



Influence of calcium, magnesium, sodium, potassium and pH on copper toxicity to barley (*Hordeum vulgare*)

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Abstract

The extent to which Ca^{2+} , Mg^{2+} , Na^+ , K^+ ions and pH independently influence copper toxicity to barley (*Hordeum vulgare*) was assessed by measuring root growth in nutrient solutions. Increased Ca^{2+} activity resulted in a sixfold decrease in $\text{EC}_{50_{\text{Cu}^{2+}}}$ values, while a positive relationship between the cation activity and the EC_{50} was expected. Increased Mg^{2+} activity resulted in a twofold increase in $\text{EC}_{50_{\text{Cu}^{2+}}}$ values. Na^+ , K^+ and H^+ activities did not significantly affect Cu^{2+} toxicity. The obtained results indicated that competition for binding sites between Cu^{2+} and cations such as Ca^{2+} , Mg^{2+} , Na^+ , K^+ and H^+ is not an important factor in determining Cu^{2+} toxicity to *H. vulgare*. However, the EC_{50} s could, with one exception, be predicted within a factor three based on the free Cu^{2+} activity, indicating that the free Cu^{2+} activity cannot only be used to predict metal toxicity to aquatic, but also to terrestrial organisms.

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1. Introduction

Copper has many industrial uses including wires and cables for transmission of electricity, water pipes and tubing, roofing and facing materials on buildings and linings in brakes of vehicles, all of which results in distribution of copper into the environment. Copper is also used as a constituent of some fungicides used in vineyards. Metals such as copper may cause environmental risks when occurring at elevated concentrations. Therefore, environmental quality criteria and standards are developed. Recently it has been recognized that standard procedures for deriving environmental quality criteria for chemical substances are inadequate for metals because they are not able to predict the potential impact on ecosystems (Fairbrother et al., 1999; Janssen et al., 2000). This is caused by the fact that current environmental quality criteria and risk assessment procedures for metals are predominantly based on total metal concentrations. However, there is increasing evidence that total metal concen-

trations do not predict metal bioavailability and toxicity very well.

To account for the observed variability in total copper toxicity, many authors have suggested that free copper ion activity is—at least in aquatic environment—a better predictor of biological response (e.g. reproduction, growth, uptake) than total copper concentration (review by Campbell, 1995). However, a considerable number of authors have concluded that free copper ion activity does not always accurately predict copper toxicity due to competition effects by other cations such as Ca, Mg and Na or bioavailability of inorganic and organic copper complexes (e.g. Pagenkopf, 1983; Blust et al., 1991; Campbell, 1995; De Schamphelaere and Janssen, 2002).

The Biotic Ligand Model concept (BLM) (Di Toro et al., 2001; De Schamphelaere and Janssen, 2002) is now considered to be the state-of-the-science metal bioavailability model/concept, because it incorporates both metal complexation and speciation in the solution surrounding the organism and interactions between metal ions and competing cations at binding sites on the organism–water interface. The main assumption of the BLM is that metal toxicity occurs as a result of free metal ions (or other

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reactive metal species) reacting with binding sites at the organism–water interface. These binding sites can be either physiologically active sites, causing a direct biological response, or transport sites, causing metal transport into the cell which is followed by a subsequent, indirect biological response. This interaction is represented as the formation of a metal–biotic ligand complex (Di Toro et al., 2001; Santore et al., 2001). The concentration of this metal–biotic ligand complex determines the magnitude of the toxic effect, independent of physical and chemical water characteristics of the test medium. Ca^{2+} , Mg^{2+} , Na^+ , K^+ and H^+ ions are able to compete with copper ions for binding sites at the organism–water interface (Pagenkopf, 1983; Di Toro et al., 2001; Santore et al., 2001). Their presence can therefore decrease toxicity of the free copper ion (Meyer et al., 1999; Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere and Janssen, 2002). In a BLM, cation–biotic ligand interactions are represented and calculated in the same way as any other reaction of a cation with an organic or inorganic ligand. To that end, the complexation capacity of the BL (analogous to the concentration of other ligands in solution) and stability constants for the metal–BL and the cation–BL complexes are required and need to be incorporated in a speciation model like WHAM (Lofts and Tipping, 2002).

Several detailed studies have already demonstrated the effect of pH and hardness cations on copper toxicity. The 96 h LC50 for fathead minnows (*Pimephales promelas*) exposed to copper increased with increasing water hardness (Meyer et al., 1999). The 48 h EC50_{Cu²⁺} for *Daphnia magna* linearly increased with higher activities of Ca^{2+} , Mg^{2+} , Na^+ and H^+ (De Schamphelaere and Janssen, 2002). However, the 21 d EC50_{Cu²⁺} (reproduction) increased with increasing Na^+ and H^+ activity, while Ca^{2+} and Mg^{2+} did not affect chronic copper toxicity (De Schamphelaere and Janssen, 2004). Free copper ion partitioning between roots of lettuce (*Lactuca sativa*) and nutrient solution was linearly related to proton and calcium concentrations in the solution (Cheng and Allen, 2001; Voigt et al., 2006). Rhizotoxicity of Cu to wheat (*Triticum aestivum*) decreased with additions of Ca and Mg (Kinraide et al., 2004) and Vulkan et al. (2004) reported that the amount of sorbed Cu in wheat decreased with increasing K, Ca and La concentrations. Critical Cu^{2+} concentrations for maize decreased with increasing pH (Lexmond and Van der Vorm, 1981).

Until present, BLMs were mainly developed to predict metal toxicity to aquatic organisms. Recently, Steenbergen et al. (2005) developed a terrestrial BLM to predict acute Cu toxicity to the earthworm *Aporrectodea caliginosa* and Lock et al. (2006) developed a BLM to predict acute Co toxicity to the oligochaete *Enchytraeus albidus*. These studies indicate that the BLM concept is also applicable in the terrestrial environment. In this study, it was examined if the BLM approach was also applicable for terrestrial plants. The aims of the present study were twofold: (1) to investigate the extent to which calcium, magnesium, sodium, potassium and hydrogen ions can

individually mitigate copper ion toxicity and (2) to develop a model that is able to predict copper toxicity to *H. vulgare* for a broad range of water characteristics, which represent ranges that can be encountered in soil pore water. The development of such a semi-mechanistic model could support efforts to improve the ecological relevance of presently applied risk assessment procedures.

2. Materials and methods

2.1. Experimental design

To assess the effect of different cations on copper toxicity, the concentration of one cation at a time was varied, while keeping all other cation concentrations constant and as low as possible. Five sets of copper bioassays were performed: Ca-set, Mg-set, Na-set, K-set and pH-set (Table 1). In each set, media were tested in which only the cation under consideration varied and for each medium, several copper levels were tested. Toxicity tests of the same set were assessed simultaneously to minimize variability. The tested cation concentrations were based on natural ranges occurring in pore waters in the field.

2.2. Preparation of the test solutions

The used chemicals were reagent grade and were purchased from VWR (Leuven, Belgium). Test media were prepared by adding different volumes of stock solutions of CaCl_2 , MgSO_4 , NaCl and KCl to carbon-filtered, deionised water. Except for the pH-sets, these media were brought to pH 7.0. pH was adjusted using MOPS-buffering (3-[*N*-morpholino] propane sulfonic acid, 0.75 g/L) and addition of NaOH. MOPS was chosen because it is completely non-complexing for metals (Kandegedara and Rorabacher, 1999). It is also recommended by US-EPA (1991) since it does not change the toxicity of effluents and sediment pore waters. Moreover, MOPS-buffering at 0.75 g/L did not affect metal toxicity to *Daphnia magna* and *Pseudokirchneriella subcapitata* (De Schamphelaere et al., 2004). At pH > 7, NaHCO_3 (2 mM) was used as a buffer, at pH 5 and 6, pH was adjusted by MES-buffering (2-[*N*-morpholino] ethane sulfonic acid, 0.75 mg/L) and addition of NaOH. pH 4.0 was adjusted with HCl only. For each bioassay, the prepared test medium was subsequently used as the dilution water to make logarithmic concentration series of copper, added as CuCl_2 . To obtain near-equilibrium situations, media were stored in the test vessels at 20 °C for 1 day before the start of the toxicity tests. The chemical characteristics of the different test-media are summarized in Table 1.

2.3. Toxicity assays

The root growth inhibition test with barley (*Hordeum vulgare* L.) was performed according to ISO guideline 11269-1 (ISO, 1993). Seeds were kindly provided by Rooney and McGrath (IACR-Rothamsted, UK). Before the start of the test, seeds were germinated on filter paper moistened with deionised water for 36 h at 20 °C in the absence of light. In the toxicity tests, the seeds were fixed in a polypropylene sheet floating on the surface of the medium. For each medium, a toxicity assay was conducted, consisting of six treatments (control + 5 copper concentrations), with a difference of 1 log-unit between the lowest and highest copper concentration tested. Each treatment was performed with 6 seeds in an acid-washed glass jar, filled with 1 L of test medium. Test media were aerated throughout the exposure time. Tests were carried out at 20 °C with a light cycle of 16:8 light:dark. The length of the roots was measured after 4 d of exposure instead of the prescribed 5 d in order to be able to use the model developed in the present study for the data obtained by Rooney and McGrath (unpublished data), who assessed copper toxicity to *H. vulgare* in 19 European field soils that were chosen to span the natural range of soil parameters affecting copper bioavailability. Roots were collected, cleaned off with paper tissue, dried at 40 °C, weighed and stored for metal analysis.

Table 1
Overview of the chemical characteristics of the test media used in the different bioassay sets with *Hordeum vulgare*

Bioassay set	pH	Ca (mM)	Mg (mM)	Na (mM)	K (mM)	Cl (mM)	SO ₄ (mM)	EC50 (nM Cu ²⁺)	ED50 (mg Cu/kg dry wt)
Ca	6.94	0.20	0.050	2.6	0.078	0.56	0.050	295	150
	7.01	2.7	0.050	2.6	0.078	5.5	0.050	199	97.2
	7.05	5.2	0.050	2.6	0.078	11	0.050	210	99.4
	7.05	7.7	0.050	2.6	0.078	16	0.050	88.1	54.7
	7.07	10	0.050	2.6	0.078	21	0.050	149	65.7
	7.09	13	0.050	2.6	0.078	26	0.050	50.0	40.4
	6.98	15	0.050	2.6	0.078	30	0.050	110	88.3
	Mg	6.77	0.20	0.050	2.6	0.078	0.56	0.050	258
6.81		0.20	1.1	2.6	0.078	0.56	1.1	311	207
6.85		0.20	2.1	2.6	0.078	0.56	2.1	327	223
6.88		0.20	3.1	2.6	0.078	0.56	3.1	346	320
6.89		0.20	4.2	2.6	0.078	0.56	4.2	414	396
6.92		0.20	5.2	2.6	0.078	0.56	5.2	515	655
Na		6.88	0.20	0.050	2.6	0.078	0.56	0.050	222
	6.82	0.20	0.050	6.9	0.078	0.9	0.050	203	127
	6.83	0.20	0.050	11	0.078	0.3	0.050	171	80.8
	6.92	0.20	0.050	16	0.078	14	0.050	158	86.3
	6.89	0.20	0.050	20	0.078	18	0.050	234	83.9
	6.94	0.20	0.050	24	0.078	22	0.050	292	151
	K	6.78	0.20	0.050	2.6	0.078	0.56	0.050	390
6.75		0.20	0.050	2.6	0.72	1.2	0.050	287	176
6.77		0.20	0.050	2.6	1.4	1.8	0.050	260	191
6.79		0.20	0.050	2.6	2.6	3.1	0.050	298	118
6.82		0.20	0.050	2.6	5.2	5.7	0.050	534	265
6.85		0.20	0.050	2.6	7.8	8.2	0.050	277	124
6.85		0.20	0.050	2.6	10	11	0.050	222	81.9
pH		7.74	0.20	0.050	2.1	0.078	0.56	0.050	82.6
	6.94	0.20	0.050	2.6	0.078	0.56	0.050	295	150
	6.23	0.20	0.050	2.4	0.078	0.56	0.050	335	179
	6.30	0.20	0.050	0.78	0.078	0.56	0.050	318	218
	5.31	0.20	0.050	0.70	0.078	0.56	0.050	357	168
	4.46	0.20	0.050	0.078	0.078	0.56	0.050	440	168

For each set, five copper concentrations and a control were tested. pH at the end of the test and the ion concentrations are interpolated for the Cu²⁺ activity at the EC50. The observed 4 d EC50s are expressed as free copper ion activity and the 4 d ED50s as internal root concentration.

2.4. Chemical measurements

Copper concentration in the test vessels were determined at the end of the test using a graphite furnace atomic absorption spectrophotometer (SpectrAA800, Varian, Mulgrave, Australia). Calibration standards (Sigma-Aldrich, Steinheim, Germany) and a reagent blank were analysed with every 10 samples. Copper concentrations in the plant roots were measured after digestion in hot acid (14N HNO₃, microwave heating). Since earlier measurements had shown that concentrations of other cations (Ca, Mg, Na, K) and anions (Cl, SO₄) were within 10% from nominal values, these ions were only analysed checkwise (once in each set) according to standard methods (Clesceri et al., 1998). pH (pH-meter P407, Consort, Turnhout, Belgium) was measured before and after the test. The pH glass electrode was calibrated daily using pH 4 and pH 7 buffers (Merck, Darmstadt, Germany).

2.5. Data treatment and statistics

Speciation calculations were conducted using WHAM 6.0.8 (Windermere Humic Aqueous Model, Lofts and Tipping, 2002) with adjusted stability constants for the inorganic copper complexes (Martell et al., 1997). The input data for WHAM were pH and concentrations of Cu, Mg,

Ca, K and Na. In open systems, CO₂ is in equilibrium with the ambient air, pCO₂ was therefore set at 10^{-3.5} atm. 4 d EC50s were calculated by fitting a sigmoid curve to the calculated copper ion activity vs. root growth relationship according to the model of Haanstra et al. (1985). Linear regressions were calculated using STATISTICA[®] software.

3. Results

Increases in Ca²⁺ activity resulted in a sixfold decrease in 4 d EC50_{Cu²⁺} values ($R^2 = 0.76$, $p = 0.011$) (Fig. 1A). Increases in Mg²⁺ activity resulted in a twofold increase in 4 d EC50_{Cu²⁺} values ($R^2 = 0.85$, $p = 0.0085$) (Fig. 1B). K⁺ activity ($R^2 = 0.041$, $p = 0.66$) (Fig. 1C), Na⁺ activity ($R^2 = 0.21$, $p = 0.36$) (Fig. 1D) and H⁺ activity ($R^2 = 0.36$, $p = 0.21$) (Fig. 1E) did not significantly affect the 4 d EC50_{Cu²⁺}.

The internal copper concentration in roots of *H. vulgare*, resulting in 50% root growth inhibition (EC50_{internal Cu}), ranged from 40.4 to 655 mg Cu/kg dry wt (Table 1).

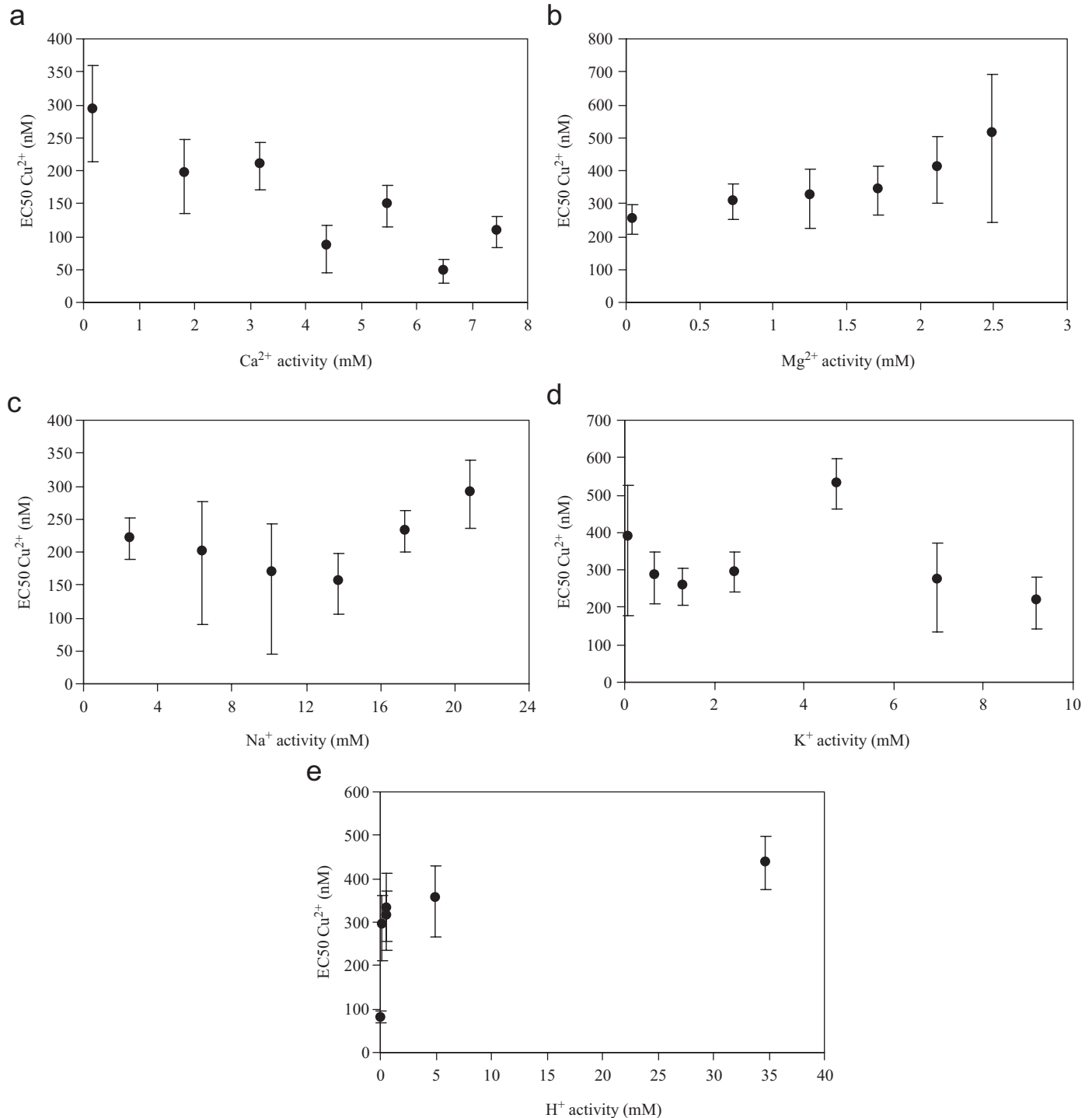


Fig. 1. The 4d EC₅₀_{Cu²⁺} for *Hordeum vulgare* as a function of the activity of Ca²⁺ (A), Mg²⁺ (B), Na⁺ (C), K⁺ (D) and H⁺ (E). Error bars indicate 95% confidence intervals.

4. Discussion

The 4d EC₅₀_{Cu²⁺} differed by more than a factor 10 between treatments, however, these differences could not be explained by positive linear relationships between the EC₅₀_{Cu²⁺} and the activity of Ca²⁺, Mg²⁺, K⁺, Na⁺ and H⁺. Increases in Ca²⁺ activity resulted in a decreased 4d

EC₅₀_{Cu²⁺}. However, if there is a linear relationship between the cation activity and the EC₅₀, the BLM concept assumes it to be positive. Increases in Mg²⁺ activity resulted in an increased 4d EC₅₀_{Cu²⁺}, but the 4d EC₅₀_{Cu²⁺} differed with less than a factor two in the Mg-set. K⁺, Na⁺ and H⁺ did not significantly affect the 4d EC₅₀_{Cu²⁺}. The results obtained in the present study did

therefore not allow to develop a BLM to predict Cu toxicity to barley. In fact, a free ion activity model (Morel, 1983)—which does not incorporate competition between metal ions and cations for binding on transport and toxic action sites on biological surfaces—was able to describe the obtained results. Internal copper concentrations in roots of *H. vulgare*, resulting in 50% root growth inhibition ($EC_{50_{\text{internal Cu}}}$), differed by a factor of more than 16, indicating that internal copper concentration was not a better indicator of copper toxicity than Cu^{2+} activity.

In most BLMs developed so far, Ca^{2+} , Mg^{2+} and H^+ activity clearly affected metal toxicity (Erickson et al., 1996; Meyer et al., 1999; De Schamphelaere and Janssen, 2002, 2004; Heijerick et al., 2002; Lock et al., 2006). For most metals, the activity of Na^+ has minor effects on toxicity (Meyer et al., 1999; Heijerick et al., 2002; Lock et al., 2006). Na^+ only affects Cu^{2+} toxicity to an important extent, which is probably due to the similar ionic radius of Cu^{2+} and Na^+ (Erickson et al., 1996; De Schamphelaere and Janssen, 2002, 2004; Steenbergen et al., 2005). It is not clear why the activity of these cations did not have a similar effect on the toxicity of Cu^{2+} to *H. vulgare*. The K^+ activity did not affect the 48 h EC_{50} for *Daphnia magna* exposed to Cu^{2+} (De Schamphelaere and Janssen, 2002) and Zn^{2+} (Heijerick et al., 2002) and in the development of most other BLMs, it is assumed that K^+ does not affect metal toxicity.

For sugar beet (*Beta vulgaris*), no effect of Ca, Mg and K was observed on copper toxicity (Saleh et al., 1999). However, free copper ion partitioning between roots of lettuce (*Lactuca sativa*) and nutrient solution was linearly related to proton and calcium concentrations in the solution (Cheng and Allen, 2001) and critical Cu^{2+} concentrations for maize decreased with increasing pH (Lexmond and Van der Vorm, 1981). Also Voigt et al. (2006) found that Ca and H inhibited rhizotoxicity of copper to lettuce (*L. sativa*). Kinraide et al. (2004) found that Ca and Mg decreased rhizotoxicity of copper to wheat (*Triticum aestivum*) and Vulkan et al. (2004) reported that the amount of sorbed copper decreased with increasing concentrations of K, Ca and La, suggesting that these cations competed with Cu for sorption at the plasma membrane of wheat root (*T. aestivum*). The above results suggest that competition for binding sites between Cu^{2+} and cations strongly depends on the considered plant species.

While toxicity is probably better predicted by pore water copper concentrations, most databases only contain total copper concentrations in soils. Sauvé et al. (1997) indicated that the soluble copper pool is closely related to the soil total copper content and that total copper concentrations in the soil can therefore easily be translated to pore water copper concentrations. However, only a very small fraction of the soluble copper in the pore water is present in inorganic forms: more than 98% of the soluble copper is bound to organic matter (Sauvé et al., 1997). Assessing the effect of dissolved organic carbon on copper speciation and

toxicity may therefore be important in the risk assessment of copper in soils. Antunes et al. (2006) also indicated that factors such as microbial activity and root exudates might affect metal bioavailability to plants that are exposed in soils, which may hamper extrapolation of results obtained in nutrient solutions to field soils.

5. Conclusion

Based on the free Cu^{2+} activity, $EC_{50_{Cu^{2+}}}$ s could be predicted—with one exception (at the one but highest Ca^{2+} activity, $EC_{50_{Cu^{2+}}}$ was almost a factor five lower than predicted)—within a factor of less than three. Applying the BLM-concept, which incorporates competition between Cu^{2+} and other cations for binding sites, could not improve the free ion activity model. Assessing the effect of dissolved organic matter on Cu speciation and toxicity and validation experiments with field soils are required to allow further improvement of the ecological relevance of the risk assessment of Cu.

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