COPPER TOXICITY THRESHOLDS IN CHINESE SOILS BASED ON SUBSTRATE-INDUCED NITRIFICATION ASSAY

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Abstract—Copper toxicity in 17 Chinese soils was screened using a substrate-induced nitrification assay to generate information for the development of a terrestrial biotic ligand model (tBLM). The leaching effect on the Cu toxicity thresholds was investigated. Both the total Cu-based median effective concentration (EC50) values (46.9–2726 mg/kg) and the solution Cu-based EC50 values (0.04–2.91 mg/L) in unleached soils varied substantially among the soils in the present study. For unleached soils, linear stepwise multiple regression analysis showed that total Ca and soil pH were the best predictors for total Cu-based EC50, while electrical conductivity (EC) and soil pH were the best predictors for solution Cu-based EC50. The variation in solution Cu-based EC50 was largely ($R^2 = 0.75$) explained by Mg but not Ca and H^+ concentration in soil solution at EC50, suggesting a protective effect of Mg^{2+} against Cu toxicity in the test soils. Leaching impacted Cu toxicity differently among the soils and apparently reduced the variations of both the total Cu-based and the solution Cu-based EC50. The predictability of the Cu EC50 by empirical models was decreased after leaching. The leaching effect on Cu toxicity, indicated by a leaching factor, was not predicted by any soil properties. There is a need to investigate quantitatively the mechanisms for the leaching effect on Cu toxicity in soils. *Environ. Toxicol. Chem.* 2010;29:294–300. © 2009 SETAC

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INTRODUCTION

Current Chinese soil guidelines for heavy metals are still based on total metal concentrations (GB 15618-1995), and there is an increasing need to incorporate bioassays in the establishment of ecologically relevant soil guidelines [1]. Deriving toxicological thresholds for heavy metals through bioassays is a key step in the establishment of new soil guidelines. However, few such studies have been reported for Cu toxicity in Chinese soils.

Efforts have been made to screen systematically for acute Cu toxicity to microbial processes [2,3] in a wide range of European and Australian soils. Results showed that Cu toxicity thresholds varied considerably among the soils. Furthermore, based on these results, terrestrial biotic ligand models (tBLMs) have been developed incorporating the free ion activity model and the protective effect of major cations in soil solution [4]. The tBLM was shown to be a reasonable framework for predicting Cu EC50 in soils. Because the controlling factors of Cu toxicity may be different in soils from different countries, there is a need to validate the tBLM based on bioassays using Chinese soils.

In addition, laboratory toxicological assays based on fresh spiking of metals in soils are not able to predict the chronic toxicity of metals in the field [5,6]. Aging processes and loss of excess salts by leaching may contribute much to this discrepancy [6].

To increase the ecological relevance of laboratory toxicological assays, leaching the soils after the metal addition has been recommended [7,8]. However, how leaching affects Cu EC50 in soils requires further investigation on a larger scale.

Therefore, the aims of the present study were to measure Cu toxicity thresholds in Chinese soils using substrate induced nitrification (SIN) assay with the objective of generating information for the development of a tBLM for Cu, to investigate the leaching effect on the Cu toxicity thresholds in soils, and to explore the relationship between Cu toxicity thresholds and soil properties.

MATERIALS AND METHODS

Soils

Seventeen uncontaminated topsoils were collected from throughout China (for detailed information of the soils, refer to Table 1). The soils were air-dried and sieved (mesh size: 2 mm), and soil properties were determined. Soil pH was measured with a soil/water ratio of 1:5. Soil organic matter content was determined by combustion analysis [9]. Total N content was determined as N_2 by thermal conductivity detection [10]. Cation exchanging capacity (CEC) was measured with 1 M ammonium chloride at pH 7.0 after pretreatment to remove the soluble salts. Soil texture was analyzed as described by Tan [11]. Elemental concentrations were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (OPTIMA 2000; PerkinElmer) or inductively coupled plasma mass spectrometry (ICP-MS) (Plasma Quad 3) after dissolution with aqua regia (HNO_3 : HCl = 1:3) [12].

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Table 1. Location, soil type, and selected soil properties of the 17 uncontaminated soils used in the present study

Site	Location	Soil type		pH	EC ($\mu\text{S}/\text{cm}$)	CEC (cmol^+/kg)	OMC %	CaCO_3 %	Background Cu Mg/kg	Total N %	Soil texture
		GSSC ^a	ST ^b								<2 μm / 2-20 μm / 20 μm -2mm
Haikou	19°55'N, 111°29'E	Humid-thermo ferralitic	Ultisols	4.93	111	8.8	1.5	—	50.5	0.12	66/18/16
Guangzhou	23°80'N, 113°17'E	Paddy soils	Inceptisols	7.30	137	8.3	1.5	0.2	10.8	0.13	25/13/62
Qiyang	26°45'N, 111°52'E	Red earths	Ultisols	5.31	74.1	7.5	0.9	—	22.2	0.09	46/35/19
Hangzhou	30°26'N, 120°25'E	Paddy soils	Inceptisols	6.80	203	12.8	2.5	—	47.2	0.25	41/42/17
Chongqing	30°26'N, 106°26'E	Purplish soils	Inceptisols	7.12	71.0	22.3	1.0	—	10.7	0.09	27/25/48
Jiaying	30°77'N, 120°76'E	Paddy soils	Inceptisols	6.70	159	19.3	1.4	—	31.3	0.15	39/36/25
Yangling	34°19'N, 108°0'E	Loessial soils	Inceptisols	8.83	83.2	8.5	0.6	8.9	21.5	0.08	16/14/70
Zhengzhou	34°47'N, 112°40'E	Fluvo-aquic soils	Inceptisols	8.86	109	8.5	1.6	0.2	13.4	0.07	28/41/31
Dezhou	37°20'N, 116°29'E	Fluvo-aquic soils	Inceptisols	8.90	112	8.3	0.7	6.2	15.0	0.08	18/18/64
Shijiazhuang	38°03'N, 114°26'E	Grey-cinnamon soils	Alfisols	8.19	302	11.7	1.0	3.8	16.5	0.11	10/4/86
Zhangye	38°56'N, 100°27'E	Irrigated desert soils	—	8.86	152	8.1	1.0	7.8	27.6	0.10	20/24/56
Langfang	39°31'N, 116°44'E	Fluvo-aquic soils	Inceptisols	8.84	5.7	6.4	0.6	2.4	9.5	0.06	21/22/57
Lingshan	39°55'N, 116°8'E	Brown earths	Alfisols	7.48	92.5	22.7	4.3	4.3	14.2	0.37	20/21/59
Gongzhuling	42°40'N, 124°88'E	Black soils	Mollisols	7.82	147	28.8	2.2	0.3	21.4	0.20	45/26/29
Urumqi	43°95'N, 87°46'E	Gray desert soils	—	8.72	226	10.3	0.9	5.1	26.5	0.10	25/23/52
Hulunber	46°03'N, 122°03'E	Chernozems	Mollisols	7.66	888	22.7	2.7	0.1	14.7	0.25	37/16/47
Hailun	47°28'N, 126°57'E	Black soils	Mollisols	6.56	153	33.6	3.0	—	18.3	0.25	40/27/33

— = not available; EC = electrical conductivity; CEC = cation exchanging capacity; OMC = organic matter content; Clay = clay content.

^a Soil type according to genetic soil classification of China (GSSC).

^b Corresponding soil order according to American soil taxonomy (ST).

Soil Cu addition and leaching

Sieved, air-dried soil was amended with CuCl_2 solution to give nominal concentrations of 37.5, 75, 150, 300, 600, 1200, 2400 mg Cu/kg dry soil for soils with $\text{pH} > 7$, 25, 50, 100, 200, 400, 800, 1600 mg/kg dry soil for soils with $\text{pH} < 7$, and 12.5, 25, 50, 100, 200, 400, 800 mg/kg dry soil for soils with $\text{pH} < 5$. The Cu-amended soils were left to equilibrate overnight at 100% maximum water holding capacity, then air-dried and sieved again through 2-mm mesh.

Aliquots of the freshly spiked soil were leached for each Cu treatment and for each soil by artificial rainwater before use. The detailed composition of the artificial rainwater and protocol for the preparation of the leached samples were described by Oorts et al. [8] Leached samples were also air-dried and sieved through 2-mm mesh. All unleached and leached soils were stored at room temperature before use.

Substrate-induced nitrification assay

The unleached and leached soils were used to establish a substrate-induced nitrification assay [2,3,13,14]. Three replicates of 7 g of amended soil for each Cu treatment were preincubated in a 50-ml centrifuge tube under dark aerobic conditions at $25 \pm 2^\circ\text{C}$, maintained at $50 \pm 5\%$ of maximum water holding capacity by addition of deionized water. There were 816 microcosm tubes in total. After the 14-d preincubation, one tube of each Cu treatment was extracted with 35 ml 1.0 M KCl and NO_2^- -N plus NO_3^- -N were determined using a flow injection auto-analyzer (SKALAR, The Netherlands). Aliquot of 0.5 mL of 0.044 M $(\text{NH}_4)_2\text{SO}_4$ was added to each of the remaining tubes in each treatment, and incubated for a further 7 d for soils with $\text{pH} > 5$, and 28 d for Haikou soil with $\text{pH} < 5$. The remaining two replicates were analyzed for NO_2^- -N plus NO_3^- -N again as above. Data were used to calculate SIN.

Soil solution extraction and analysis

Soil solutions in all treatments (one replicate for each treatment) were extracted at the beginning of the SIN assay using the method of Thibault and Sheppard [15]. The dissolved elemental concentrations including Cu were measured either by ICP-AES or ICP-MS (mass spectrometry) depending on their concentrations in the soil solutions, following the methods of Khan et al. [16]; pH, electrical conductivity and dissolved organic carbon (DOC) were measured following conventional procedures [11].

Data analysis

Potential nitrification rate was calculated from the linear increase in nitrate plus nitrite after substrate addition. Substrate-induced nitrification assay was calculated from the difference between the concentrations of nitrite plus nitrate at day 0 and day 7 after ammonium addition [17]. The SIN data were used to generate log-log dose-response curves automatically by SigmaPlot 9.0 (SPSS) for each soil. Copper EC50 based on both the measured total Cu concentration (total Cu-based EC50) and the dissolved Cu concentration in soil solution (solution Cu-based EC50) was calculated for both the unleached and the leached soils. Toxicity thresholds were related to soil properties with multiple regressions (SPSS 11.5). The corresponding concentrations of pH, Ca, Mg, Mn, and Zn in the soil solutions at Cu EC50 were estimated by linear interpolation between the measured values [2].

Copper speciation in soil solution at EC50 was estimated using MINTEQ 2.6 following the assumptions by Oorts et al. [2] Briefly, the input parameters included solution Cu-based EC50 and corresponding soil solution pH, DOC, Ca, Mg, K, Na, Al, Fe, SO_4^{2-} and Cl^- . The SO_4^{2-} was calculated from dissolved S and Cl^- was set at a concentration estimated to balance the cations. It was assumed that 65% of the dissolved organic matter

(DOM) (DOC/2) was fulvic acid. CO₂ pressure was specified as 3.8×10^{-4} atm.

RESULTS

Soil physiochemistry

Selected soil properties of the 17 soils used in the present study are listed in Table 1. These soils represent the majority of the Chinese soil types except for those in Taiwan and Tibet. The total concentrations of other potentially toxic elements (e.g., Cr, Cd, Zn, As, Pb) in studied soils generally fall within the normal range of background levels in Chinese soils [18].

Potential nitrification rates in uncontaminated soils

Potential nitrification rates in uncontaminated soils (PNR0) ranged from 0.74 mg N/kg/d (Haikou) to 10.85 mg N/kg/d (Urumqi). Stepwise multiple regression between the PNR0 values and the soil parameters showed that pH was the major factor controlling the PNR0 ($R^2 = 0.70$). A curve showing the relationship between PNR0 and soil pH and its 95% confidence interval is illustrated in Figure 1. A better prediction for PNR0 in the studied soils was obtained by multiple regression between PNR0 and soil pH, total Ca, CEC, clay content, and electrical conductivity ($R^2 = 0.82$) (Table 2).

Cu concentration

Measured total Cu concentrations (Cu_{total}) were in good agreement with the nominal dose applications in almost all treatments. Generally, the dissolved Cu concentration ([Cu]) in unleached soil solution increased exponentially with applied Cu dose. For the unleached treatments, the [Cu] ranged from <0.01 mg/L to 0.081 mg/L (Jiaxing soil) in unamended soil solutions, and varied from 0.52 mg/L (Yangling soil) to 225.3 mg/L (Guangzhou soil) at the highest Cu dose. Furthermore, Cu amendments changed the other chemical composition of the soil solutions in unleached treatments (data not shown), leading to a remarkable increase in H⁺, Ca²⁺, and Mg²⁺ for all soils. Stepwise multiple regression showed that the original soil pH was the dominant factor ($R^2 = 0.41$; $p = 0.008$; R^2 is a determination coefficient) controlling [Cu] in soil solution at the highest Cu dose, secondly organic matter content ($p = 0.07$), and overall 67% of its variation can be explained by soil pH, CEC, organic matter content, clay content, and total Ca.

Fig. 1. The potential nitrification rate (PNR) in the 17 unleached control soils as a function of soil pH.

Table 2. Simple linear regressions between potential nitrification rate in the 17 unleached control soils and soil properties

Regression equation	R^2	p value
PNR0 = 2.14 pH - 9.17	0.70	<0.001
PNR0 = 2.80 pH - 0.91 Ca - 13.03	0.74	<0.001
PNR0 = 3.04 pH - 1.48 Ca - 0.12 CEC - 0.005 EC - 12.40	0.82	<0.001

PNR0 = potential nitrification rate in uncontaminated soils; Ca = total calcium content; CEC = cation exchanging capacity; EC = electrical conductivity.

Leaching effects on Cu_{total} in the soils and [Cu] in the soil solutions were shown by plotting the Cu_{total} in the unleached against the leached soils, and [Cu] in the unleached against the leached soil solutions, respectively. Leaching did not cause a significant loss of Cu_{total} in any treatment and any soil. In contrast, although a similar result as Cu_{total} was found for [Cu] in soil solution at lower [Cu] (< 2 mg/L), at higher [Cu] the loss of [Cu] in soil solution was significant and generally increased with [Cu] (Fig. 2). The other chemical composition of the soil solutions was strongly impacted by leaching (data not shown). For instance, up to 94.4% of Ca in soil solution at the highest Cu dose was removed by leaching in Langfang soil. Unfortunately, no correlation was found between the removal rates of soil solution Cu at the highest Cu doses and the soil parameters of uncontaminated soils.

Cu toxicity threshold

All the dose-response curves based on the SIN assays were described well by a log-logistic model ($R^2 > 0.7$). Both the total Cu-based EC50 and the solution Cu-based EC50 for Cu in unleached and leached soils were calculated (Fig. 3). Nitrification was inhibited by more than 50% at the highest doses in most soils except for the Zhangye soil (unleached, 42% inhibition), the Shijiazhuang soil (leached, 29% inhibition), and the Urumqi soil (leached, 34% inhibition).

The EC50 values varied largely among soils for both the total Cu-based and the solution Cu-based EC50. In unleached soils, the total Cu-based EC50 values varied 58-fold and the solution Cu-based EC50 varied 73-fold. In leached treatments,

Fig. 2. Leaching effect on the dissolved Cu concentration in soil solution ([Cu]) in the 17 studied soils.

Fig. 3. Total Cu-based and solution Cu-based median effective concentrations (EC₅₀) of Cu in the 17 unleached and leached soils subjected to the substrate-induced nitrification assay.

the total Cu-based EC₅₀ values varied 43-fold and the solution Cu-based EC₅₀ varied 23-fold.

Leaching factor is defined as the ratio of EC₅₀ in leached soil to EC₅₀ in unleached soil [8]. Results showed that there was no consistent pattern for the difference in the EC₅₀ values between leached and unleached soils, leaching factors ranging from 0.4 (Yangling) to 4.9 (Shijiazhuang) for total Cu-based EC₅₀ and from 0.5 (Yangling) to 14.0 (Chongqing) for solution Cu-based EC₅₀. In addition, no significant correlation was found between the leaching factor and the soil parameters.

Linear regressions between the Cu EC₅₀ values and the soil parameters were carried out for both unleached and leached soils (see Table 3). In unleached soils, total Ca and soil pH were the best predictors for the total Cu-based EC₅₀, while electrical conductivity and soil pH were the best predictors for the solution Cu-based EC₅₀. A best prediction equation for the solution Cu-based EC₅₀ was obtained with a correlation coefficient up to 0.92. On the other hand, leaching weakened the predictability of both the total Cu-based and the solution Cu-based EC₅₀ with the soil parameters.

Because a toxicity of CuOH⁺ in solution culture has been suggested [19], both free Cu²⁺ and CuOH⁺ were considered as toxic Cu species in the present study. Results showed that most of the dissolved Cu in soil solution was bound by DOM (>95% in the present study). Estimated CuOH⁺ activity was at the same level as free Cu²⁺ in most soils, and a strong positive correlation ($R^2 = 0.78$) was found between them in alkaline soil solutions.

To investigate the protection effect of major cations (e.g., H⁺, Ca²⁺, Mg²⁺, Na⁺, K⁺) against Cu toxicity, solution Cu-based EC₅₀ and free Cu²⁺ activity together with CuOH⁺ activity at EC₅₀ were related to pH, Ca, Mg, Na, and K in soil solution at EC₅₀. Solution Cu-based EC₅₀ for unleached soils was not correlated with pH, Ca, Na, or K in soil solution at EC₅₀, but significantly correlated with Mg in soil solution at EC₅₀ ($R^2 = 0.75$). The free Cu²⁺ activity and the CuOH⁺ activity were both positively correlated with Mg in unleached soil solution at EC₅₀ (Fig. 4). For leached soils, no significant correlation was found between solution Cu-based EC₅₀ and pH, Ca, Mg, Na, or K in soil solution at EC₅₀.

Table 3. Simple linear regressions between Cu toxicity thresholds based on total Cu in soils (mg/kg) and free Cu concentration in soil solutions (mg/L) and soil properties

	Regression equation	R^2	p value
Unleached soils	Based on total Cu ($n = 17$)		
	EC ₅₀ = 468.7 Ca + 83.4	0.61	<0.001
	lg EC ₅₀ = 0.3 pH + 0.37	0.55	0.001
	EC ₅₀ = 13.8 pH + 601.0 Ca - 6.0 CEC + 8.7 Clay + 166.4 OMC - 645.3	0.67	0.02
	Based on soil solution Cu ($n = 17$)		
	EC ₅₀ = 0.003 EC + 0.33	0.56	0.001
	EC ₅₀ = 0.23 pH + 0.003 EC - 1.42	0.71	<0.001
Leached soils	EC ₅₀ = 0.34 pH - 0.36 Ca - 0.05 CEC - 0.02 Clay + 0.27 OMC + 0.003 EC + 0.014 Cu - 1.14	0.92	<0.001
	Based on total Cu ($n = 17$)		
	EC ₅₀ = -39.9 Clay + 2290.1	0.23	0.05
	EC ₅₀ = -60.0 pH - 109.7 Ca - 15.5 CEC - 46.0 Clay - 231.3 OMC + 3343.5	0.33	0.58
	Based on soil solution Cu ($n = 17$)		
	EC ₅₀ = 0.002 EC + 0.72	0.29	0.02
	EC ₅₀ = 0.002 EC - 0.038 CEC + 1.20	0.52	0.01
	EC ₅₀ = 0.20 pH - 0.46 Ca - 0.06 CEC - 0.02 Clay + 0.09 OMC + 0.002 EC - 1.08	0.70	0.03

EC₅₀ = median effective concentration; Ca = total calcium content; CEC = Cation exchanging capacity; Clay = clay content; OMC = organic matter content; EC = electrical conductivity; Cu = background Cu concentration.

Fig. 4. Positive correlations between activities of the potentially toxic Cu species (pCu^{2+} and pCuOH^+) and the dissolved Mg concentration (pMg) in unleached soil solution at median effective concentration (EC50). Two acidic soils from Haikou and Qiyang were excluded.

DISCUSSION

Both the total Cu-based EC50 values and the solution Cu-based EC50 values in unleached soils varied considerably among the soils in the present study. This is in agreement with previous studies. Oorts et al. [2] and Rooney et al. [20] found a variation of 15- to 90-fold for total Cu-based EC50 according to various microbial and plant assays in European soils, and Broos et al. [3] found a 20-fold variation for total Cu-based EC50 according to microbial assays in 12 Australian soils. All these results indicated that it is more reasonable to establish site-specific guidelines for Cu in soils rather than a single value.

Empirical models were developed to predict Cu EC50 in Chinese soils using easily determined soil parameters. In the linear regression models shown in Table 3, the best predictors for total Cu-based EC50 were total Ca and soil pH. This is different from European and Australian soils where CEC provides the best correlations for Cu toxicity using the SIN assay [2,3]. This difference can be due to the chemical compositions of the studied soils. Because there was a strong correlation between Ca content and soil pH, it is not possible to distinguish between Ca effect and pH effect on Cu toxicity in the present study. Basically, soil pH was one of the key factors controlling the expression of Cu toxicity to soil biota [21]. Yet, in the studied soils, soil pH may be largely controlled by Ca minerals. As shown in Table 1, nearly half of the soils used in the present study are calcium-rich, containing $>2.6\%$ of CaCO_3 and with a $\text{pH} > 8$. Therefore, it is not surprising to see a correlation between soil Ca content and total Cu-based EC50. In addition, Ca-rich minerals can strongly impact the ageing process of freshly added Cu [22]. The proposed mechanisms for such an influence include the formation of cupric complexation on calcite surface and CuCO_3 precipitation [23–25].

A linear model, $[\text{EC50} = 468.7 \text{ Ca} + 83.4 \text{ (} R^2 = 0.61 \text{)}]$, was able to predict the total Cu-based EC50 for 14 of the 17 unleached soils within a twofold range of the measured values in the present study (Fig. 5). The predictive ability of the models for total Cu-based EC50 was generally at the same level as the models defined by Oorts et al. [2] and Broos et al. [3] Leaching decreased the predictability of the Cu EC50 by empirical

Fig. 5. The predicted versus observed total Cu-based median effective concentration (EC50) based on substrate induced nitrification assay (SIN) in the 14 unleached Chinese soils.

models with a single predictor (Table 3). Whether a semi-mechanistic tBLM is able to explain the variation in Cu EC50 in leached soils needs further study.

The total Cu-based EC50 was not explained by the solution Cu-based EC50 or the calculated free Cu^{2+} -based EC50, which is consistent with previous studies [2,3]. This means that solution Cu alone is not able to predict the Cu toxicity to soil nitrification. The variation in the solution Cu-based EC50 was largely, but not completely explained by the dissolved Mg at EC50. The dissolved Mg at EC50 was also positively correlated with the estimated free Cu^{2+} activity and CuOH^+ activity at EC50 (Fig. 4). These results were consistent with the theory of a protective effect of coexisting abundant cations [26]. Many studies suggested a protective effect of H^+ and Ca^{2+} in soil solution against metal toxicity, including Cu [2,4,8]. However, no such effect of H^+ and Ca^{2+} was indicated in the present study. Soil nitrification is very sensitive to environmental factors, like pH, as shown in Figure 1 and previous studies [17], and osmotic potential [27]. Therefore, negative effects of excessive Ca^{2+} and H^+ in soil solution might exist and counteract their protective effect on Cu toxicity, leading to inconsistent overall effect of Ca^{2+} and H^+ on Cu EC50 in different soils. Second, evidence [19,28–32] has been obtained to challenge the free ion activity model (FIAM). The FIAM predicts free Cu^{2+} in soil solution, seemingly the major toxic species responsible for the observed Cu toxicity in soil [33]. However, a toxicity of both CuOH^+ and Cu-DOM complex in soil have been suggested [19,29,34]. Because CuOH^+ and Cu-DOM complex were important species in alkaline and high DOC-content soils, they might contribute to the variation in the observed solution Cu-based EC50. Third, the increases in Zn and Al in soil solutions from some acidic soils reached a potentially toxic dose [14,35], up to 18.3 mg/L and 27.9 mg/L at the highest Cu doses, respectively. These covarying toxic metals may contribute synergistically or antagonistically to the observed toxicity in some soils. Taken together, these factors may all reduce the power of the models based on the free ion activity model and the protective effect of cations in soil solution to predict EC50 for Cu in soils covering such a wide range of soil properties. Therefore, more variables other than free Cu^{2+} and protective cations need to be involved in the tBLM for Cu in soils.

Leaching apparently reduced the variations in both the total Cu-based and the solution Cu-based EC50. As indicated by the leaching factor, leaching impacted Cu EC50 differently for different soils. The inconsistent impact of leaching on the total Cu-based EC50 among soils can be attributed to the two counteracting effects of leaching on the soil solution compositions [8]. As shown in Figure 2, leaching obviously reduced [Cu] at high Cu doses. For acidic soils with high [Cu] in soil solution and thus low EC50, like the Chongqing and Hainan soils, leaching can increase the total Cu-based EC50. On the other hand, the loss of a large amount of protective cations induced by leaching may negatively decrease the EC50. Therefore, for alkaline soils with a high loss of abundant cations, like soils from Yangling, Zhangye, and Zhengzhou, leaching may decrease the total Cu-based EC50, and thus the variations in the total Cu-based EC50 values. To this end, leaching may overcome the complexity of soil chemistry caused by Cu amendments and increase the ecological relevance of the SIN assay.

Unfortunately, the smaller variation of EC50 values after leaching led to a decrease rather than an increase in the predictability of both the total Cu-based and solution Cu-based EC50 by empirical models shown in Table 3. Such a decrease is not surprising considering the inconsistent impact of leaching on the Cu toxicity in different soils. The decrease in correlation between EC50 and soil parameters may result from the decrease in the predictability of [Cu] in soil solutions. The linear relationship between [Cu] in soil solutions at the highest Cu doses and soil parameters disappeared after leaching, while a strong correlation was found between [Cu] in unleached soil solutions at the highest doses can be explained by primary soil parameters ($R^2 = 0.67$). In addition, the leaching effect on the loss of salts in soil solutions may be inconsistent between soils. The original purpose of leaching was to remove the excess counterions which can exert a direct toxic effect or an indirect effect by raising the salinity [7,8]. However, similar to the study by Lock et al. [7], conductivities of more than 4 mS/cm were observed in the highest Cu treatments of 8 soils. As natural processes, the partitioning and ageing of the soluble Cu added to soils, together with the covarying solution chemistry, are controlled by soil properties [36–39]. Yet, the artificial leaching disturbed these processes and the leaching effect on Cu toxicity may largely depend on the leaching process (e.g., the duration of leaching, the composition of leaching solution, and the total volume of leaching solution) as well as soil properties. Therefore, the leaching effect for different soils may be incomparable, and there is a need to quantitatively investigate the mechanisms for the leaching effect on the Cu toxicity in soils.

In conclusion, large variations in total Cu-based EC50 values and solution Cu-based EC50 values in unleached soils were observed. The observed Cu toxicity in the 17 studied soils may be controlled and affected by a suite of factors, and the future models for predicting Cu thresholds in soils should take into account more variables other than free Cu^{2+} and protective cations (e.g., H^+ , Ca^{2+} , and Mg^{2+}). In addition, leaching procedures may need to be standardized through the quantitative study of the mechanisms controlling the leaching effect on Cu toxicity.

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