH-set:

$$\begin{aligned}\frac{1}{\mathrm{EC50}\{\mathrm{Cu}^{2+}\}} &= \frac{1-f^{50\%}_{\mathrm{CuBL}}}{f^{50\%}_{\mathrm{CuBL}}\left(1+\sum K_{\mathrm{XBL}}\{X^{n+}\}\right)} \\ &\quad \times \left(K_{\mathrm{CuBL}} + K_{\mathrm{CuHCO_3BL}}K_{\mathrm{CuHCO_3^+}}\{\mathrm{HCO_3^-}\}\right. \\ &\quad \left. + K_{\mathrm{CuCO_3BL}}K_{\mathrm{CuCO_3}}\left\{\mathrm{CO_3^{2-}}\right\} + K_{\mathrm{CuOHBL}}K_{\mathrm{CuOH^+}}\{\mathrm{OH^-}\}\right)\end{aligned} \tag{6}$$

where $K_{\mathrm{CuHCO_3}}$, $K_{\mathrm{CuCO_3}}$ and $K_{\mathrm{CuOH}}$ are stability constants for the formation of the $\mathrm{CuHCO_3^+}$, $\mathrm{CuCO_3(aq)}$ and $\mathrm{CuOH^+}$ complexes, respectively (M). Based on equilibrium equations of $\mathrm{Cu^{2+} + H^+ + CO_3^{2-} = CuHCO_3^+}$, $\mathrm{Cu + CO_3^{2-} = CuCO_3(aq)}$ and $\mathrm{Cu^{2+} + OH^- = CuOH^+}$, Eq. (6) could be transformed to Eq. (7):

$$\begin{aligned}\frac{1}{\mathrm{EC50}\{\mathrm{Cu}^{2+}\}} &= \frac{1-f^{50\%}_{\mathrm{CuBL}}}{f^{50\%}_{\mathrm{CuBL}}\left(1+\sum K_{\mathrm{XBL}}\{X^{n+}\}\right)}\left(K_{\mathrm{CuBL}} + K_{\mathrm{CuHCO_3BL}}\frac{\{\mathrm{CuHCO_3^+}\}}{\{\mathrm{Cu}^{2+}\}}\right. \\ &\quad \left. + K_{\mathrm{CuCO_3BL}}\frac{\{\mathrm{CuCO_3}\}}{\{\mathrm{Cu}^{2+}\}} + K_{\mathrm{CuOHBL}}\frac{\{\mathrm{CuOH^+}\}}{\{\mathrm{Cu}^{2+}\}}\right)\end{aligned} \tag{7}$$

Eq. (7) can be transformed to a linear equation with 1/EC50$\{\mathrm{Cu}^{2+}\}$ as a dependent variable as well as $\mathrm{CuHCO_3^+/Cu^{2+}}$, $\mathrm{CuCO_3(aq)/Cu^{2+}}$ and $\mathrm{CuOH^+/Cu^{2+}}$ as independent variables. This trans-

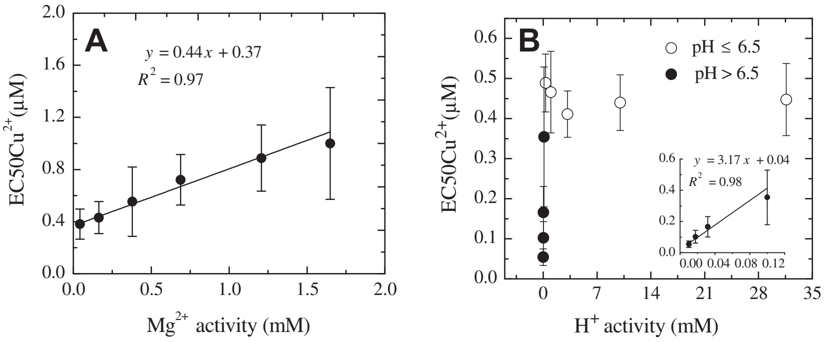

**Fig. 2.** The EC50 values expressed as free $Cu^{2+}$ activity for barley root elongation as a function of the activity of $Mg^{2+}$ (A) and $H^+$ (B). Error bars indicate 95% confidence intervals. Significant correlations are represented by a solid line.

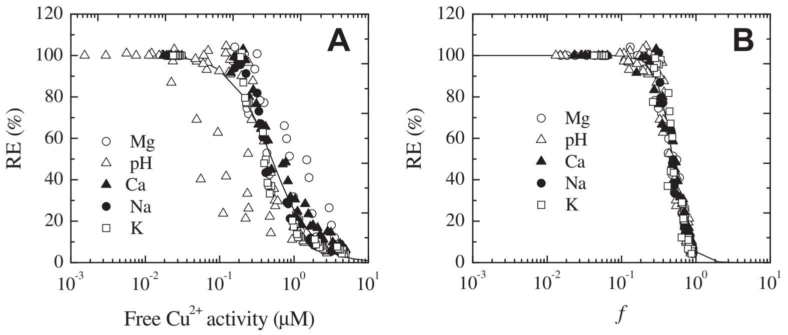

**Fig. 3.** Toxicity of Cu to barley root elongation expressed as different dose–response curves: the dose as only $Cu^{2+}$ activity (A) and the fraction (*f*) of the total biotic ligand sites occupied by toxic $Cu^{2+}$, $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ (B). The lines are the fitted logistic curves based on all sets.

formation is in accordance with not allowing proton competition in the calculations, since proton competition would not be significant at pH greater than 6.5 (Li et al., 2009). Then, the multiple linear regression between 1/EC50{$Cu^{2+}$} and $CuHCO_3^+/Cu^{2+}$, $CuCO_3(aq)/Cu^{2+}$ as well as $CuOH^+/Cu^{2+}$ was calculated as:

$$\frac{1}{\mathrm{EC50}\{\mathrm{Cu}^{2+}\}} = 2.098(\pm 0.141) + 0.496(\pm 0.058)\frac{\{\mathrm{CuHCO}_3^+\}}{\{\mathrm{Cu}^{2+}\}} + 0.476(\pm 0.09)\frac{\{\mathrm{CuCO}_3\}}{\{\mathrm{Cu}^{2+}\}} + 2.419(\pm 0.281)\frac{\{\mathrm{CuOH}^+\}}{\{\mathrm{Cu}^{2+}\}} \quad (8)$$

$K_{\mathrm{CuHCO_3BL}}/K_{\mathrm{CuBL}} = 0.24$, $K_{\mathrm{CuCO_3BL}}/K_{\mathrm{CuBL}} = 0.23$, $K_{\mathrm{CuOHBL}}/K_{\mathrm{CuBL}} = 1.15$, which from a mechanistic point of view, means that $CuHCO_3^+$ and $CuCO_3$(aq) are less toxic, but $CuOH^+$ is more slightly toxic than $Cu^{2+}$.

### 3.3. Estimation of BLM parameters

When toxicity of $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ was considered, Eq. (4) could be transformed to Eq. (9) (De Schamphelaere and Janssen, 2002):

$$f = \frac{K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\} + K_{\mathrm{CuHCO_3BL}}\{\mathrm{CuHCO}_3^+\} + K_{\mathrm{CuCO_3BL}}\{\mathrm{CuCO}_3\} + K_{\mathrm{CuOHBL}}\{\mathrm{OH}^-\}}{1 + K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\} + K_{\mathrm{CuHCO_3BL}}\{\mathrm{CuHCO}_3^+\} + K_{\mathrm{CuCO_3BL}}\{\mathrm{CuCO}_3\} + K_{\mathrm{CuOHBL}}\{\mathrm{OH}^-\} + \sum K_{\mathrm{XBL}}\{X^{n+}\}} \quad (9)$$

and then barley root elongation can be written as:

$$\mathrm{RE} = \frac{100}{1 + \left(\frac{K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\}+K_{\mathrm{CuHCO_3BL}}\{\mathrm{CuHCO}_3^+\}+K_{\mathrm{CuCO_3BL}}\{\mathrm{CuCO}_3\}+K_{\mathrm{CuOHBL}}\{\mathrm{CuOH}^+\}}{1+K_{\mathrm{CuBL}}\{\mathrm{Cu}^{2+}\}+K_{\mathrm{CuHCO_3BL}}\{\mathrm{CuHCO}_3^+\}+K_{\mathrm{CuCO_3BL}}\{\mathrm{CuCO}_3\}+K_{\mathrm{CuOHBL}}\{\mathrm{CuOH}^+\}+K_{\mathrm{CaBL}}\{\mathrm{Ca}^{2+}\}+K_{\mathrm{MgBL}}\{\mathrm{Mg}^{2+}\}}\right)^{\beta}} \quad (10)$$

In Eq. (8), the intercept, coefficient of $CuHCO_3^+/Cu^{2+}$, $CuCO_3(aq)/Cu^{2+}$ and $CuOH^+/Cu^{2+}$ were significant at $p < 0.01$ level, which demonstrated that the Cu toxicity to barely root elongation could be caused by $Cu^{2+}$ plus $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ when they exists in solution at certain pH values. Furthermore, according to Eqs. (7) and (8), the ratio of conditional binding constants can be obtained for $CuHCO_3^+$, $CuCO_3$(aq), $CuOH^+$ and $Cu^{2+}$ at BL:

From Eq. (10), barley root elongation was affected by {$Cu^{2+}$}, {$CuHCO_3^+$}, $CuCO_3$(aq), $CuOH^+$, {$Ca^{2+}$}, {$Mg^{2+}$} where $Na^+$ and {$K^+$} were omitted from Eq. (10) because its effects on Cu toxicity were insignificant in the present study. Based on Eq. (10) and using the ratio of conditional binding constants of $CuHCO_3^+$, $CuCO_3$(aq), $CuOH^+$ and $Cu^{2+}$ at BL, the parameters $K_{\mathrm{CuBL}}$, $K_{\mathrm{CuHCO_3BL}}/K_{\mathrm{CuBL}} = 0.24$, $K_{\mathrm{CuCO_3BL}}$, $K_{\mathrm{CuOHBL}}$, $K_{\mathrm{CaBL}}$, $K_{\mathrm{MgBL}}$, $f_{\mathrm{CuBL}}^{50\%}$ and $\beta$ can be obtained by

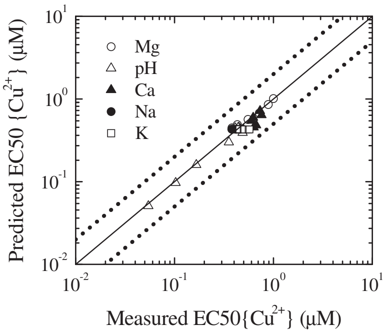

**Fig. 4.** Relationship between the measured and predicted EC50{$Cu^{2+}$} based on the BLM developed in the present study. The solid line indicates a perfect match between measured and predicted EC50{$Cu^{2+}$} values, and the dashed lines indicate the range of a factor of 1.5 between observed and predicted EC50{$Cu^{2+}$} values.

data fitting until the predicted RE (% of control) with minimal *RMSE* and maximal $R^2$ for all sets using DPS statistic software. The conditional binding constants were obtained as follows: $\log K_{\mathrm{CuBL}}$ 6.33, $\log K_{\mathrm{CuHCO_3BL}}/K_{\mathrm{CuBL}} = 0.24$ 5.71, $\log K_{\mathrm{CuCO_3BL}}$ 5.70, $\log K_{\mathrm{CuOHBL}}$ 6.39, $\log K_{\mathrm{CaBL}}$ 1.96 and $\log K_{\mathrm{MgBL}}$ 2.92. These results indicated that the toxicity across the wide range of pH and concentrations of cations was closely related to the activities of $Cu^{2+}$, $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ as well as the competition with $Ca^{2+}$ and $Mg^{2+}$ for barley root binding sites, which should be incorporated in the Cu-BLM. So, the dose–response curves were plotted in terms of *f* (fraction of the total barley root sites occupied by toxic $Cu^{2+}$, $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ species) based on the BLM, and in terms of free $Cu^{2+}$ activity based on FIAM (Fig. 3). When considering the influences of $Cu^{2+}$, $CuHCO_3^+$, $CuCO_3$(aq), $CuOH^+$, $Ca^{2+}$ and $Mg^{2+}$, the BLM was better able to predict Cu toxicity than the FIAM based on *RMSE* and $R^2$ values. The *RMSE* decreased from 13.7 for FIAM to 6.8 for BLM and the $R^2$ value increased from 0.87 for the FIAM to 0.97 for the BLM. The results indicated that BLM could predict barley root elongation much better than FIAM when $Cu^{2+}$ plus $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ as toxic species and the competition of $Ca^{2+}$ and $Mg^{2+}$ with the binding sites of barley root were incorporated into the BLM.

### 3.4. Validation of BLM

Finally, the derived Cu-BLM was used to predict EC50s for the media in this study. The predicted equation of EC50{$Cu^{2+}$} can be expressed as Eq. (11) based on Eq. (3):

$$\mathrm{EC50}\{\mathrm{Cu}^{2+}\} = \frac{f^{50\%}_{\mathrm{CuBL}}\left(1 + K_{\mathrm{MgBL}}\{\mathrm{Mg}^{2+}\}\right)}{(1 - f^{50\%}_{\mathrm{CuBL}})(K_{\mathrm{CuBL}} + K_{\mathrm{CuHCO_3BL}}K_{\mathrm{CuHCO3}}\{\mathrm{HCO}_3^-\} + K_{\mathrm{CuCO_3BL}}K_{\mathrm{CuCO3}}\left\{\mathrm{CO}_3^{2-}\right\} + K_{\mathrm{CuOHBL}}K_{\mathrm{CuOH}}\{\mathrm{OH}^-\}} \tag{11}$$

Using the solution activities of $Ca^{2+}$, $Mg^{2+}$, $CO_3^{2-}$, $HCO_3^-$, $OH^-$ and the conditional binding constants in Eq. (11), EC50{$Cu^{2+}$} can be predicted. The predicted EC50s differed from the measured EC50s by less than a factor of 2 in the present study (Fig. 4), indicating that the BLM can be used to predict Cu toxicity to barley root elongation.

## 4. Discussion

Though in most BLMs developed so far, cations such as $Ca^{2+}$ and $Mg^{2+}$ clearly affected Cu toxicity (Erickson et al., 1996; Meyer et al., 1999; De Schamphelaere and Janssen, 2002; Heijerick et al., 2002; De Schamphelaere et al., 2004), sometimes the measured effects varied. Cheng and Allen (2001) found that Ca and H inhibited rhizotoxicity of Cu to lettuce (*Latuca sativa*) in nutrient solution. Thakali et al. (2006a) developed a terrestrial BLM using European noncalcareous soils and barley root elongation bioassay, and showed that only $H^+$ could decrease $Cu^{2+}$ toxicity significantly. For wheat (*Triticum aestivum*), Mg and Ca had a protected effect against Cu toxicity (Parker et al., 1998; Kinraide et al., 2004; Luo et al., 2008). However, $Ca^{2+}$ and $Mg^{2+}$ had no effect on the Cu toxicity to sugar beet (*Beta vulgaris*) (Saleh et al., 1999). The above results suggest that competition for binding sites between toxic metal ions and cations depends on the plant species and toxicants tested. In our study, protective effects of $Ca^{2+}$ and $Mg^{2+}$ on $Cu^{2+}$ toxicity to barley was found, similar to the results reported by Parker et al. (1998), Kinraide et al. (2004) and Luo et al. (2008). Although the effect of Na on copper toxicity was observed for *Daphnia magna* (De Schamphelaere and Janssen, 2002) and *fathead minnow* (Erickson et al., 1996) for aquatic ecosystems, but for most metals, the activity of $Na^+$ has minor effects on toxicity to terrestrial plants (Lock et al., 2007a,c; Luo et al., 2008; Li et al., 2009; Wang et al., 2010). The $K^+$ activity did not affect the EC50 for barley exposed to $Ni^{2+}$ (Li et al., 2009) and $Cu^{2+}$ (Lock et al., 2007a,b,c) and in the development of most other BLMs, it is assumed that $K^+$ does not affect metal toxicity. In the present study, $Na^+$ and $K^+$ did not affect $Cu^{2+}$ toxicity to barley root elongation.

There is little doubt that free metal ions are the dominant form available to biota; however, this does not imply other inorganic species of metals are non-toxic. When BLMs for freshwater organisms were developed, the toxicity of $CuOH^+$, $CuCO_3$(aq) and AgCl(aq) to freshwater organisms were supposed (Niyogi and Wood, 2004). For example, De Schamphelaere and Janssen (2002) studied the acute Cu toxicity for *Daphnia magna* in the pH range 5.98–7.92 and found that the relation between $H^+$ and EC50{$Cu^{2+}$} should rather be explained in terms of toxicity of $Cu^{2+}$ plus $CuOH^+$ than in terms proton competition. For terrestrial plants, inorganic species of metals such as $ZnHCO_3^+$, $NiHCO_3^+$ were found to be toxic to barley root elongation at relatively high pH range in solution culture (Li et al., 2009; Wang et al., 2010). In the present study, the regression coefficient ($R^2$) between the measured and the predicted EC50{$Cu^{2+}$} values was as high as 0.97 when $Cu^{2+}$ plus $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ as toxic species and the competition by $Ca^{2+}$ and $Mg^{2+}$ with the binding sites of BL were incorporated into the BLM. It should be recognized that incorporating the toxicity of $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ by allowing direct reacting with biological BLs may be an over-simplification of the real mechanisms taking place at the organism–water interface. There are some other possible mechanisms that need to be considered, such as the lability of metal complexes and differences between the physico-chemistry of the bulk solution and that of the organism's micro-environment (Ryan et al., 2009). However, as long as these suggestions are only indicative of possible processes, bioavailability models will always tend to over-simplify.

The conditional binding constants derived in this study for barley root elongation (5 d EC50) of *Hordeum vulgare*, can be compared with those reported in Cu-tBLM (Thakali et al., 2006a,b) for root growth inhibition (*H. vulgare*, 4 d EC50) of barley, and for root growth inhibition of wheat (*T. aestivum*, 2 d EC50). The value of

$\log K_{CuBL}$ (6.33) in the present study was found to be lower than that ($\log K_{CuBL} = 7.41$) reported by Thakali et al. (2006a) in soil solutions, whereas it was similar to that ($\log K_{CuBL} = 6.25$) reported by Luo et al. (2008) in culture solutions. The binding constants $\log K_{CaBL}$ (1.96) and $\log K_{MgBL}$ (2.92) in the present study are also similar to the results ($\log K_{CaBL} = 2.43$, $\log K_{MgBL} = 3.34$) of Luo et al. (2008). Differences in binding constants may, for example, result from different exposure duration, endpoint, target tissue or BL, or mechanisms of Cu uptake and/or toxicity (Lock et al., 2006). Clearly more research with Cu is necessary to determine and explain differences and similarities across organisms, endpoints and exposure duration.

The present study indicates the inorganic complexes of $CuHCO_3^+$ and $CuCO_3$(aq) have 4-fold lower binding affinity than $Cu^{2+}$, it is similar to the results of Niyogi and Wood (2004), who reported that the affinities of inorganic complexes for the BL are approximately 4–10-fold lower relative to $Cu^{2+}$. However, in the present study, the affinity of $CuOH^+$ is a little higher than $Cu^{2+}$. It has been found that $CuOH^+$ is about 1.3-fold more toxic than $Cu^{2+}$ in the study of Ryan et al. (2009), which is same as the result in the present study. Similarly, higher affinity of monohydroxylated metal ions for adsorption on soils and soil components than hydrated metal ions has been suggested (James and Healy, 1972). Actually, the hydrated radius of $CuOH^+$ is much smaller than that of hydrated $Cu^{2+}$, which may result in $CuOH^+$ being easier to move or bind to ligands with higher binding constant. However, the binding of inorganic Cu complexes with BL needs further investigation and direct evidence.

The EC50{$Cu^{2+}$} values varied by a factor of almost 20, which clearly indicates the FIAM-which does not incorporate competition between metal ions and cations and bioavailability of $CuHCO_3^+$, $CuCO_3$(aq) and $CuOH^+$ -was not able to describe the obtained results. The BLM developed in this study could predict EC50s accurately (difference of factor of two), indicating it can be used to predict metal toxicity to terrestrial plants. However, our study was based on nutrient solutions but other factors, such as metal solid–liquid distribution, microbial activity and root exudates, which accumulate in the rhizosphere affect Cu toxicity to plants (Antunes et al., 2006). So, the application of our results is limited to soil solution. When this result applied to soils, the above processes should be considered. Thus, such a semi-mechanistic model needs for further refinement when applied to soils.

## 5. Conclusions

In this study, a BLM was developed that can accurately predicting the toxicity of Cu to barley (*H. vulgare*) in nutrient solutions, which indicated that modeling Cu toxicity to terrestrial plants by BLM is promising. However, further refinement with various natural/field soils with broad ranges of properties is necessary before BLMs can be used for risk assessments of metal-contaminated soils. The BLM, therefore, may initiate a promising tool for improving the ecological relevancy of risk assessment procedures for metals in soils.

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