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Chapter II

Predicting Copper Toxicity To Different Earthworm Species Using A Multicomponent Freundlich Model

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Abstract

This study aimed to develop bioavailability models for predicting Cu toxicity to earthworms (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*) in a range of soils of varying properties. A multicomponent Freundlich model, complying with the basic assumption of the biotic ligands model, was used to relate Cu toxicity to the free Cu^{2+} activity and possible protective cations in soil porewater. Median lethal concentrations (LC50s) of Cu based on the total Cu concentration varied in each species from soil to soil, reaching differences of approximately a factor 9 in *L. rubellus*, 49 in *A. longa* and 45 in *E. fetida*. The relative sensitivity of the earthworms to Cu in different soils followed the same order: *L. rubellus* > *A. longa* > *E. fetida*. Only pH not other cations (K^+, Ca^{2+}, Na^+, and Mg^{2+}) were found to exert significant protective effects against Cu toxicity to earthworms. The Freundlich-type model in which the protective effects of pH were included, explained 84%, 94%, and 96% of variations in LC50s of Cu (expressed as free ion activity) for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. Predicted LC50s never differed by a factor of more than 2 from the observed LC50s. External validation of the model showed a similar level of precision, even though toxicity data for other soil organisms and for different endpoints were used. The findings of the present study showed the possibility of extrapolating the developed toxicity models for one earthworm species to another species. Moreover, the Freundlich-type model in which the free Cu^{2+} activity and pH in soil porewater are considered can even be used to predict toxicity for other soil invertebrates and plants.

2.1 Introduction

Soil contamination with metals poses a serious threat to soil functions and sustainability of ecosystems (De Boer et al., 2011). A large amount of Cu discharge comes with the widespread use of this metal, for example, in mining industry as a floatation reagent and in agriculture as a fungicide or fertilizer (Gimeno-García et al., 1996). This strengthens the need to develop appropriate quality criteria and predictive models to evaluate to what extent and what magnitude Cu poses risks to soil organisms.

Numerous studies have shown that uptake and effects of metal depend on species, soil type and metal bioavailability (Nahmani et al., 2007; Peijnenburg et al., 2007; Van Gestel et al., 2004). To induce potential effects, metal should be bioavailable for being taken up by soil organisms (Peijnenburg et al., 2007). The porewater hypothesis proposes that exposure takes place via the porewater or that uptake of metal is mediated by a porewater related route (Van Gestel and Koolhaas, 2004; Vijver et al., 2003). The free metal ion in soil porewater is supposed to be the potential toxic species that is actually taken up by soil organisms (Morel, 1983; Peijnenburg et al., 1999). This forms the theoretical basis of using the free metal ion to predict uptake and toxicity and leads to the free ion activity model (Morel, 1983). The development of the biotic ligand model (BLM) is an extension of the free ion activity model (Campbell, 1995; De Schamphelaere and Janssen, 2002). It considers metal speciation and competition with other cations (e.g., H^+, Na^+, K^+, Ca^{2+}, Mg^{2+}). Toxicity is assumed to be proportional to the fraction of the total biotic ligand sites (transport sites or physiologically active sites) occupied by the toxic metal (De Schamphelaere and Janssen, 2002; Niyogi and Wood, 2004). Initially BLMs were proposed as a tool to quantitatively predict metal toxicity for aquatic organisms. However, the principles underlying aquatic BLMs are likely to be valid also for terrestrial species (Plette et al., 1999), especially when exposure is predominantly via the dermal route and soil organisms (such as earthworms) are in close contact with the soil porewater (Steenbergen et al., 2005; Vijver et al., 2003).

Van Gestel and Koolhaas (2004) studied the toxicity of Cd to the springtail *Folsomia candida* and found that besides the free Cd ion activity, pH in soil porewater or in water extractable fraction influenced toxicity while calcium had a mitigating effect on toxicity. This suggested that the BLM approach may be applicable for soil-dwelling organisms. A Cu-BLM was developed for the earthworm *Aporrectodea caliginosa*, exposed in solution-sand system (Steenbergen et al., 2005). Increasing H^+ and Na^+ activities mitigated Cu toxicity while increasing Ca^{2+} and Mg^{2+} activities had inconsistent effects. The final Cu-BLM developed incorporated the effects of H^+ and Na^+. The verification of the BLM in artificially contaminated soil showed that, in some cases, predictions were not within the 95% confidence interval. To predict Co toxicity to the potworm *Enchytraeus albidus*, a Co-BLM, which considered the effects of H^+, Ca^{2+}, and Mg^{2+}, was developed using the exposure system with solution and quartz sand (Lock et al., 2006). The developed model was validated in a standard artificial soil and a standardized field soil. LC50s of Co were accurately predicted, that is, by an error of less than a factor of 2. Based on toxicity tests using a range of European soils of varying properties (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006), Thakali and co-workers have developed terrestrial BLMs for predicting Cu and Ni toxicity to soil invertebrates, plants, and microbial processes (Thakali et al., 2006a; 2006b).

Some cases suggested the usefulness of the BLM to predict metal toxicity in soil, while others showed exceptions (Lock et al., 2006; Steenbergen et al., 2005; Thakali et al., 2006a; 2006b). This raises the question of whether the BLM concept is too simplified for complex soil processes.

When developing BLMs for terrestrial organisms, solution system, instead of the real soil, is often used in order to simplify the complex soil processes (Lock et al., 2006; Steenbergen et al., 2005). There may be two problems hindering the development of BLMs directly from soil system and the interpretation of data. Unlike the solution system, it is difficult to univariately modify the parameters that affect metal toxicity in soil. Another difficulty is that the intercorrelation among parameters in soil culture, for example, the amount of H^{+}, Ca^{2+}, and Mg^{2+} released to the soil porewater covaried with the amount of metal added in soil (Wang et al., 2011a). Even so, the soil exposure system was chosen in the present study considering the environmental reality. A multicomponent Freundlich model (Plette et al., 1999), rather than the BLM, was proposed to link Cu toxicity in different species of earthworms to free Cu^{2+} and possible protective cations in soil porewater. This model complies with the basic assumptions of BLM but requires fewer parameters than the BLM (Mertens et al., 2007; Ore et al., 2010), facilitating the application of BLM principles in soil exposure system.

The main objectives of the present study were to examine whether the multicomponent Freundlich model, which complies with the BLM concept and incorporates cations competition, can effectively predict Cu toxicity across different earthworm species (*Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida*), and to explore the possibility of extrapolating the study results to other studies reported in literature.

2.2 Materials and methods

Soil spiking

Soils of varying properties were collected from six different agricultural sites (Valkenswaard, Boxtel, Woerden, Drimmelen, Vlaardingen, and Mook) in The Netherlands. The soil was air-dried, homogenized and passed through a 2 mm sieve before use. These soils were spiked with Cu acetate (Acros Chemicals; purity 98%) to achieve designed levels of concentrations (from 12.5 to 4000 mg/kg depending on soils; for details see Figure S2.1 in the Supporting Information (SI)) including a control. After spiking, the soils were subjected to alternation of wetting and drying at 35 °C in a temperature cabinet for two months to eliminate acetate by mineralization. A previous study showed that the net results of hydrolysis of released Cu^{2+} and acetate mineralization exerts unnoticeable effects on soil pH (Qiu et al., 2011).

Organisms

Earthworm species used in the toxicity tests were *Eisenia fetida*, *Lumbricus rubellus*, and *Aporrectodea longa*. These species were selected because they represent a range of earthworm ecotypes. *E. fetida* (OECD recommended species) is not a species inhabiting soils from nature but they are cultured and released into the natural soils. They inhabit only organic matter-rich locations, such as animal manure or compost heaps. *L. rubellus* is epigeic, living in the uppermost 5 cm of soil and litter layers (Spurgeon et al., 2000). *A. longa* is anecic and lives in deep, permanent burrows. *E. fetida* was purchased from the Earthworm Cultivation Farm (Regenwormen, NL). Mature earthworms *L. rubellus* and *A. longa* were collected from an unexploited grassland soil located in Leiden, The Netherlands. Prior to the experiments the earthworms were acclimated in the unspiked soils for at least one week in the laboratory at 15 ± 2 °C.

Toxicity tests

Adult earthworms with a clearly developed clitellum were used. The earthworms with weight ranging from for 600 to 800 mg for *E. fetida*, 800 to 1000 mg for *A. longa*, and 700 to 900 mg for *L. rubellus* were selected for testing. Exposures were conducted in a climate room at 15 °C with an 8h-light: 16 h-dark cycle. Four earthworms were put into a plastic jar containing 500 g soil of different treatments. Each treatment was performed in triplicate. All soils were maintained at 80% maximum water holding capacity. Deionized water was added every week to compensate for water evaporation. The earthworms were fed with organic-rich food (5 g of cow manure per jar per week) during the experiment. Soil was aerated and mortality was checked every week and the dead worms were removed. After 28 days of exposure, the surviving number and fresh body weights of earthworms in each treatment were recorded (OECD, 2004). In all unspiked soils, mortality of the earthworms was less than 10% and no significant weight loss ($p > 0.05$) was observed.

Chemical analysis

Total Cu concentrations in soil samples were determined after digestion with *aqua regia*. Soil porewater was collected by means of suction over a 0.45 μm acetate filter of soil samples stored for one week at 15 °C at 100% of their maximum water holding capacity. Soil pH in 0.01 M $CaCl_2$ extracts and in porewater samples was measured using a pH meter (691, Metrohm AG) at the end of the test. Some soil samples were taken before, during, and after the tests. No noteworthy differences in soil pH were observed among different sampling periods. At the end of the test, soil texture, organic matter content (OM), and cation exchange capacity (CEC) were determined following the methods described by Pansu and Gautheyrou (2006). Dissolved organic matter in soil porewater was determined by a TOC/DOC analyzer (TOC-VCSH, Shimadzu). A copper ion-selective electrode coupled with a voltmeter with 0.1 mV resolution (Cole-Palmer, Copper Electrode) were used to measure the free Cu^{2+} activity (denoted $\{Cu^{2+}\}$) in the soil porewater. Standard stock solutions of $Cu(NO_3)_2$ of known activities at pH 2 were used to generate calibration curves for measuring $\{Cu^{2+}\}$ according to the Nernst equation. Concentrations of dissolved Cu, K, Ca, Na, and, Mg were measured by flame atomic absorption spectrophotometry (AAS, Perkin-Elmer 1100B). The detection limits of flame AAS for Cu, K, Ca, Na, and, Mg were 1.5 μg/L, 3 μg/L, 1.5 μg/L, 0.3 μg/L, and 0.15 μg/L, respectively. Standard reference material for soil (ISE 989) were used for each of 30 samples for the purpose of analytical quality control. Measured concentrations by flame AAS generally were in good agreement (±10%) with certified reference values.

Data analysis

Median lethal concentrations (LC50s) of Cu for each earthworm species in each soil were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). The Windermere Humic-Aqueous Model (WHAM VI) (Tipping, 1998) was used to calculate the activities of Cu^{2+} and other cations in soil porewater. Input data include porewater pH, colloidal fulvic acid (FA), dissolved Cu, major cation and anion concentrations (Ca, Mg, Na, K, Cl^{-} and SO_4^{2-}). It was assumed that 65% of DOC was active (available for metal binding) as the colloidal FA, while the remaining 35% was inert (Tipping et al., 2003). Presence of dissolved Cl^{-} and SO_4^{2-} in a molar ratio of 6:1 was assumed to maintain electroneutrality (Thakali et al., 2006a). For all the calculations in the present study, measured concentrations were used unless otherwise stated.

Modelling theory

A multicomponent Freundlich model was used to describe the Cu binding to the biotic ligand as it has conceptual and practical advantages over the BLM (Mertens et al., 2007; Ore et al., 2010; Plette et al., 1999). The Freundlich type model considers site heterogeneity, while in the BLM biotic ligands are considered to be chemically homogeneous with a single ligand-binding constant. Furthermore, it is flexible to describe, for example, that the effect of cations on Cu toxicity is pH dependent. By incorporating the competitive effect of protons and protective ions on Cu sorption to the biotic ligands, this model reads:

$$[\mathrm{CuBL}] = k\ \{\mathrm{Cu}^{2+}\}^{n\mathrm{Cu}}\ \{\mathrm{H}^{+}\}^{n\mathrm{H}}\ \prod\{\mathrm{C}_i^{z+}\}^{n\mathrm{Ci}} \qquad (2\text{-}1)$$

where [CuBL] is the amount of Cu assumed to be bound to the biotic ligands, k, n_{Cu}, n_{H}, and n_{Ci} are the Freundlich parameters, and $\{Cu^{2+}\}$, $\{H^{+}\}$, and $\{C_i^{z+}\}$ (i.e., $\{Na^{+}\}$, $\{Ca^{2+}\}$, $\{Mg^{2+}\}$, and $\{K^{+}\}$) are ion activities (mol/L) in soil porewater. The free Cu^{2+} activity inducing 50% mortality (LC50$\{Cu^{2+}\}$) of earthworms in different soils is assumed to be associated with a given constant [CuBL] according to the BLM theory (De Schamphelaere and Janssen, 2002). Therefore, equation 2-1 can be transformed as follows:

$$\log\mathrm{LC50}\{\mathrm{Cu}^{2+}\} = \alpha\ \mathrm{pH} - \sum \beta_i \log\{\mathrm{C}_i^{z+}\} + \gamma \qquad (2\text{-}2)$$

where the coefficients α (= $n_{\mathrm{H}}/n_{\mathrm{Cu}}$), β_i (= $n_{\mathrm{Ci}}/n_{\mathrm{Cu}}$), and γ are constants. Stepwise multiple linear regression analysis was carried out using SPSS 16.0 (IBM) to decide which toxicity-modifying factors (H^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, and K^{+}) need to be included in the model. This model was further applied to predict Cu toxicity to a range of other soil organisms with different endpoints using the underlying data in the literature (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b).

The general practice in applying the toxicity model to the data is to calculate individual toxic endpoints (LC50 or EC50) for each species in each soil (i.e., point estimates of toxicity), as the best test of a model's predictive ability is how well it predicts LC50 or EC50 (Thakali et al., 2006a). However, it may also be possible to extend the multicomponent Freundlich model (equation 2-2) to consider the entire dose–response curve. Although γ is constant at a given effect level, it will vary according to the effect level being described. The coefficient α and β_i, describing the effects of cations on Cu toxicity, are assumed to be independent of the effect level (Lofts et al., 2004). Generalizing to any effect level, the model reads

$$\log\{\mathrm{Cu}^{2+}\}_{\mathrm{EFFECT}} = \alpha\ \mathrm{pH} - \sum\beta_i \log\{\mathrm{C}_i^{z+}\} + \gamma_{\mathrm{EFFECT}} \qquad (2\text{-}3)$$

or:

$$\gamma_{EFFECT} = \log\{Cu^{2+}\}_{EFFECT} - \alpha\, pH + \sum \beta_i \log\{C_i^{z+}\} \quad (2\text{-}4)$$

here, γ_{EFFECT} can be interpreted as the effect dose that incorporates not only the $\{Cu^{2+}\}$, but also the terms describing the effects of bioavailability, and differences in inherent sensitivity of organisms to Cu. $\{Cu^{2+}\}_{EFFECT}$ is the corresponding value of $\{Cu^{2+}\}$ at any given effect level.

The entire data set for each earthworm species were fitted with a logistic dose−response curve (equation 2-5) (Haanstra et al., 1985) using total Cu concentration, $\{Cu^{2+}\}$, and γ_{EFFECT} as dose, respectively.

$$R = \frac{R_0}{1 + (\frac{x}{x_{50}})^{\beta}} \quad (2\text{-}5)$$

where R = response, R_0= control response, x = total Cu concentration, $\{Cu^{2+}\}$, and γ_{EFFECT}, x_{50} = concentration (dose) at the 50% effect level, and β = shape parameter. R was plotted against x to fit the parameters x_{50} and β. The model parameters were estimated by minimizing the RMSE (root-mean-square error) using the SOLVER program in Microsoft Excel 2010.

2.3 Results and discussion

Soil and porewater properties

The most important properties of the unspiked soils are presented in Table 2.1. These soils represented a range of soil types and varied in soil pH, OM, and CEC, etc. The selected properties of soil porewater are listed in Table S2.1 in the SI. Soil porewater pH prior to spiking ranged from 5.0 ± 0.2 to 8.0 ± 0.3. The addition of different amounts of Cu (0-4000 mg/kg) only induced marginal effects (usually < 0.3 units) on the soil porewater pH (Figure S2.1 in the SI). It has been reported that the effects of Cu spiking on soil properties can be reduced to a minimum by using Cu acetate instead of other Cu salts (e.g., $CuCl_2$, $Cu(NO_3)_2$, and $CuSO_4$) (Qiu et al., 2011). Dissolved Cu concentrations in soil porewater increased with increasing total Cu concentrations. Significant linear relationships between porewater Cu and total Cu were found for all soils ($p < 0.0001$) (Table S2.2 in the SI). The significance of the regression equations was not improved by the inclusion of porewater pH or DOC as explanatory variables. The relationship between calculated and measured pCu ($-\log\{Cu^{2+}\}$) in the porewater of all soils is shown in Figure S2.2 in the SI. The free Cu^{2+} activities spanned almost 6 orders of magnitude. A significant correlation between calculated and measured pCu was observed ($R^2 = 0.85$, $n = 75$, $p < 0.0001$, $F = 369.6$). WHAM VI provided robust predictions of free Cu^{2+} activities even for the alkaline soils. According to a soil solid-solution partition model for metals (Lofts et al., 2004), pCu in the porewater of all soils conformed to a equation: pCu = 0.714pH − 0.211log(total Cu) + 3.565log(OM) + 5.203, ($R^2 = 0.954$, $p < 0.001$; total Cu in mg/kg, organic matter content (OM) in %), as obtained by multivariate linear regression. This suggested that metal partitioning and speciation was to a large extent determined by the soil pH and organic matter content.

Table 2.1 Selected soil properties of the unspiked soils. All values are given as means of three replicates.

	Valkenswaard	Boxtel	Woerden	Drimmelen	Vlaardingen	Mook
pH[a]	5.5±0.2	6.2±0.3	5.8±0.2	7.9±0.1	7.3±0.2	4.5±0.2
Total Cu[b] (mg/kg)	9.0±0.5	10.6±0.44	26.2±1.7	25.7±1.1	29.1±0.5	8.2±0.3
Texture[c]	loamy sand	sandy loam	silt loam	clay loam	loam	sandy
Clay (%)	5.9	7.0	16.5	33.8	27.1	0.8
Silt (%)	8.9	22.5	53.2	46.1	40.9	2.9
Sand (%)	85.2	70.5	30.3	20.1	32.0	96.3
OM[d] (%)	6.4±0.9	5.7±0.2	21.7±1.3	10.2±0.6	12.3±0.7	2.0±0.4
CEC[e] (cmol/kg)	8.3±0.4	10.1±0.3	38.8±2.2	16.5±1.2	30.9±2.2	1.7±0.3
WHC[f] (%)	36.5±3.3	53.5±4.6	69.3±5.7	47.7±2.9	52.1±4.6	25.1±3.2

[a]pH in 0.01M $CaCl_2$ extract. [b]*aqua regia* digestion. [c]Determined by the hydrometer method (Pansu and Gautheyrou, 2006). [d]Organic matter content determined by the ignition method (Pansu and Gautheyrou, 2006). [e]Cation exchange capacity determined by ammonium acetate method (Pansu and Gautheyrou, 2006). [f]Maximum water-holding capacity determined by the saturation and gravity drainage method (Pansu and Gautheyrou, 2006).

Entire dose−response relationships

The relationships between survival rate of earthworms and three different expressions of exposure (total Cu concentration, $\{Cu^{2+}\}$, andγ_{EFFECT}) are shown in Figure 2.1. Earthworm survival rate decreased with increasing total Cu concentrations (Figure 2.1, first column). Copper toxicity to each earthworm species varied widely in the different soils. When expressed as total concentration, LC50 of Cu ranged from 32.4 to 284 mg/kg for *L. rubellus*, from 39.5 to 1942 mg/kg for *A. longa*, and from 82.8 to 3717 mg/kg for *E. fetida* (Table 2.2). Apart from calculating LC50s in six individual soils, an overall LC50 for each species was also obtained by fitting the toxicity data in all soils together with equation 2-5 (Table 2.2). In case of using the total Cu concentration as the expression for Cu toxicity, poor fits were observed with R^2 of 0.35 and RMSE of 33.9 for *L. rubellus*, R^2 of 0.32 and RMSE of 36.9 for *A. longa*, and R^2 of 0.27 and RMSE of 33.0 for *E. fetida* (Figure 2.1, first column). The large differences between the individual LC50 values and the overall LC50 values showed that total soil concentration failed to explain the variation in Cu toxicity among soils.

When using the computed $\{Cu^{2+}\}$ as dose, the goodness of fit was considerably improved (Figure 2.1, second column). The R^2 and RMSE values were, respectively, 0.79 and 18.7 for *L. rubellus*, 0.89 and 14.1 for *A. longa*, and 0.57 and 25.9 for *E. fetida* (Figure 2.1). LC50$\{Cu^{2+}\}$ for different earthworms in each soil are shown in Table 2.2. There are extensive evidence showing that total concentrations of metals in soils are poor indicators for toxicity and may result in either underestimation or overestimation of the actual risks (Mertens et al., 2007; Peijnenburg et al., 2007; Thakali et al., 2006b; Van Gestel and Koolhaas, 2004), while free ion activities are the immediately available metal fractions and can represent the bioavailability much better (Hobbelen et al., 2006). The results of the present study confirmed what could be expected: free Cu^{2+} in soil porewater, rather than total Cu in soil, is the dominant toxic species for earthworms.

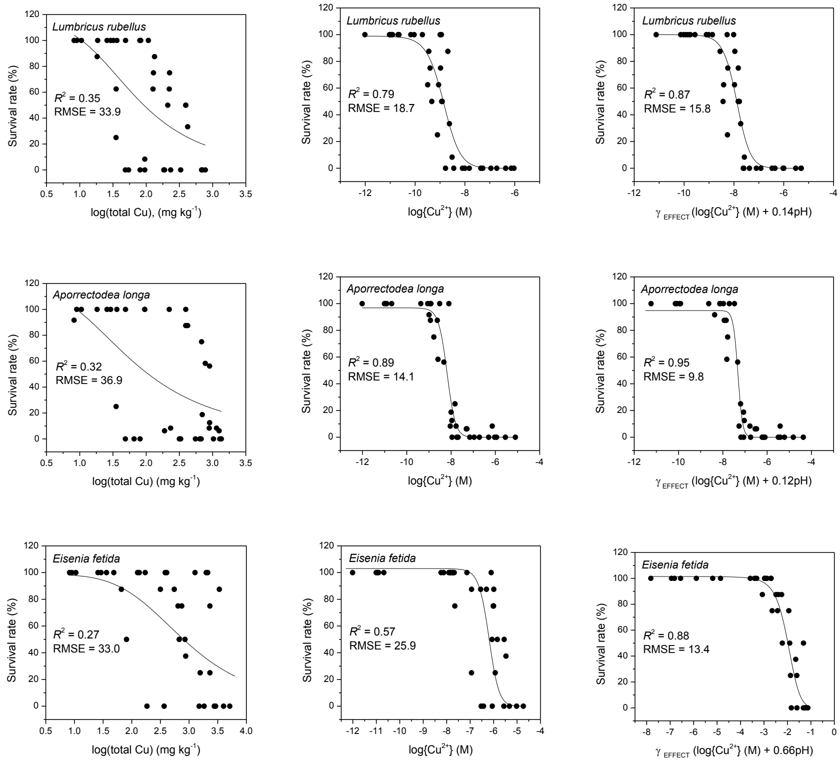

Figure 2.1 Dose−response relationships between survival rate and total Cu concentration in soil (first column), free Cu^{2+} activity in soil porewater (second column), effect dose γ_{EFFECT} (third column) for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six tested soils after 28 days of exposure. The solid lines represent the log-logistic model fits (equation 2-5). R^2 indicates the coefficient of determination of the linear regression between the predicted and observed survival rate. RMSE indicates root mean square error of the predicted survival rate.

Table 2.2 Median lethal concentrations of Cu expressed as total Cu concentrations (LC50[Cu], (mg/kg)) and free Cu^{2+} activities (LC50{Cu^{2+}}, (M)) for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* exposed in different soils for 28 days (*n* = 3).

Soils	LC50[Cu] [a] for *L. rubellus*	logLC50{Cu^{2+}} for *L. rubellus*	LC50[Cu] for *A. longa*	logLC50{Cu^{2+}} for *A. longa*	LC50[Cu] for *E. fetida*	logLC50{Cu^{2+}} for *E. fetida*
Boxtel	72.2	-8.65	111.1	-7.89	1322	-6.14
	(64.7~80.4) [b]	(-8.52~-8.80)	(80.2~153.9)	(-7.42~-8.17)	(1033~1692)	(-5.89~-6.31)
Drimmelen	279.2	-8.94	753.2	-8.37	2486	-7.41
	(193.8~402.2)	(-8.82~9.22)	(559.2~1014)	(-8.18~-8.52)	(2390~2586)	(-7.18~-7.67)
Mook	32.4	-7.69	39.5	-6.94	82.8	-5.46
	(28.1~37.3)	(-7.50~-7.83)	(34.9~44.7)	(-6.71~-7.16)	(68.5~100.1)	(-5.24~-5.59)
Valkenswaard	36.1	-8.09	156.8	-7.12	667.0	-5.56
	(30.4~42.9)	(-7.91~-8.23)	(142.5~172.5)	(-6.84~-7.43)	(574.5~774.4)	(-5.33~-5.72)
Vlaardingen	238.3	-9.30	618.0	-8.44	2233	-7.13
	(162.9~348.6)	(-9.11~-9.42)	(548.9~695.7)	(-8.12~-8.70)	(2144~2326)	(-7.01~-7.38)
Woerden	283.6	-8.36	1942	-7.68	3717	-6.05
	(208.4~385.8)	(-8.25~-8.62)	(1779~2119)	(-7.34~-7.81)	(3678~3756)	(-5.85~-6.26)
All Soils	139.1 (NA) [c]	-8.86 (NA)	175.9 (NA)	-7.32 (NA)	1001 (NA)	-6.17 (NA)

[a] Individual LC50 for each species in each soil was calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). The overall LC50 for each species was obtained by fitting the toxicity data in all tested soils together with a logistic dose-response curve (equation 2-5).
[b] 95% confidence intervals. [c] Not applicable

When applying the logistic dose−response model to fit all data with the effect dose ($\gamma_{EFFECT} = \log\{Cu^{2+}\}_{EFFECT} - \alpha$ pH), the model provided superior fits in comparison to the fits obtained with other models for *L. rubellus* (R^2 = 0.87, RMSE = 15.8), for *A. longa* (R^2 = 0.95, RMSE = 9.8), and especially for *E. fetida* (R^2 = 0.88, RMSE = 13.4) (Figure 2.1, third column). The effect dose term was then extended to consider an extra cation (Na^+, Ca^{2+}, Mg^{2+}, and K^+) as well as H^+, the corresponding R^2 values for *L. rubellus* were 0.87, 0.85, 0.87, and 0.86, respectively, for *A. longa* were 0.96, 0.97, 0.95, and 0.93, respectively, and for *E. fetida* were 0.89, 0.86, 0.90, and 0.87, respectively. This indicated that the predictive capacity of the model was not enhanced by accounting for the effect of other cations. According to the BLM theory, it was postulated that metal toxicity is dependent on the free ion activity in soil porewater and that cations competition can modify metal toxicity (De Schamphelaere and Janssen, 2002; Thakali et al., 2006a). By accounting for the bioavailability-modifying factor pH only, the application of the effect dose γ_{EFFECT} provided a feasible approach for modelling the entire dose−response curves of Cu toxicity to earthworms.

Species sensitivities

Table 2.2 provides more information on species variation. The species variation was observed in terms of sensitivity (LC50), decreasing in the order: *L. rubellus* > *A. longa* > *E. fetida*. Spurgeon et al. (2000) found that earthworms *L. rubellus* and *A. caliginosa* were more sensitive to Zn than *L. terrestris* and *E. fetida.* Langdon et al. (2005) reported that the sensitivity of three earthworm species to Pb followed the decreasing order: *L. rubellus* > *A. caliginosa* > *E. fetida*. Species-specific differences in ecological strategies and physiological characteristics (detoxification and elimination strategies) might account for the differences in earthworm sensitivity (Morgan et al., 2002; Nahmani et al., 2007). The epigeic *E. fetida* feeds almost entirely on the soil surface on organic matter (cow manure in the present study), whereas endogeic *A. caliginosa* and anecic *A. longa* would be more exposed to metals as they live and feed in the soil (Langdon et al., 2005). In addition, the activity of calcium glands in earthworms may partially account for the differences in sensitivity as calcium involves in the sequestration and elimination of many metals. It was found that the more tolerant species *E. fetida* has more active calcium secretion glands than the other sensitive species (Piearce, 1972; Spurgeon et al., 2000).

Cu toxicity to earthworm was soil and species specific. It is worth to note that although soil factors influenced to different extents the LC50s of Cu for different earthworm species, the relative sensitivity of these species did not change in different soils, that is, all earthworm species tested, irrespective of their ecological characteristics and ecotypes, gave the same ranking in sensitivity to Cu. These findings provided the possibility of extrapolating the results for one earthworm species to other earthworm species, which is significant for further developing a multispecies toxicity model for the purpose of environmental risk assessment.

Prediction of LC50{Cu^{2+}}

Stepwise multiple-linear-regression analysis was used to generate equations to predict the LC50{Cu^{2+}} in relation to soil porewater properties. The soil porewater properties at LC50s for each soil were interpolated from the measured values (Table S2.3−S2.5 in the SI). For all three earthworm species (*L. rubellus*, *A. longa, and E. fetida*), only pH was identified as the explanatory variable of Cu toxicity and incorporated into the multicomponent Freundlich model (equation 2-2). Other cations were the excluded variables as they did not improve the model fit significantly (when the Freundlich-type model comprising pH and one of the variables Na^+, Ca^{2+}, Mg^{2+}, and K^+, the corresponding R^2 values for *L. rubellus* were 0.80, 0.83, 0.79, and 0.85, respectively, for *A. longa* were 0.92, 0.93, 0.93, and 0.92, respectively, and for *E. fetida* were 0.95, 0.98, 0.98, and 0.98, respectively). The estimated parameters for the final model are given in Table 2.3. In all cases, model parameter α for pH was negative, indicating increased toxicity with increasing pH (i.e., decreasing H^+), which is in agreement with the competitive concept of the BLM. Model parameter α revealed that an increase in pH with one unit results in a 2.6-fold, 4.6-fold and 6.0-fold decrease of LC50{Cu^{2+}} for *L. rubellus*, *A. longa*, and *E. fetida*, respectively. The model obtained explained, respectively, 84%, 94%, and 96% of the variance in logLC50{Cu^{2+}} for *L. rubellus*, *A. longa*, and *E. fetida*.

Table 2.3 Parameter estimates for the multicomponent Freundlich Model (equation 2-2) using the data of the present study on Cu toxicity to earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six soils tested and the literature data on Cu toxicity to other soil invertebrates, plants and microbial processes in a range of European soils. The parameters were estimated by multiple-linear-regression.

	α	*γ*	R^2_{adj} [h]
L. rubellus	−0.42 (0.09)[g]	−5.84 (0.61)	0.84
A. longa	−0.66 (0.07)	−3.27 (0.47)	0.94
E. fetida	−0.78 (0.07)	−1.24 (0.46)	0.96
ECP[a]	−0.93 (0.09)	−0.94 (0.42)	0.94
FJP[b]	−0.56 (0.08)	−2.35 (0.40)	0.86
HRE[c]	−0.82 (0.07)	−2.46 (0.38)	0.93
LSY[d]	−0.87 (0.09)	−1.99 (0.47)	0.91
GIR[e]	−1.27 (0.24)	1.52 (0.91)	0.79
PNR[f]	−0.69 (0.12)	−2.15 (0.74)	0.74

[a]ECP is *Eisenia fetida* cocoon production (Criel et al., 2008; Thakali et al., 2006b). [b]FJP is *Folsomia candida* juvenile production (Criel et al., 2008; Thakali et al., 2006b). [c]HRE is *Hordeum vulgare* root elongation (Rooney et al., 2006; Thakali et al., 2006a), [d]LSY is *Lycopersicon esculentum* shoot yield (Rooney et al., 2006; Thakali et al., 2006b). [e]GIR is glucose induced respiration (Oorts et al., 2006; Thakali et al., 2006b). [f]PNR is potential nitrification rate (Oorts et al., 2006; Thakali et al., 2006b). [g]Standard errors are indicated in brackets. [h]R^2_{adj} indicates the coefficients of determination adjusted for the degrees of freedom for observed versus predicted values.

The accuracy of model predictions is shown in Figure 2.2A. In general, the model gave good predictions for Cu toxicity to all different species of earthworms tested. Predicted values were in good agreement with the observed values, with an error of less than a factor of 2, indicating that the multicomponent Freundlich can be used to predict Cu toxicity to earthworms irrespective of their ecotypes in a range of soils of varying properties.

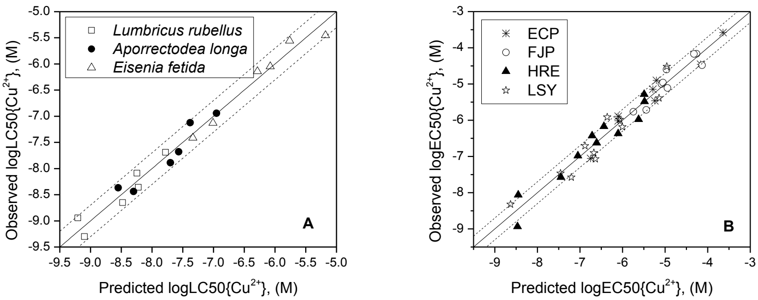

Figure 2.2 Relationships between the observed and predicted logL(E)C50{Cu^{2+}} for the earthworms *Lumbricus rubellus*, *Aporrectodea longa*, and *Eisenia fetida* in all six tested soils after 28 days of exposure (A), and for ECP, FJP, HRE, and LSY in a range of European soils (B). ECP, FJP, HRE, and LSY are *Eisenia fetida* cocoon production (Criel et al., 2008; Thakali et al., 2006b), *Folsomia candida* juvenile production (Criel et al., 2008; Thakali et al., 2006b), *Hordeum vulgare* root elongation (Rooney et al., 2006; Thakali et al., 2006a), and *Lycopersicon esculentum* shoot yield (Rooney et al., 2006; Thakali et al., 2006b), respectively. The predictions were based on the multicomponent Freundlich model (equation 2-2) using the parameters given in Table 2.3. The solid line represents the 1:1 line, and the dashed lines represent a factor of 2 differences between the observed and predicted values.

To evaluate if the existing BLMs for one earthworm species can be extrapolated toward other earthworm species, the first step is to qualitatively validate if the toxicity-modifying effects (protective cations) are similar for different earthworm species. For *A. caliginosa*, significant competition of H^+ and Na^+ with Cu^{2+} has been observed with mortality as endpoint in a solution-sand system (Steenbergen et al., 2005). For *E. fetida*, only the protective effect of H^+ (not Ca^{2+} and Mg^{2+}) on Cu toxicity was found with cocoon production as endpoint in soil (Thakali et al., 2006b). In the present study, the same Cu toxicity-modifying factor (pH) was identified for *L. rubellus*, *A. longa*, and *E. fetida*, which was consistent with the findings of Thakali et al. (2006b). The extrapolation of the results from solution to soil is difficult as the exposure is different and soil processes are far more complex than the simple solution. A structural underestimation of toxicity was found when directly applying the model developed from solution-sand system to soils (Koster et al., 2006). The multicomponent Freundlich model in which the competitive effects of pH were included, well predicted logLC50{Cu^{2+}} for all the test species. With regard to the physical meaning of the model parameters, the exponent n_{Cu} (equation 2-1) reflects the sensitivity of species to Cu. It could probably be used as an indicator of toxic strength of metals when different metals are assessed. The exponents n_H and n_{Ci} (equation 2-1) describe to what extent Cu interactions with the earthworms is dependent on pH and on other cations. These parameters are soil and organism specific as the amount and the type of binding sites will vary (Plette et al., 1999). The deduced coefficients α (= n_H/n_{Cu}) and β_i (= n_{Ci}/n_{Cu}) (equation 2-2) quantify the competition of H^+ and other cations with Cu^{2+} at the site of toxic action. Thus, the α values differed for different species, suggesting that the effects of pH on Cu toxicity differed per species. In our study, the obtained α values did reflect the actual sensitivity of earthworms to Cu by taking the toxicity-modifying factors into account and the γ values were constants at the given effect level.

In the literature, no consistent effects of cations (K^+, Na^+, Ca^{2+}, and Mg^{2+}) on Cu toxicity are found. Protective effects of H^+, Ca^{2+}, and Mg^{2+} against Cu toxicity to bioluminescence of *Nitrosomonas europaea* were observed in a study by Ore et al.(2010). Le et al. (2012) developed a BLM to estimate Cu toxicity to lettuce (*Lactuca sativa*) in terms of root elongation. They found that only H^+ can be integrated into the BLM, and competitive effects of the cations (K^+, Na^+, Ca^{2+}, and Mg^{2+}) were insignificant. A protective effect of Ca^{2+} and Mg^{2+} and no effect of H^+, Na^+ and K^+ were found for wheat root (Luo et al., 2008). The appearance of the inconsistencies might be explained from the following aspects. First, it is most likely that binding constants for different cations differ across species. Second, the covariance between H^+ and other cations may mask the effects of cations competition in soil solution. Such covariance was observed in the study of Thakali et al. (2006a; 2006b). They showed that major cations (such as Ca^{2+} and Mg^{2+}) tend to increase with increasing Cu concentrations and decreasing pH. Third, the difference in the endpoints used is a possible explanation. For example, an obvious effect on reproduction does not mean a visible effect on the mortality (Santorufo et al., 2012). Even ranking of species sensitivity can change with the endpoints selected. Last, the narrow range of dissolved cations concentrations in the porewater of the soils tested may also limit the assessment of the effects of cations on Cu toxicity.

Extrapolation of study results to other studies

An important question in generalizing the toxicity model within a regulatory framework is whether or not the model developed for one species can be extrapolated to other species with different endpoints. In case of Cu, there are existing data sets (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b) that are suitable for external validation of the applicability of the multicomponent Freundlich model. These data sets consist of six toxicity tests, covering soil invertebrates, plants and microbial processes, in a range of European soils. The levels of K^+, Na^+, Ca^{2+}, and Mg^{2+} in soil porewater (Criel et al., 2008; Oorts et al., 2006; Rooney et al., 2006; Thakali et al., 2006a; 2006b) were comparable to that of the present study. It should be realized that when other studies and other species were used, they may be exposed to different soils with different properties. Previously, these toxicity data were used for developing Cu-BLMs (Thakali et al., 2006a; 2006b) and a pH dependence of Cu^{2+} toxicity was found for all species. In the present study, equation 2-2 with only pH of the soil porewater as the explanatory variable was applied to the underlying data from the literature. Table 2.3 shows the estimated model parameters for *E. fetida* cocoon production (ECP), *Folsomia candida* juvenile production (FJP), *Hordeum vulgare* root elongation (HRE), and *Lycopersicon esculentum* shoot yield (LSY). The pH exerted similar effects on Cu toxicity for ECP, HRE and LSY as reflected by the parameter α. More than 86% of the variation in toxicity was explained by the models for all these species. Nearly all the predicted values of $\log EC50\{Cu^{2+}\}$ were within a factor of 2 of the observed values (Figure 2.2B). This level of precision in prediction of EC50 is similar to that of the BLM developed by Thakali et al. (2006a; 2006b). For the microbial processes glucose induced respiration (GIR) and potential nitrification rate (PNR), the toxicity variances explained by the model were 79% and 74%, respectively (Table 2.3). The model performed better in predicting Cu toxicity to soil invertebrates and plants than to soil microorganisms. This finding could be explained by the varying microbial communities among soils and the fact that they are less mobile as soil microorganisms are usually attached to the soil particles, suggesting that the microbe bathing solution may differ from the porewater that can often be used to assess metal bioavailability for soil invertebrates and plants (Mertens et al., 2007; Ore et al., 2010). Therefore, care should be taken when extrapolating the results to microbial processes. The generalized model ($\log L(E)C50\{Cu^{2+}\} = \alpha\ pH + \gamma$) accurately predicted toxicity for soil invertebrates and plants across different soils, suggesting that the same processes of the soil (i.e., the impact of pH) played a role in ranking Cu toxicity for these soil organisms and that the model can be extrapolated to other soil organisms except for microorganisms.

2.4 Implications

In the context of implementing the use of bioavailability models into environmental risk assessment of Cu for soil organisms, it is important to assess the effects of the variability of porewater chemistry parameters on Cu toxicity across different species. To produce a theoretical function for soil Cu toxicity, a terrestrial BLM is an option. However, unlike the case of a hydroponic system, it is difficult to provide direct evidence for the protective effects of cations in soils. The prerequisite of the BLM in requiring linear relations between the protective effects of the cations and metal toxicity was not met by the results of the present study. The multicomponent Freundlich model, requiring fewer parameters than the BLM, proved to be a feasible framework for directly linking the porewater chemistry to Cu toxicity in soils for various soil dwelling organisms and plants with different endpoints. Based on the results of our study, the developed models can be used to evaluate environmental risks associated with the specific Cu-contaminated soil for the corresponding species as long as the Cu^{2+} activity and pH in the soil porewater are known. In practice, soil solutions tend to exhibit negative covariance of pH and other cations since pH is controlled by the concentrations of the other ions (Lofts et al., 2004; Thakali et al., 2006b; Wang et al., 2011a). Although Na^+, Ca^{2+} and Mg^{2+} may be important factors in modifying metal toxicity by means of competition, the equation ($\log LC50\{Cu^{2+}\} = \alpha\ pH + \gamma$) does not imply that the H^+ alone exerts a protective effect but rather that the overall protective effect of all free cations may be expressed as a function of pH alone (Lofts et al., 2004). It is thus possible that the need to consider the effects of other cations implicitly rather than explicitly is not a source of significant uncertainty in the model derived. Even so, for future users of the model, it is suggested that where free cation activities (especially Ca^{2+} and Mg^{2+}) are controlled by solid phases, an explicit consideration of these cations (whether or not to be included in the extended model) may be appropriate. In those cases, the Freundlich-type model can be easily extended to incorporate the effect of those cations ($\log LC50\{Cu^{2+}\} = \alpha\ pH - \sum \beta_i \log\{C_i^{z+}\} + \gamma$). Thus, the extrapolation is possible in both cases.

Supporting information

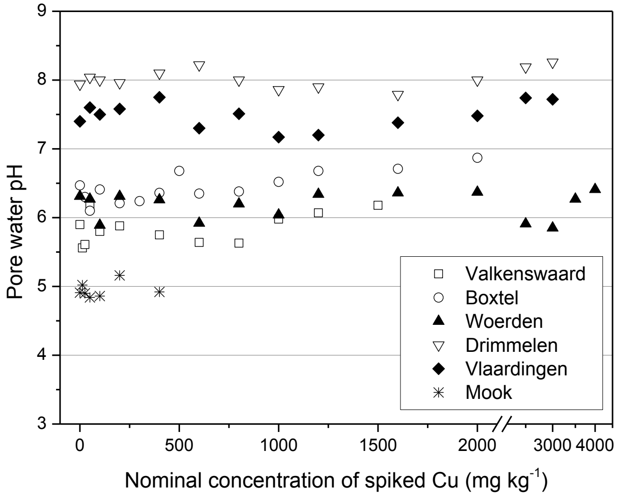

Figure S2.1 Relationships between soil porewater pH and nominal concentrations of Cu spiked into the soils tested. For soil Valkenswaard, 12 levels of Cu concentrations (0-1500 mg/kg) were spiked; for soil Boxtel, 14 levels of Cu concentrations (0-2000 mg/kg) were spiked; for soil Woerden, 15 levels of Cu concentrations (0-4000 mg/kg) were spiked; for soil Drimmelen and soil Vlaardingen, 13 levels of Cu concentrations (0-3000 mg/kg) were spiked; for soil Mook, 8 levels of Cu concentrations (0-400 mg/kg) were spiked.

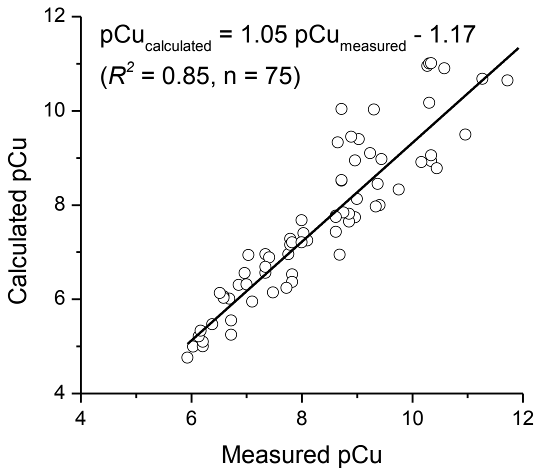

Figure S2.2 Relationship between the calculated free Cu^{2+} activity (expressed as pCu) by the Windermere Humic-Aqueous Model and the measured pCu by copper ion-selective electrode in the porewater of all six tested soils.

Table S2.1 Selected properties of the porewater of unspiked soils (Valkenswaard, Boxtel, Woerden, Drimmelen, Vlaardingen, and Mook). All values are given as means of three replicates.

	Valkenswaard	Boxtel	Woerden	Drimmelen	Vlaardingen	Mook
pH	5.8	6.4	6.2	8.0	7.4	5.0
Dissovled Cu (mg/L)	0.07	0.03	0.05	0.04	0.03	0.04
DOC[a] (mg/L)	149.4	181.7	502.5	192.3	342.4	188.6
Dissovled Na (mg/L)	41.3	66.1	119.1	97.4	45.0	43.8
Dissovled K (mg/L)	190.9	68.8	39.0	15.3	31.7	96.8
Dissovled Ca (mg/L)	120.3	198.7	509.2	369.7	336.7	101.0
Dissovled Mg (mg/L)	92.6	71.5	141.0	56.3	51.5	79.4

[a]Dissolved organic carbon determined with TOC analyzer (TOC-VCSH, Shimadzu)

Table S2.2 Linear regression relationships between dissolved Cu concentrations in soil porewater (Cu_{pw}, mg/L), total soil Cu concentrations (Cu_{total}, mg/kg), pH of the porewater, and dissolved organic carbon (DOC, mg/L) in the six tested soils

Soils	Equations	R^2_{adj}[a]	n	F	p
Valkenswaard	$\log Cu_{pw} = 0.84\ (\pm 0.04) \log Cu_{total} - 1.88\ (\pm 0.09)$	0.978	12	507.7	<0.0001
	$\log Cu_{pw} = 0.84\ (\pm 0.04) \log Cu_{total} - 0.04\ (\pm 0.05)\ pH - 1.63\ (\pm 0.29)$	0.978	12	251.1	<0.0001
	$\log Cu_{pw} = 0.91\ (\pm 0.04) \log Cu_{total} - 0.08\ (\pm 0.04)\ pH + 0.002\ (\pm 0.0006)\ DOC - 1.82\ (\pm 0.24)$	0.986	12	272.9	<0.0001
Boxtel	$\log Cu_{pw} = 1.01\ (\pm 0.04) \log Cu_{total} - 2.49\ (\pm 0.10)$	0.979	14	630.4	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm 0.04) \log Cu_{total} - 0.16\ (\pm 0.06)\ pH - 1.52\ (\pm 0.41)$	0.985	14	444.6	<0.0001
	$\log Cu_{pw} = 0.99\ (\pm 0.05) \log Cu_{total} - 0.20\ (\pm 0.07)\ pH + 0.001\ (\pm 0.0008)\ DOC - 1.32\ (\pm 0.41)$	0.986	14	325.9	<0.0001
Woerden	$\log Cu_{pw} = 1.09\ (\pm 0.06) \log Cu_{total} - 3.16\ (\pm 0.22)$	0.941	15	222.7	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm 0.07) \log Cu_{total} - 0.12\ (\pm 0.07)\ pH - 2.25\ (\pm 0.57)$	0.948	15	128.7	<0.0001
	$\log Cu_{pw} = 0.92\ (\pm 0.05) \log Cu_{total} - 0.06\ (\pm 0.05)\ pH + 0.0004\ (\pm 0.0001)\ DOC - 2.53\ (\pm 0.36)$	0.979	15	229.0	<0.0001
Drimmelen	$\log Cu_{pw} = 0.84\ (\pm 0.07) \log Cu_{total} - 2.45\ (\pm 0.19)$	0.926	13	153.2	<0.0001
	$\log Cu_{pw} = 0.83\ (\pm 0.07) \log Cu_{total} - 0.34\ (\pm 0.31)\ pH - 5.12\ (\pm 2.44)$	0.928	13	78.7	<0.0001
	$\log Cu_{pw} = 0.75\ (\pm 0.03) \log Cu_{total} - 0.15\ (\pm 0.13)\ pH + 0.002\ (\pm 0.0002)\ DOC - 1.33\ (\pm 1.02)$	0.990	13	402.7	<0.0001
Vlaardingen	$\log Cu_{pw} = 1.04\ (\pm 0.07) \log Cu_{total} - 2.82\ (\pm 0.20)$	0.948	13	220.3	<0.0001
	$\log Cu_{pw} = 1.04\ (\pm 0.07) \log Cu_{total} - 0.10\ (\pm 0.14)\ pH - 2.09\ (\pm 1.07)$	0.946	13	105.2	<0.0001
	$\log Cu_{pw} = 0.91\ (\pm 0.03) \log Cu_{total} - 0.14\ (\pm 0.04)\ pH + 0.0009\ (\pm 0.0001)\ DOC - 1.74\ (\pm 0.33)$	0.994	13	760.1	<0.0001
Mook	$\log Cu_{pw} = 0.92\ (\pm 0.09) \log Cu_{total} - 1.40\ (\pm 0.16)$	0.936	8	104.0	<0.0001
	$\log Cu_{pw} = 0.99\ (\pm 0.08) \log Cu_{total} - 0.19\ (\pm 0.10)\ pH - 0.56\ (\pm 0.45)$	0.956	8	77.7	<0.0001
	$\log Cu_{pw} = 1.08\ (\pm 0.06) \log Cu_{total} - 0.24\ (\pm 0.07)\ pH + 0.001\ (\pm 0.0004)\ DOC - 0.70\ (\pm 0.29)$	0.982	8	129.9	<0.0001

[a] R^2_{adj} is the coefficient of determination adjusted for the degrees of freedom; n indicates the number of data points; F is the value of F test; p indicates the statistical significance level; Standard errors are indicated in brackets.

Table S2.3 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Lumbricus rubellus* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater (mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	72.2	6.39	161.4	252.5	74.5	73.4	66.3	0.29
Drimmelen	279.2	8.00	168.9	419.2	69.2	103.4	15.6	0.50
Mook	32.4	4.95	220.8	138.2	92.8	53.5	137.8	0.94
Valkenswaard	36.1	5.98	186.3	105.9	69.5	46.4	171.7	0.29
Vlaardingen	238.3	7.54	260.0	332.5	65.9	41.1	33.0	0.63
Woerden	283.6	6.21	323.4	526.0	161.0	118.3	43.1	0.28

Table S2.4 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Aporrectodea longa* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater(mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	111.1	6.43	176.9	267.9	82.4	77.5	71.2	0.50
Drimmelen	753.2	8.03	263.4	438.3	76.8	111.0	17.7	1.22
Mook	39.5	4.95	226.9	147.6	102.8	57.8	141.9	1.23
Valkenswaard	156.8	5.66	171.5	182.2	125.0	66.1	234.1	0.92
Vlaardingen	618.0	7.44	423.2	437.6	92.8	56.1	37.7	1.77
Woerden	1942	6.19	533.1	496.3	152.4	119.4	39.0	2.90

Table S2.5 The soil porewater properties (pH, dissolved organic carbon (DOC), and dissolved cations concentrations) interpolated at LC50 values of Cu (expressed as total soil concentration) for *Eisenia fetida* in the six soils tested. The function TREND in Microsoft Excel 2010 was used for interpolation based on the measured values.

Soil	LC50 (mg/kg)	Porewater pH	DOC (mg/L)	Dissolved cations in soil porewater (mg/L)				
				Ca	Mg	Na	K	Cu
Boxtel	1322	6.64	217.6	198.7	57.8	69.8	63.7	3.87
Drimmelen	2486	7.94	196.0	341.2	36.2	86.8	13.2	2.10
Mook	82.8	5.05	218.4	100.9	143.9	54.4	140.4	2.31
Valkenswaard	667.0	5.72	130.8	120.3	112.1	62.3	147.5	3.07
Vlaardingen	2233	7.49	369.0	336.6	45.7	39.3	29.5	3.56
Woerden	3717	6.17	559.0	420.9	136.5	122.8	38.6	6.14