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## Preface

This report is written in the scope of the EU-project LC-IMPACT workpackage 2.1, which started in 2009 and a workpackage milestone of May 2011.

Metal emissions commonly dominate the life cycle impact assessment results for human and ecotoxicity. These results are, however, heavily criticized, due to the fact that metal emissions from landfills and bioavailability in the environment are suspected to be highly overestimated by the current models. On-going research under UNEP-SETAC Life Cycle Initiative is focusing on improving estimation of metal impacts on freshwater ecosystems with partner 1 (RU) and partner 10 (CML) as participants. We will build upon the knowledge gained in this project for freshwater ecotoxicity in the further development of operational and scientifically sound characterisation models for the terrestrial ecotoxicity of metal compounds.

This study is classified as an experimental study under laboratory conditions. The objective was to develop a BLM that is able to predict copper toxicity to *Lactuca sativa* (lettuce) for a broad range of water characteristics. The cations under consideration were: $H^+$, $Na^+$, $Ca^{2+}$ and $Zn^{2+}$. The development of such a model could support efforts to improve the ecological relevance of presently applied risk assessment procedures. The method development for predicting toxicity to lettuce of Cu is a semi-mechanistic/empiric illustration that can be used to derive more generic and simple approaches for more metals that is consistent with the practice of LCIA.

As follow up on this report, some extended data will be gathered in our laboratory (see also recommendations in this report). Consequently, this report will be rewritten as a manuscript in order to submit it to a peer-reviewed scientific journal. This step in the progress will not be done within the EU-project anymore, but the results can be send to the team-members of LC-IMPACT on request.

Sincerely
Martina Vijver, Reinout Heijungs, Willie Peijnenburg

## Abstract

The extent to which hydrogen, sodium, calcium and zinc ions independently mitigate copper toxicity to seedlings of lettuce (*Lactuca sativa*) in nutrient solutions was examined. Increasing pH linearly increased the 4d $EC_{50}$ (expressed in units of pCu), supporting the concept that some ions can compete with $Cu^{2+}$ for binding to the active sites at the terrestrial organism–solution interface (i.e., the biotic ligand, BL). For sodium, calcium and zinc ions a similar relationship was not found. According to the biotic ligand model (BLM), the stability constants for the binding of $Cu^{2+}$ and $H^+$ to the BL were derived from the toxicity data. They were 7.72 for $\log K_{CuBL}$, and 6.91 for $\log K_{HBL}$. It was calculated that on average 50.1% of BL sites need to be occupied by $Cu^{2+}$ to induce 50% root growth inhibition.

## Contents

## Introduction

Metals are natural components of the earth and exist in many forms; they each have their own specific chemical characteristics that define their interactions with the environment and with living organisms. Pollution by heavy metals is a problem in many areas in the world and is often caused by human activities. Because metals may cause environmental risk, their concentrations in soil and water are managed through the establishment of environmental quality criteria. Current environmental quality criteria and risk assessment procedures for metals are predominantly based on total metal concentrations. However, accumulating evidence indicates that total metal concentrations are poor predictors of metal bioavailability and toxicity (Paquin et al., 2002). Many different factors that are driven by environmental parameters steer the bioavailability and toxicity of metals. The environmental parameters are often site specific or water type specific. To make things more pragmatic for environmental risk assessment or for Life Cycle Analysis goals, archetypes based on important environmental parameters (such as pH, salinity or dissolved organic carbon content) that describe certain regions are derived for aquatic ecosystems (see e.g. Diamond et al 2010). At the moment not much affort is done to incorporate these site-specific effect models for terrestrial soil systems. Here in this report the first building blocks are created to come to a terrestrial Biotic Ligand Model that can prioritize regions that are more increased or less decreased at risk.

## Life Cycle Assessment

Life cycle assessment (LCA) is a tool for the “compilation and evaluation of the inputs, outputs and the potential environmental impacts of a product system throughout its life cycle” (ISO, 2006). An important part of the procedure is the life cycle impact assessment (LCIA) phase, in which aims “at understanding and evaluating the magnitude and significance of the potential environmental impacts for a product system throughout the life cycle of the product” (ISO, 2006). It aggregates world-wide emissions of different greenhouse gases into one score for climate change, using the global warming potentials (GWPs), numbers that reflect the potential to contribute to climate change per mass unit of greenhouse gas. Such GWPs are based on a combination of substance properties (persistence, hazard, mobility, etc.) and environmental conditions (temperatures, wind speeds, aerosol concentrations, etc.). For aggregating different toxic releases into a small number of toxic impact categories, similar exercises must be done, combining substance parameters (DT50, Kow, etc.) with environmental conditions. The exposure of aquatic organisms to toxics proceeds through the presence of the toxic in the aquatic phase. For some chemicals the amount of the substance in the aquatic phase is not without ambiguity. Especially for metals, there is an important difference between the amount of metal, the dissolved amount, the available amount, and so on.

Traditional multi-media models for toxicity assessment were primarily derived for organics, although with some creativity they worked also for metals. Application of these models in LCIA, however, soon made clear that the aggregated toxicity scores were strongly dominated by metals (Ligthart et al, 2004). The assumption of steady-state in Mackay level III-models, combined with the absence of a degradation pathway for metals, was responsible for this. Indeed, metals do not degrade, but they do become unavailable. The BLM has been identified as one promising way to reduce the error in the assessment of metal emissions in LCA (Ghandi et al., 2010).

Terrestrial concentrations of toxics strongly depend on the aquatic concentrations. As a result, the BLM is an important object of study for terrestrial ecotoxicity as well, both outside and within LCA. This report derives a terrestrial BLM for copper.

Unlike with greenhouse gases, the effect of toxic releases is different for different emission locations. Ambient conditions in terms of pH, temperature, cations, etc. co-determine the distribution of a substance, including its bioavailability. Product life cycles, and thereby the associated emissions, are never on one location, but are dispersed over entire globe. Potatoes cultivated in the Netherlands need pesticides and fertilizer that is produced in a range of other countries, and the diesel that is needed at the farm is produced in even more countries. Even when the emissions of the agricultural process iself would be known exactly, many of the upstream locations are not known. Oil just comes from the world market. Hence, in an LCA it is not possible to use a too specifically defined environment, and a balance must be found between two little accuracy and too much detail. A framework for including spatial differentiation in a feasible has been elaborated in the GLOBOX model (Wegener Sleeswijk & Heijungs, 2010). GLOBOX does not contain a BLM, but it may contain one in future. This report serves as a step forward in the development of BLMs for the important metals, with an eye on the dependence on environmental conditions.

## Water type specific effect assessment of metals

The Biotic Ligand Model (BLM) (De Schamphelaere and Janssen, 2002; Di Toro et al., 2001) is now considered a much better predictor of metal bioavailability and toxicity in water. BLMs incorporate metal complexation and speciation in the solution surrounding the organism with dissolved (in)organic compounds and acidic ions, as well as interactions between metal ions and competing cations at the binding sites on the organism-water interface. The main assumption of the BLM is that the organism is in equilibrium with its environment and that metal toxicity occurs as the result of free metal ions interacting with the biotic ligand. The magnitude of the toxic effect is determined by the concentration of the metal-biotic ligand complex.

### Aquatic to terrestrial BLMs

Originally BLMs were developed for aquatic organisms, with the gill as the biotic ligand. At present however, it has been proven possible to extend the applicability of the BLM by considering the biotic ligand as a more general binding site. There have already been successful implementations of the model for terrestrial organisms such as *Aporrectodea calliginosa* (earthworm), (Steenbergen et al., 2005), *Enchytraeus albidus* (potworm), (Lock et al., 2006), *Hordeum vulgare* (barley) (Lock et al., 2006) and *Folsomia candida* (springtail), (Van Gestel and Koolhaas 2004). The precondition for the application of the BLM is that the organism is predominantly exposed to metals through the watery solution, so exposure via the dietary route should play an insignificant role.

### Aims

This study is classified as an experimental study under laboratory conditions. The objective was to develop a BLM that is able to predict copper toxicity to *Lactuca sativa* (lettuce) for a broad range of water characteristics. The cations under consideration were: $H^+$, $Na^+$, $Ca^{2+}$ and $Zn^{2+}$. The development of such a model could support efforts to improve the ecological relevance of presently applied risk assessment procedures. This study is confined to hydroponically grown lettuce, so the result will be an aquatic BLM. However in the end, the purpose of this study is to create a terrestrial BLM for lettuce.

## Materials and methods

### Test organism

Lettuce, *Lactuca sativa*, is part of the family of Compositae. Lettuce seed is about 3-4 mm in length, 0.8-1.0 mm broad and 0.3-0.5 mm thick. Lettuce requires light to germinate and can be grown on all possible soils. Lettuce needs a relatively high pH. Lettuce has a rather short growing period; between 30-65 days for a full-grown plant (PAGV, 1985). Lettuce is a hyper accumulating plant for metals, because lettuce has a relatively large possibility for translocation and absorption of nutrients (Garate et al., 1993; McKenna et al., 1993). In general lettuce will accumulate more metals in its roots than in its leaves (Garate et al., 1993; McKenna et al., 1993; Florijn, 1993; Xue and Harrison, 1991). The RIVM has broad experience with lettuce in toxicity studies. They found that lettuce can be grown quite easily under conditioned circumstances. Lettuce is recommended as test plant by the OECD (Organization for Economic Cooperation and Development).

Before the start of the test, seeds of lettuce were germinated for four days at 15 ºC during a normal light cycle, on gauze in Steiner solution. During the toxicity tests with nutrient solutions, seeds were fixed in a parafilm sheet and their roots were in the medium. For each medium, a toxicity assay was conducted, consisting of five treatments (control + 4 metal concentrations). Each treatment was performed with 4 seeds fixed on the surface of 150 ml test solution in an acid-washed glass. Root lengths were measured after 4 days of exposure. Root growth, the difference in length between the first and the last day of exposure, was the endpoint.

### Experimental design of the toxicity tests

*Lactuca sativa* was tested in nutrient solutions, in which it is easier to adjust conditions (such as stable concentrations) than in real soil. To examine the effects of water chemistry on toxicity of copper for *Lactuca sativa*, there were a Na-set, a pH-set, a Ca-set and finally a Zn-set (Table 1). The concentrations of these cations were set in naturally occurring ranges (Oorts et al., 2006), to prevent either deficiency or toxicity from respectively too little or too many cations. All sets consisted of a series of media in which only the concentration of the cation or metal under consideration varied, the other cations were kept at the same concentration as in the Steiner nutrient conditions.

**Table 1: Bioassay set characteristics**

| Bioassay set | pH | Na (mM) | Ca (mM) | Zn (mM) |
|---|---|---|---|---|
| pH | 7 | 0 | 0 | 0 |
|  | 6,5 | 0 | 0 | 0 |
|  | 6 | 0 | 0 | 0 |
|  | 5,5 | 0 | 0 | 0 |
|  | 5 | 0 | 0 | 0 |
| Na (mM) | 7 | 20 | 0 | 0 |
|  | 7 | 15 | 0 | 0 |
|  | 7 | 10 | 0 | 0 |
|  | 7 | 5 | 0 | 0 |
|  | 7 | 0 | 0 | 0 |
| Ca (mM) | 7 | 0 | 15 | 0 |
|  | 7 | 0 | 10 | 0 |
|  | 7 | 0 | 5 | 0 |
|  | 7 | 0 | 2 | 0 |
|  | 7 | 0 | 0 | 0 |
| Zn (mM) | 7 | 0 | 0 | 25 |
|  | 7 | 0 | 0 | 20 |
|  | 7 | 0 | 0 | 10 |
|  | 7 | 0 | 0 | 5 |
|  | 7 | 0 | 0 | 0 |

*** 0= Steiner condition**

Preparation of the test media

Steiner solution (Appendix 1) was the default test medium that was used in the experiments. The standard test medium was slightly modified for all experimental sets.

Except for the pH-sets, these media were adjusted to pH 7.0. pH was controlled using MOPS-buffering (3-[N morpholino] propane sulfonic acid, 0.75 gram per liter). MOPS was chosen because it is completely non-complexing for metals (Kandegedara and Rorabacher, 1999). MOPS is also recommended by US-EPA (1991) because it does not change the toxicity of effluents and sediment pore waters. In addition, MOPS-buffering did not influence metal toxicity to algae and daphnia (De Schamphelaere et al., 2004). In solutions with pH 5 to 6, pH was controlled using

MES-buffering (2-[N-morpholino] ethane sulfonic acid, 0.75 mg/L) and adding NaOH.

The free copper ion activity and pH of the solutions were checked daily and adjusted when necessary, allowing a maximum pH variation of 0.3 units and a maximum pCu variation of 0.5 units in five minutes. The copper activity was adjusted by adding copper nitrate Cu $(NO_3)_2$ to the Steiner nutrient solution. The pH was adjusted by adding either HCl or KOH for respectively decreasing or increasing pH. All cations were added as chloride salts.

## Chemical measurements

An Ion-selective electrode was used to measure the free copper ion activity, in combination with a voltmeter of 0.1 mV resolution (Cole- Palmer Copper Electrode). The Ion-selective electrode was calibrated in the relevant pCu range by means of stock solutions of $Cu(NO_3)_2$ of known activities at pH 2, using $NaNO_3$ to adjust the ionic strength. The maximum deviation of the slope of the calibration curve that was considered acceptable was 3% of the theoretical value calculated with the Nernst equation (Appendix 2).

## Data treatment and statistics

Four days $EC_{50}$'s were calculated by fitting a sigmoid dose-response curve to the measured "pCu versus root growth" relationship using Graphpad Prism software.

## Short mathematical description of the BLM and derivation of BLM parameters

This section gives a short mathematical description of the BLM, in terms of the equations required to understand the calculations performed in the present study. A more detailed description and the derivation of the equations are given elsewhere (De Schamphelaere and Janssen, 2002). The toxic effect is, according to the BLM-hypothesis, determined by the fraction of the total number of copper binding sites occupied by copper ($f_{CuBL}$), regardless of test solution (or pore water) chemistry. By multiplying this fraction with the Cu-binding capacity of the BL, i.e. the total number of BL sites, the concentration of Cu bound to the BL can be obtained. This fraction, i.e. $f_{CuBL}$, equals:

$$f_{\mathrm{CuBL}} = K_{\mathrm{CuBL}}(\mathrm{Cu}^{2+}) / 1 + K_{\mathrm{CuBL}}(\mathrm{Cu}^{2+}) + K_{\mathrm{X1BL}}(\mathrm{X1}^{n+}) + K_{\mathrm{X2BL}}(\mathrm{X2}^{n+}) \qquad [1]$$

Where $K_{\mathrm{X1BL}}$ is the stability constant for binding of cation X1 to the biotic ligand and ($\mathrm{X1}^{n+}$) denotes the chemical activity of cation $\mathrm{X1}^{n+}$. Stability constants are defined as, for example for Cu:

$$K_{\mathrm{CuBL}} = [\mathrm{CuBL}^{+}] / [\mathrm{Cu}^{2+}] [\mathrm{BL}^{-}] \qquad [2]$$

Where $K_{\mathrm{CuBL}}$ is the stability constant for $\mathrm{Cu}^{2+}$ binding to BL sites, [$\mathrm{Cu}^{2+}$] the activity of the free cobalt ion, [$\mathrm{CuBL}^{+}$] the concentration of Cu bound to the BL, and [$\mathrm{BL}^{-}$] the concentration of free BL, unoccupied by any cation. Similar equations can be written for the other cations. According to the BLM-concept, $f_{\mathrm{CuBL}}$ is constant at 50% effect $f50\%_{\mathrm{CuBL}}$ and Equation [1] can be re-organized to:

$$\mathrm{EC}_{50\mathrm{Cu2+}} = f50\%_{\mathrm{CuBL}} / (1 - f50\%_{\mathrm{CuBL}})\, K_{\mathrm{CuBL}} \{1 + K_{\mathrm{X1BL}}(\mathrm{X1}^{n+}) + K_{\mathrm{X2BL}}(\mathrm{X2}^{n+})\} \qquad [3]$$

Where $\mathrm{EC}_{50\mathrm{Cu2+}}$ is the free copper ion activity resulting in 50% effect in *Lactuca sativa* after 4 days of exposure. With Equation [3], $\mathrm{EC}_{50\mathrm{Cu2+}}$ can be predicted when ($\mathrm{X1}^{n+}$) and ($\mathrm{X2}^{n+}$) are known, provided that the values of $K_{\mathrm{CuBL}}$, $K_{\mathrm{X1BL}}$, etc. are known (see further). Equation [3] shows that, if the BLM concept is correct, linear relationships should be observed between $\mathrm{EC}_{50\mathrm{Cu2+}}$ and the activity of one cation when other cation activities are kept constant. The slopes and the intercepts of those relationships can then be used to derive the stability constants of competing cations according to the method described by De Schamphelaere and Janssen (2002). The calculation of $K_{\mathrm{CuBL}}$ and $f50\%_{\mathrm{CuBL}}$ is based on the optimization of the logittransformed effect versus $f_{\mathrm{CuBL}}$ for varying $K_{\mathrm{CuBL}}$ (see results section).

## Results

Figure 1 displays the 4-day $EC_{50}$, expressed in pCu, with increasing pH and increasing sodium, calcium and zinc concentration respectively.

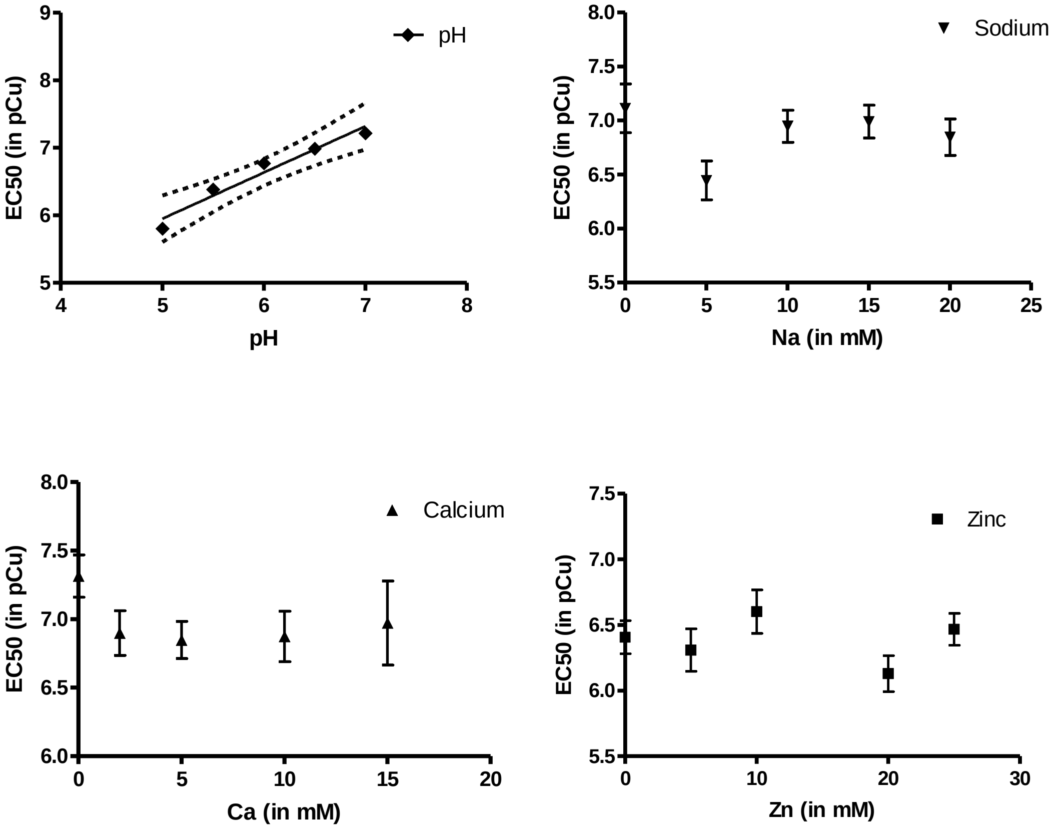

Figure 1: The 4 d $EC_{50}$ for *Lactuca sativa* as a function of pH and the concentrations of, Na, Ca and Zn. Error bars indicate 95% confidence intervals. The solid line in the pH-graph is the linear regression line ($R^2 = 0, 95$), the dotted line represents the 95% confidence band.

In the pH experiment, a significant effect of the assumed competing ion, in this case $H^+$, on Cu toxicity was found. The 4-day $EC_{50}$ (in pCu) decreased with decreasing pH, which means that with more $H^+$ ions in the solution, the 50% effect concentration contains more $Cu^{2+}$. In the cases of sodium, calcium and zinc no such relationship could be seen.

Development of the BLM

Equation [1] denotes the mathematical description of the BLM. For the development of the BLM for copper toxicity to lettuce, there are three parameters that have to be calculated: $K_{\mathrm{CuBL}}$, $K_{\mathrm{HBL}}$, and the fraction of the biotic sites occupied at the $EC_{50}$-value (*f*). $K_{\mathrm{HBL}}$ can be calculated by deriving the slope and intercept from the graph in which $EC_{50}$ (expressed in $Cu^{2+}$ activity) is plotted against the $H^+$-activity (Figure 2) (De Schamphelaere and Janssen, 2002).

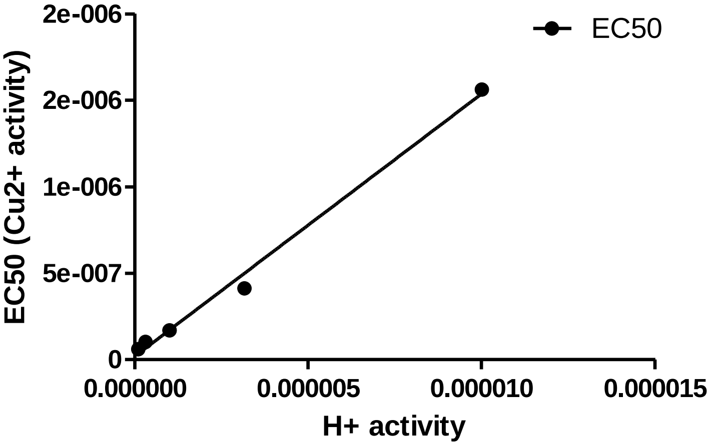

Figure 2: $EC_{50}$ set against $H^+$ activity

The slope that is obtained this way is equal to:

$$f50\%_{\mathrm{CuBL}} / [(1- f50\%_{\mathrm{CuBL}}) * K_{\mathrm{CuBL}}] * K_{\mathrm{HBL}} \qquad [4]$$

With $f50\%_{\mathrm{CuBL}}$ = fraction of the Biotic Ligand occupied at 50 % effect

The intercept is equal to:

$$f50\%_{\mathrm{CuBL}} [(1- f50\%_{\mathrm{CuBL}}) * K_{\mathrm{CuBL}}] \qquad [5]$$

Given the calculated slope of 0.1518 and the calculated intercept of $1.882 * 10^{-8}$, a value of log $K_{\mathrm{HBL}}$ equal to 6.91 is obtained when applying equations [4] and [5].

Values of $f50\%_{\mathrm{CuBL}}$ and $K_{\mathrm{CuBL}}$ can be obtained by applying equation [3]: optimization of the fit between experimental $EC_{50}$-values and calculated $EC_{50}$-values using equation [3] yields the optimum values of $f50\%_{\mathrm{CuBL}}$ and $K_{\mathrm{CuBL}}$. The optimized value of $f50\%_{\mathrm{CuBL}}$ turns out to be 0.501, whereas log $K_{\mathrm{CuBL}}$ is equal to 7.72.

The final BLM incorporating the effect of $H^+$-ions on root growth of lettuce thus is as follows:

$$EC_{50\mathrm{Cu}2+} = 4.72 * 10^{-9} * (1 + 8128305 * [H^+]) \qquad [6]$$

## Discussion and recommendations

Whereas a protective effect is expected from the addition of cations due to competition at the biotic ligand, this was not the case for all cations that were used in this study (Figure 1). In this study the 4-day $EC_{50}$ (in pCu) decreased with decreasing pH, which means that with more $H^+$ ions in the solution, the 50% effect concentration contains more $Cu^2$. Thus $H^+$ ions compete with $Cu^{2+}$ ions for binding with the biotic ligand and therefore protect lettuce from copper toxicity. Sodium, calcium and zinc did not have a significant effect on the $EC_{50}$-values obtained (Figure 1).

For obvious reasons it is unfair to compare BLMs made for plants with the BLMs developed for aquatic organisms such as fish and terrestrial organisms such as worms, as they are different organisms, living in different compartments. However when we consider the biotic ligand as a more general binding site, such as calcium and sodium transporters, which are inherent to every living cell, the comparison among different organisms appears to be valid.

Niyogi and Wood (2004) make a division in three different sites of action at the biotic ligand: $Na^+$ transport sites, $Ca^{2+}$ transport sites and a thus far unknown site of action (Figure 2).

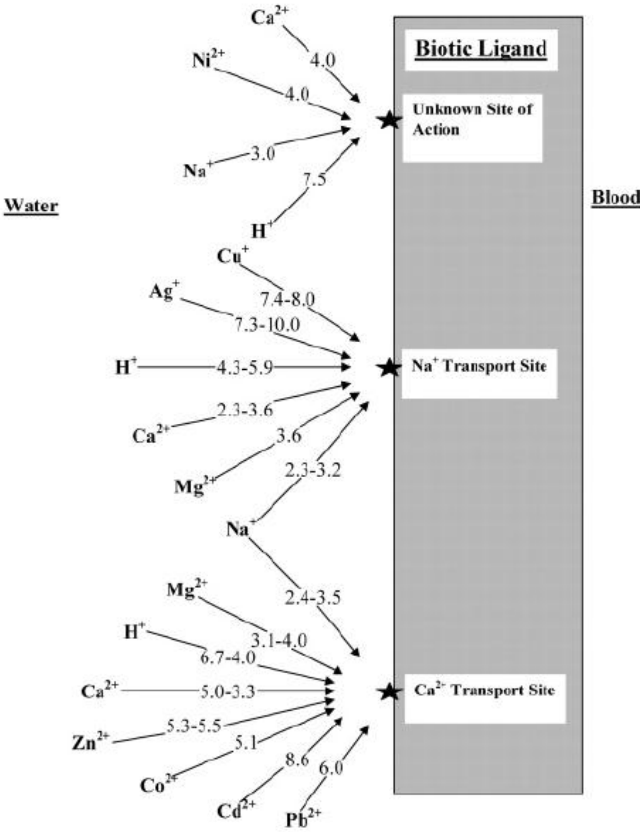

Figure 3: Binding constants (log *K*) of free ions of different metals (copper, silver, cadmium, cobalt, lead, zinc, and nickel) and environmental cations, interacting at different sites of toxic action on the biotic ligand. (Niyogi and Wood, 2004)

On the basis of Figure 3, it is to be expected that calcium, sodium and hydrogen compete with copper for sorption at the biotic ligand, in this case the sodium transport site. And no competition with zinc, since this heavy metal targets the calcium transport site. What can be seen from our experimental study is that only pH has an effect on copper toxicity. This might be explained by the binding affinity of the cations in question. Niyogi and Wood found that $H^+$ is a much better competitor (log $K$= 4.3 – 5.9) with $Cu^{2+}$ (log $K$= 7.4-8.0), than $Ca^{2+}$ (log $K$= 2.3-3.6) and $Na^+$ (log $K$= 2.3-3.2). It is to be expected that when testing higher concentrations of calcium and sodium, competition most likely would occur. This was not done however, because these concentrations would lie outside the range of naturally occurring values in Dutch soils, and they would be beyond the ecological preferences of lettuce; higher concentrations would risk seeing the effects of toxicity of too high calcium or sodium concentrations.

Although the representation by Niyogi and Wood might explain the results found in this study, it is not able to explain all variation in experimental data. When comparing BLMs developed for copper toxicity to plants, no consistent results are found. For example Lock et al. (2007) found a protective effect of $Mg^{2+}$, but a harmful effect of $Ca^{2+}$, and they observed no effect of pH, $Na^+$ and $K^+$ for Barley roots. Luo et al. (2007) found a protective effect of $Ca^{2+}$ and $Mg^{2+}$, and no effect of pH, $Na^+$ and $K^+$ to Wheat roots. Thakali et al. (2006) found no protective cations at all to Tomato shoots; they tested: pH, $Ca^{2+}$ and $Mg^{2+}$. It also happens that BLMs made for the same species, concerning the same toxic metal, but studied by different researchers, display different results (wheat: Luo et al., 2007; Vulkan et al., 2004; barley: Lock et al., 2007; Thakali et al., 2006). More examples are possible, however this shows that there is still ground for discussion on this topic.

Presuming there were no mistakes made in any of the studies, there should be another explanation for these inconsistencies. One possible explanation is the use of cation-concentrations. Since addition of more cation X, increases the chance you can see the result of cation X competing with the toxic metal ion in question. Another possible explanation might be the difference in use of endpoints; there is a chance that while you can see an effect in the roots it might not be visible in the shoots, the same accounts for exposure duration. The last three explanations are most likely the reasons for the occurrence of different results for the same BLMs; however they can not clarify the appearance of all inconsistencies. A better explanation is most likely that binding constants for different cations differ per organism. For example: while for one organism it might be that $H^+$-ions bind stronger than for example $Ca^{2+}$ ions, it might be the other way around for another organism.

To develop the BLM for copper toxicity to lettuce, three parameters had to be calculated. Two binding constants: log $K_{\mathrm{CuBL}}$ = 7.72 and log $K_{\mathrm{HBL}}$ = 6.91, and $f$ (the fraction of the biotic sites occupied at the $\mathrm{EC}_{50}$-value) = 0.501. These parameters may directly be compared with parameters found in other BLM studies. However, it is not justified to compare log $K_{\mathrm{HBL}}$ with BLMs investigating the effect of cadmium, cobalt, lead, zinc, and nickel, because these metals are expected to target other transport sites, where $H^+$-ions have different binding constants for different binding sites.

Santore et al. (2001) found log $K_{\mathrm{HBL}}$ = 5.40, log $K_{\mathrm{CuBL}}$= 7.40 and $f$ = 0.33 for freshwater fish and water fleas. De Schamphelaere & Janssen (2002) found log $K_{\mathrm{CuBL}}$ = 8.02 and $f$ = 0.39 for *Daphnia magna* (water flea). Steenbergen et al. (2005) found log $K_{\mathrm{HBL}}$ = 4.61, log $K_{\mathrm{CuBL}}$= 5.89 and $f$ = 0.19 for *Aporrectodea caliginosa* (earthworm). Thakali et al. (2006) found $K_{\mathrm{HBL}}$ = 6.48, log $K_{\mathrm{CuBL}}$= 7.41 for *Hordeum vulgare* (barley), $K_{\mathrm{HBL}}$ = 4.38, log $K_{\mathrm{CuBL}}$= 5.65 for *Lycopersicon esculentum* (tomato), $K_{\mathrm{HBL}}$ = 2.97, log $K_{\mathrm{CuBL}}$= 4.62 for *Folsomia candida* (springtail), log $K_{\mathrm{HBL}}$ = 5.90, log $K_{\mathrm{CuBL}}$= 6.50 for is *Eisenia fetida* (redworm). Luo et al. (2007) found log $K_{\mathrm{CuBL}}$= 6.28 and $f$ = 0.436 for *Triticum aestivum* (wheat).

Thus, the range found for log $K_{\mathrm{HBL}}$ is 2.97 - 6.91, whereas log $K_{\mathrm{CuBL}}$ is reported to vary between 4.62 - 8.02. The values found in this study are clearly in the upper part of the range, particularly log $K_{\mathrm{HBL}}$ appears to be on the high side. Log $K_{\mathrm{CuBL}}$ is in line with previous findings.

It must be said that Thakali et al. (2006) used a different method to deduce the parameter values, so that less importance should probably be given to their data in comparison with the rest. It should also be noticed that in this study we used Figure 2 to calculate the binding constants, however in this figure it can be seen that the point in the far right corner has a high leverage, which might give a distorted reflection of what is really happening.

To optimize the BLM for *Lactuca sativa* it is important to see the effects of cations such as magnesium and silver, which, if the picture created by Niyogi and Wood really fits this BLM, should be able to compete with copper for binding at the sodium transport sites. Of course it is also necessary to validate the model in real soils, since the goal of a BLM is actually to be able to make predictions of toxicity in real soils. This latter step is now work in progress as collaboration between our laboratory and the laboratory of Michael Hauschildt. The work is done by Willie

Peijnenburg, Martina Vijver and PhD-student Karen Søgaard Christiansen and will be writing a paper on this verification in real soil systems.

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## Appendices

***Appendix 1:*** Composition of the Steiner solution.

The Steiner nutrient solution is composed by adding 10 ml of solution A and B and 1 ml of solution C and D to one litre of tap water and then aerating the solution during 16 hours. Afterwards the pH is measured and adjusted at pH 6.5 ± 0.5 using no more than 158 µl concentrated sulphuric acid per litre solution. The precipitation that might have been produced during the aeration will disappear while adjusting the pH.

Solution A: 68 g $Ca(NO_3)_2$ * 4 H2O

62 g $KNO_3$

per 1litre water.

Solution B: 46 g $MgSO_4$ * 7 $H_2O$

13.6 g $KH_2PO_4$

per 1 litre water.

Solution C: 2.69 g $H_3BO_3$

2.00 g $MnSO_4$ * $H_2O$

0.506 g $ZnSO_4$ * 7 $H_2O$

0.126 g $Na_2MoO_4$ * 2 $H_2O$

0.078 g $CuSO_4$ * 5 $H_2O$

per 1 litre water.

First dissolve these chemicals separately in 100 ml of tap water, where $H_3BO_3$ is dissolved in hot water (± 70 °C). Then join the solutions of the first 4 chemicals, supplement with water to ± 850 ml, add the solution of $CuSO_4 * 5 H_2O$ and supplement again until 1 litre. If there is a precipitation in the $CuSO_4 * 5 H_2O$-solution or in solution C after adding of the $CuSO_4 * 5 H_2O$-solution, then add 0.5 or1 M $H_2SO_4$ until the precipitation disappears.

Solution D1: 16.659 g Titriplex III ($C_{10}H_{14}O_8N_2Na_2 *2 H_2O$)

2.91 KOH

per 500 ml distilled water

Dissolve the Titriplex in hot distilled water (± 70 °C).

Solution D2: 12.44 g $FeSO_4 *7 H_2O$

2 ml 0.5 M $H_2SO_4$

per 200 ml distilled water

Solution D: Join solutions D1 and D2, supplement until 900 ml with distilled water, aerate the solution during ca. 16 hours and supplement until 1litre with distilled water. After aeration the solution is coloured reddish brown.

**Appendix 2:** How to get from measured copper activity in millivolt to pCu

The copper activity corresponds to the measured potential, which is described by the Nernst equation:

$$E = E^0 + (RT/zF) \cdot \ln a_{Cu^{2+}}$$

Where:

$E$ = measured electrode potential (mV)

$E^0$ = reference potential (constant = 0,34 mV)

$R$ = gas constant (8,3143 J.mol-1.K-1)

$T$ = temperature (290 K)

$z$ = ion-valence (+2)

$F$ = Faraday constant (96487 C/equivalent)

$a_{Cu^{2+}}$ = free copper ion activity

This equation shows a linear relation between the measured electrode potential and the free copper ion activity expressed as pCu.

The free copper ion activity is related to concentration of free copper ions in solution:

$$a_{Cu^{2+}} = y_{Cu^{2+}} \cdot c_{Cu^{2+}}$$

where $y_{Cu^{2+}}$ is the activity coefficient and $c_{Cu^{2+}}$ the free copper ion activity.

$y_{Cu^{2+}}$ can be calculated with the Debye-Hückel equation:

$$-\log y_{Cu^{2+}} = \frac{A \cdot z^2 \cdot \sqrt{I}}{(1 + B \cdot å \cdot \sqrt{I})}$$

where $A$, $B$ and å are constants dependent on temperature and size of the copper ion, $I$ is the total ionic strength of the solution:

$$I = ½ \sum c_i \cdot z_i^2$$

From these equations it can be concluded that $y_{Cu^{2+}}$ will be practically constant in solutions with a high background concentration of ions. To adjust the background ionic strength to a high and constant value, ionic strength adjuster (ISA, 5M $NaNO_3$) was added to the three copper nitrate standard solutions used for the calibration as specified in table 1.

| **Activity** | **Concentration** | **ISA** | **pH** |
|---|---|---|---|
| pCu 5 | $10^{-5}$ M $Cu(NO_3)_2$ | 5 M $NaNO_3$ | 3 |
| pCu 6 | $10^{-6}$ M $Cu(NO_3)_2$ | 5 M $NaNO_3$ | 3 |
| pCu 7 | $10^{-7}$ M $Cu(NO_3)_2$ | 5 M $NaNO_3$ | 3 |

Table 2. Standard solutions used for the calibration of the electrodes.

The standard solutions were used to generate calibration curves for measuring pCu. An example is given in figure 4.

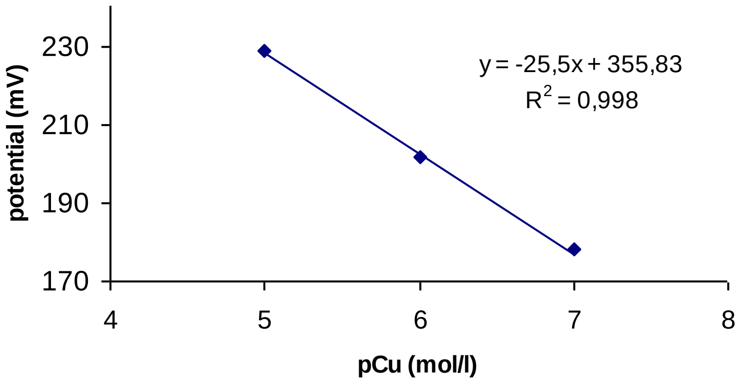

Figure 4: An example of a calibration curve used to calculate copper activity from the measured potential.

As temperature influences the measured potential (Nernst equation) all measurements were carried out at 20 ± 2 ºC.