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Copper Speciation and Impacts on Bacterial Biosensors in the Pore Water of Copper-Contaminated Soils

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Knowledge of heavy metal speciation and its relationship with biological responses is important for the derivation of effects-based soil quality criteria. We determined soluble Cu concentrations and free Cu^{2+} activities in the pore waters from 22 soils with total Cu varying from 19 to 8645 mg kg^{-1}. Pore water pCu^{2+} (= −log (Cu^{2+} activity)) varied from 3.9 to 10.5 and was controlled by soil pH and total Cu concentration. The percentage of free Cu^{2+} in total soluble Cu varied from 0.02 to 96% and was influenced strongly by pore water pH and, to a lesser extent, by dissolved organic C. In the pore waters with pH >6, the percentage of free Cu^{2+} in total soluble Cu was lower than 1%. Using the default data base and with the fulvic acid content of DOC optimized at 69%, the equilibrium speciation program WHAM/Model VI gave estimates of pCu^{2+} that agreed closely with measured values. Pore water samples were analyzed by two bioluminescence-based bacterial biosensors: *Escherichia coli* HB101 pUCD607 and *Pseudomonas fluorescens* 10586r pUCD607. The response of *P. fluorescens* correlated more closely with soil pore water pCu^{2+} than with soluble Cu concentration, whereas pCu^{2+} and soluble Cu fitted the response of *E. coli* equally well. The effect concentrations (EC_{25} and EC_{50} values) of pCu^{2+} for the two biosensors were about 5.8 and 5.0, respectively. This is the first time that threshold values for Cu have been obtained for bacterial biosensors exposed to soil pore water from well-equilibrated contaminated soils.

Introduction

Although copper is an essential element for all life, at high concentrations it is potentially toxic to soil microorganisms, soil fauna, and higher plants. Numerous studies have reported adverse effects of heavy metals on soil microbial biomass, populations of individual species, microbial biodiversity, and microbial mediated processes (*1*−*3*). In most studies involving soil microorganisms or plants in soils, toxicity of heavy metals has been expressed in terms of total soil concentrations or some operationally defined extractable fractions (*4*). Existing legislation or guidelines on heavy metals in soils are also based on total concentrations (*5*). However, it is well-established that total metal concentrations in soils do not reflect bioavailability, and the relationships between bioavailability and operationally defined fractions of metals are often influenced by other soil properties (*4, 6*).

Following the paradigm that free metal activity is the main factor controlling metal toxicity to aquatic organisms (*7*−*10*), recent studies on soil metals have increasingly focused on the linkage between metal speciation in soil pore water and toxicological responses (*4, 11*−*13*). Speciation is particularly important for metals such as Cu and Pb for which organic ligands in the liquid phase have high affinities. Soil systems are usually more complex than the aquatic environment because of the heterogeneity of soil properties and the usually large spatial variation. Nevertheless, evidence is accumulating that free ion activity in soil pore water is one of the key factors controlling metal bioavailability to soil microorganisms and plants (*4, 11, 14*−*16*). Also, soil microorganisms or microbial processes appear to be more sensitive to Cu toxicity than higher plants (*4*). Recent development of bioluminescence-based biosensors, using soil bacteria that have had the *lux* genes introduced into them using molecular cloning techniques, has provided a rapid and sensitive tool for soil ecotoxicological studies (*17*−*19*). Our previous work showed that these biosensors responded to free Zn^{2+} in pore waters of sewage sludge-amended soils (*11, 12*). However, no data like these are available for Cu.

For derivation of soil quality criteria, it is important that toxicological tests on soil heavy metals are linked directly to chemical speciation. So far, the information linking heavy metal speciation, bioavailability, and toxicological responses in soils is still very limited. Ideally, this type of research should avoid experiments where unpolluted soils are spiked with metal salts but should use well-equilibrated soils obtained from contaminated environments and preferably with a single heavy metal. In this study, we used a series of soils that varied greatly in the degree of Cu contamination but showed much smaller variations in the concentrations of other heavy metals. We determined both Cu speciation in the soil pore water and the bioluminescence responses of two bacterial biosensors. The main aim was to link Cu speciation in soil pore waters with biological effects. In addition, we investigated factors that influence Cu solubility and speciation and evaluated the performance of a humic ion-binding model, WHAM/model VI (*20*) in predicting Cu speciation in soil pore waters.

Materials and Methods

Soils Used. Three sets of soil samples were used. The first set included seven soil samples collected from within 100 m of a copper rod rolling factory near Prescot, Merseyside, UK (*21*). The same uncontaminated soil was imported onto the factory site in 1975 and deposition of particulate Cu (mainly oxides) since that time has resulted in a gradient of Cu concentrations. The second set included 12 soils from Cachapoal and Santiago Basins in Chile, which were contaminated with Cu to varying degrees due to the incorporation of Cu mine tailings. The third set of samples was collected

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from a paddy field in Jiangsu, China. This included one uncontaminated soil and two soils contaminated with a waterborne spill of $CuSO_4$ from a factory discharge. All soils were taken from the upper soil horizon (0−20 cm), and the moist samples were sieved to <2 mm.

Analyses of Soil Properties. Portions of moist soils were air-dried, ground, and sieved to either <2 or <0.45 mm for chemical analyses. Total concentrations of heavy metals in soils were determined by inductively coupled plasma atomic emission spectrometer (ICP-AES; Fisons-ARL Accuris, Ecublens, Switzerland), following a digestion with aqua regia (*22*). Soil pH was determined in a mixture of 1:2.5 soil:water (w/v). Total concentrations of carbon and nitrogen were determined using a LECO combustion analyzer (LECO CNS 2000, St. Joseph, MI).

Extraction and Analysis of Soil Pore Water. Moist soil samples equivalent to 0.8 kg dry weight were put into 1-L plastic pots. The soils were brought to 100% water holding capacity (WHC) by adding deionized water via a saucer underneath the pot. The soils were then allowed to dry to 75% of their WHC. The soils were incubated at 18 °C for 4 weeks with the moisture content being maintained at 75% WHC, before soil solutions were extracted. Soils were replicated in two pots each, except Prescot No. 1 and No. 3 and Chilean Cachapoal Basin No. 1 of which there was only enough soil for one pot each.

Soil pore waters were extracted using Rhizon soil moisture samplers (Rhizosphere Research Products, Wageningen, The Netherlands, e-mail: info@rhizosphere.com). These samplers consist of a length of porous plastic tube, capped with nylon membrane (<0.2 μm) at one end, and attached to a 5-cm length of polyethylene tubing at the other end. A nylon strengthening rod is present inside the porous tube, and the polyethylene tube is joined to a female Luer lock. A previous study showed that the samplers were suitable for trace metal research (*23*). All the samplers were washed by forcing 60 mL of 5% HNO_3 through the probe, followed by 60 mL of deionized water and then dried at 30 °C before use. Two samplers were placed in each pot while the soils were being packed. Acid-washed disposable syringes were used to extract soil pore water directly from the soil through the soil moisture samplers. Soil pore water was extracted twice from each pot within a 6-month period.

Aliquots of soil pore water were used for determining dissolved organic carbon (DOC), pH, electrical conductivity (EC), free Cu^{2+} activity, and inorganic anions by ion chromatography. Another set of aliquots of soil pore water were acidified with 5% HCl and used to determine total soluble Cu and other major cations by ICP-AES. The concentrations of Cd were determined by graphite furnace atomic absorption spectrometry (GF-AAS; Perkin-Elmer ZL 4100, Norwalk, CT). Pore water ionic strength (IS) was calculated from EC using the empirical relationship IS = 0.13EC (*24*).

Determination of Free Cu^{2+} Activity. Free Cu^{2+} activity was determined using a Cu^{2+} ion selective electrode (Cu-ISE, ORION 94-29) coupled with a reference electrode (ORION 900200) at 20 °C. The Cu-ISE was polished for 30 s with 3 μm aluminum oxide strips before use each day. Both Cu-ISE and the reference electrode were soaked successively for 5 min in 0.025 M H_2SO_4 and 0.1 M Na_4EDTA after use. A calibration curve was established according to Sauvé et al. (*25*). In the pCu^{2+} (negative log of Cu^{2+} activity) range of 4−13, calibration buffers were made with 1 mM IDA (iminodiacetic acid), 0.1 mM $Cu(NO_3)_2$, 6 mM NaOH, 2.5 mM $KHC_8H_4O_4$ (potassium acid phthalate), and 0.01 M $CaCl_2$, and the pH was varied by incremental additions of HNO_3. In the pCu^{2+} range of 2−4, calibration buffers were made with 0.1 M $NaNO_3$ and 0.1−10 mM $Cu(NO_3)_2$. The electrode potential (EP) determined by Cu-ISE was then plotted against pCu^{2+} calculated by GEOCHEM-PC (*26*). This showed a linear relationship with the following regression equation:

$$pCu^{2+} = 7.041 - 0.0347 \times EP \quad (n = 66, r^2 = 0.99)$$

The slope of the electrode response obtained in the calibration (28.8 mV/pCu^{2+}) was close to the theoretical Nernstian slope of 29.6. Free Cu^{2+} concentrations were calculated from free Cu^{2+} activities and the activity coefficients. The latter were calculated from the ionic strength using the extended Debye−Hückel equation.

Calculation of Free Cu^{2+} by WHAM/Model VI. WHAM/model VI is based on the chemical equilibrium program WHAM (*20*) but modified by substitution of Humic Ion-Binding Model VI (*20*) for model V. Model VI is a discrete site/electrostatic model of the interactions of protons and metal cations with humic substances: it differs from model V principally by including representations of high affinity sites for metals, present in small amounts. In addition, the distributions of proton affinity and metal affinity are no longer fully correlated in model VI, as is the case in model V. The program was used to calculate free Cu^{2+} activities of soil pore waters, with pore water pH, DOC, total soluble cations, and anions as inputs. We further examined the effects of pCO_2 and Fe on the model calculations of Cu speciation. For the latter, we assumed that Fe^{3+} was in equilibrium with $Fe(OH)_3$ (aged ferrihydrite) having K_{SO} of 2.5. K_{SO} is the solubility product for the reaction $Fe^{3+} + 3H_2O = Fe(OH)_3 + 3H^+$.

Bioassays Using Lux-Based Bacterial Biosensors. Pore water samples from the first extraction were used. Two lux-based bacterial biosensors were used: *Escherichia coli* HB101 pUCD607 (*27*) and *Pseudomonas fluorescens* 10586r pUCD607 (*28*). Both biosensors were constructed by inserting the genes encoding bioluminescence, *lux*CDABE, via the multicopy plasmid pUCD607. The biosensors were stored as freeze-dried cultures (−20 °C), which were prepared according to standard laboratory protocols (*19*). *E. coli* HB101 pUCD607 was resuscitated for 1 h in 10 mL of 0.1 M KCl. *P. fluorescens* 10586r pUCD607 was resuscitated in 10 mL of Luria Bertani broth (10 tryptone, 5 yeast extract, and 10 NaCl g L^{-1}), and the cells were harvested by centrifugation and resuspended in 5 mL of 0.1 M KCl. The bioassay consisted of adding 50 μL of cells to 450 μL of a pore water sample in a cuvette at 15-s intervals. The bioluminescence of the cells was then measured on a Bio-Orbit 1253 luminometer after an exposure time of 45 min. Bioluminescence results from the individual soil solutions were expressed as percentages of the light output of the appropriate uncontaminated soil solution at each site (soil no. 1 from each site in Table 1). The bioluminescence assays were replicated three times for each pore water sample.

Statistical Analyses. Regression analysis was performed using Genstat 5 (*29*). Where necessary, the data were normalized before analysis by log transformation. A four-parameter Gompertz model was fitted to the relationships between bioluminescence data and soluble Cu concentration or free Cu^{2+} activity in the soil solutions. The equation for the model is $y = y_0 + a\,(\exp(-\exp(-(x - x_0)/b)))$, where y_0, a, x_0, and b are parameters to be fitted. It is a nonsymmetrical curve about the inflection point of $x = x_0$ and has asymptotes at $y = y_0$ and $y = y_0 + a$.

Results and Discussion

Soil Solid-Phase Properties. Selected properties of soil solid phase are shown in Table 1. Soil pH varied from 5.5 to 8.0, with most samples within ±1 unit of neutrality. The ranges of total C and N were 9.8−69.8 and 1.1−5.1 mg g^{-1}, respectively. There was a large variation in the concentration of total Cu (19.4−8645 mg kg^{-1}). This range covers both

TABLE 1. Soil Solid Phase Properties

sampling site	sample no.	soil pH	total C (mg g^{-1})	total N (mg g^{-1})	total concn (mg kg^{-1})				
					Cu	Zn	Cd	Ni	Pb
Prescot, U.K.	1	6.65	19.4	1.3	61.6	60.6	0.3	16.1	29.7
	2	5.53	37.0	2.3	165.1	98.0	2.3	28.0	58.6
	3	6.18	26.8	1.6	736.9	100.5	3.4	20.6	51.4
	4	6.03	27.1	1.5	1045	122.9	2.0	24.9	62.4
	5	6.08	35.0	1.8	1341	161.7	4.8	25.7	87.2
	6	5.71	51.5	3.4	5146	166.2	5.4	29.8	100.5
	7	6.11	69.8	5.1	8645	176.6	2.5	31.3	114.3
Cachapoal Basin, Chile	1	6.13	25.3	2.6	158.4	115.6	3.3	9.0	9.8
	2	7.06	18.9	1.8	143.6	115.8	3.4	4.6	11.8
	3	6.77	20.5	2.2	193.4	123.4	2.7	10.1	15.5
	4	7.50	16.4	1.6	914.5	125.2	3.0	12.1	17.3
	5	6.78	10.5	1.1	436.3	137.7	3.4	11.9	26.3
	6	6.73	13.7	1.3	614.4	121.2	2.8	10.4	14.5
Santiago Basin, Chile	1	7.78	21.8	1.5	62.2	111.4	2.4	12.6	18.5
	2	8.00	11.2	1.2	19.4	49.8	2.2	4.8	7.7
	3	7.54	14.4	1.5	173.5	147.0	2.9	8.9	21.4
	4	7.77	26.6	2.7	412.6	154.2	3.7	18.1	15.6
	5	6.98	9.8	1.1	137.6	112.4	2.4	10.3	15.0
	6	7.48	27.9	2.9	1111	299.3	4.0	23.4	87.9
Jiangsu, China	1	6.26	14.5	1.4	23.5	71.1	2.4	20.0	22.3
	2	6.22	20.7	1.5	90.3	56.4	2.3	20.3	20.5
	3	7.05	14.6	1.5	158.5	64.0	2.4	23.0	19.2

TABLE 2. Soil Pore Water Properties[a]

sampling site	sample no.	pH	electrolytic conductivity (S m^{-1})	DOC (mg L^{-1})	soluble Zn (mg L^{-1})	soluble Cd (mg L^{-1})	soluble Cu (mg L^{-1})	pCu^{2+} acty	free Cu^{2+} concn (μg L^{-1})	% free Cu
Prescot, U.K.	1	7.22	0.13	13.0	0.08	0.004	0.021	9.6	0.03	0.12
	2	4.72	0.20	30.8	0.62	0.043	0.15	6.7	20.1	13.8
	3	5.38	0.15	8.9	0.37	0.009	0.28	6.0	96.0	33.8
	4	5.28	0.10	6.8	1.02	0.022	0.84	5.3	450.2	58.2
	5	5.24	0.14	9.3	1.51	0.032	1.66	5.0	975.4	66.5
	6	5.40	0.17	16.0	2.54	0.060	8.40	4.3	5640	71.6
	7	5.49	0.20	16.5	3.51	0.066	16.8	3.9	15562	95.5
Cachapoal Basin, Chile	1	5.36	0.31	71.1	0.47	0.001	0.15	7.5	3.9	2.40
	2	7.19	0.19	22.5	0.19	<DL	0.049	9.4	0.046	0.09
	3	6.97	0.15	18.7	0.21	<DL	0.062	9.4	0.041	0.08
	4	7.57	0.13	12.2	0.16	0.001	0.10	8.6	0.25	0.24
	5	6.95	0.09	7.6	0.17	0.001	0.058	7.6	2.7	4.86
	6	6.93	0.21	28.3	0.22	<DL	0.35	7.9	1.5	0.51
Santiago Basin, Chile	1	7.90	0.22	22.8	0.13	<DL	0.032	10.3	0.006	0.02
	2	7.78	0.36	35.4	0.16	0.003	0.019	10.5	0.004	0.03
	3	7.48	0.30	24.0	0.13	0.003	0.048	9.9	0.014	0.03
	4	7.61	0.35	28.7	0.14	0.001	0.061	10.2	0.007	0.02
	5	6.90	0.19	38.4	0.11	<DL	0.065	8.8	0.19	0.23
	6	7.20	0.18	20.0	0.21	<DL	0.12	8.9	0.14	0.13
Jiangsu, China	1	6.12	0.15	8.8	0.25	<DL	0.009	8.6	0.30	4.66
	2	5.36	0.13	8.9	0.14	<DL	0.027	7.0	10.5	32.3
	3	6.74	0.14	7.8	0.23	0.001	0.022	8.5	0.31	1.84

[a] Data are means of 3−4 replicates for each soil. DL, detection limit (~0.001 mg L^{-1}).

uncontaminated soils (about 20−50 mg of Cu kg^{-1}; *30*) and severely contaminated soils. Even though total Cu concentrations varied by more than 2 orders of magnitude between the soil samples, other heavy metals varied less than 1 order of magnitude and, in most cases, were within the background ranges reported for soils (*30*).

Soil Pore Water Properties and Cu Speciation. Some of the soil pore water properties are shown in Table 2. Soil pore water pH was generally lower than the bulk soil pH measured in the 1:2.5 soil to water suspension, probably due to a higher solute concentration in the former (soil pore water was extracted from soils held at about 1:0.3 solid:solution ratio, depending on the soil). Dissolved organic C (DOC) varied widely from 6.8 to 71 mg L^{-1}. The range of ionic strength calculated from electrical conductivity was 0.01−0.05 M. Variation within this range has been shown to cause little effect on the electrode potential of Cu-ISE (*25*).

The concentrations of soluble Cu in the pore water samples varied by more than 3 orders of magnitude, from 0.01 to 16.8 mg L^{-1} (Table 2). These are much lower than the calculated solubility products of copper hydroxy and carbonate minerals (*31*), indicating that these mineral phases were unlikely to be controlling the concentrations in soil pore waters. Therefore, the distribution of Cu between the solid and aqueous phases is best described by means of a partition coefficient, K_d (solid phase concentration/solution phase concentration). Figure 1a shows the relationship between the concentration of soluble Cu in pore waters and

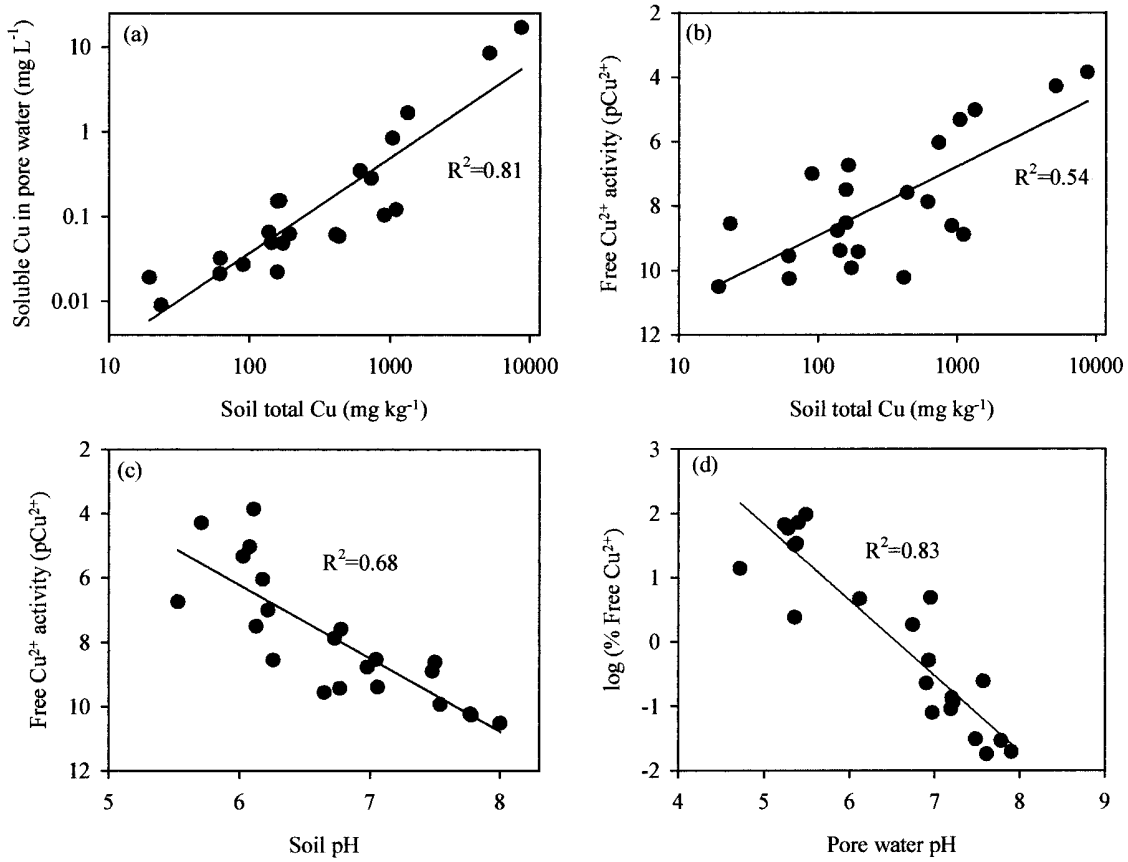

FIGURE 1. Relationships between (a) soluble Cu in pore water and soil total Cu. (b) Free Cu^{2+} activity in pore water and soil total Cu. (c) Free Cu^{2+} activity in pore water and soil pH. (d) Percent of free Cu^{2+} in total soluble Cu and pore water pH. Each data point represents the mean of 3−4 replicates.

the concentration of total Cu in soils, both of which are expressed on the log scale. Although there was a fairly good correlation between soluble Cu and soil total Cu, the K_d for Cu ranged widely from 515 to 9294 L kg^{-1}. Soil pH and pore water DOC were the two significant factors influencing K_d. The influence of pH and DOC on K_d was in opposite directions, as can be seen in the following regression equation:

$$\log(K_d) = 1.74 + 0.34\ \text{pH} - 0.58\ \log(\text{DOC})$$

($R^2_{adj} = 0.42$; pH and DOC were significant at $p < 0.01$ and $p < 0.05$, respectively).

The rather low coefficient for the pH term in the above equation is consistent with Sauvé et al. (*32*), who obtained a value of 0.27 from a compiled data set incorporating both spiked and field-collected contaminated soils. The above equation explained only 42% of the variability of the K_d for Cu, indicating a large uncertainty in the attempt to predict K_d from simple soil properties.

In comparison with soluble Cu, the variations of soluble Zn and Cd were much smaller. In most soils, soluble Zn in the pore waters was <1.0 mg L^{-1}. Four highly contaminated soils from Prescot had soluble Zn in the pore water between 1 and 3.5 mg L^{-1}. Soluble Cd varied from below the detection limit to 0.066 mg L^{-1}.

pCu^{2+} [$-\log(Cu^{2+}$ activity)] in all pore water samples ranged from 3.9 to 10.5. This range was much greater than that reported by Sauvé et al. (*25*), mainly because some of the soils used in this work were far more contaminated with Cu than those used by Sauvé et al. (*25*). Several low Cu soils had pCu^{2+} of >10, approaching values expected of uncontaminated, neutral pH soils (*33*). Pore water pCu^{2+} was influenced by two main factors: soil pH and total Cu concentration. The following multiple regression equation was obtained:

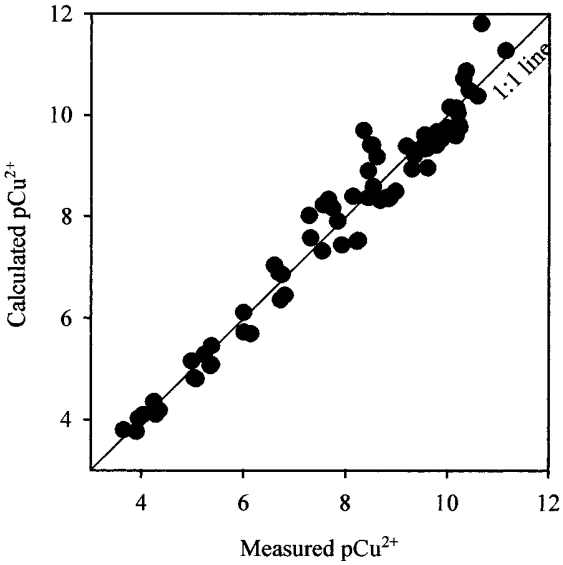

FIGURE 2. Comparison between measured and WHAM/Model VI calculated pCu^{2+}. Data points represent individual replicates of the extracted pore water.

$$pCu^{2+} = 1.79\ \text{pH} - 1.47\ \log(\text{total Cu}) - 0.53$$

($R^2_{adj} = 0.89$, $p < 0.001$, pH and total Cu were both significant

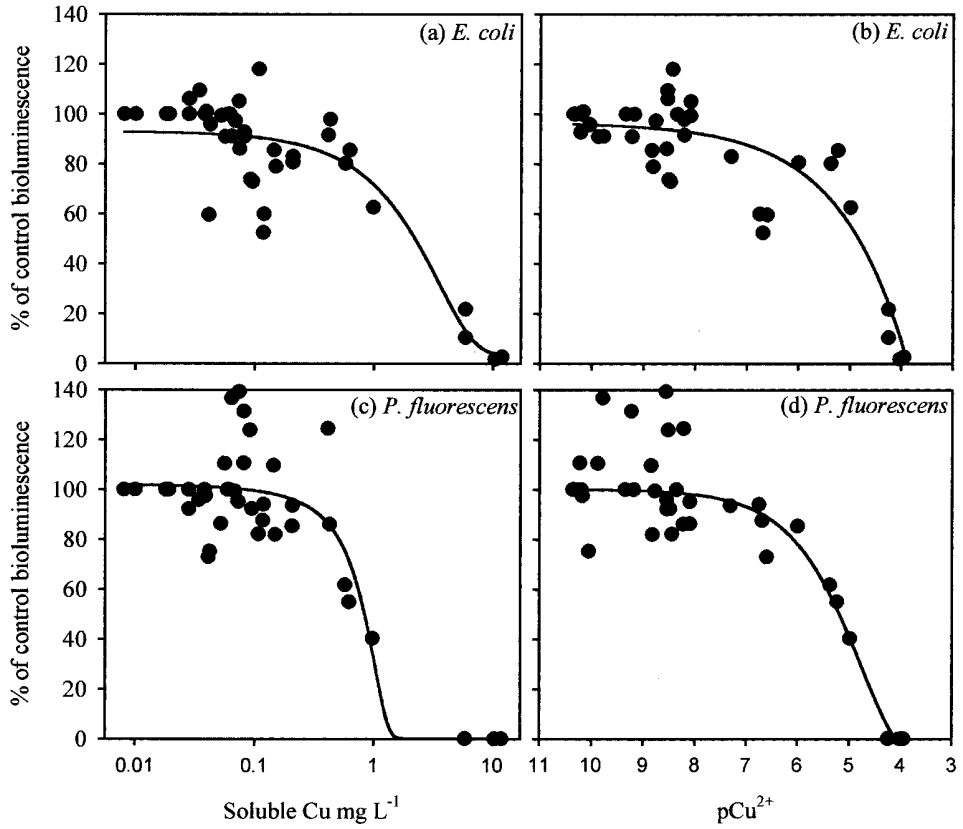

FIGURE 3. Relationships between bioluminescence of *E. coli* HB101 pUCD607 and *P. fluorescens* 10586r pUCD607 and soluble Cu concentration in pore water (a and c) or free Cu^{2+} activity in pore water (b and d). Data for all individual soil replicates from the first extraction are included.

TABLE 3. Fitted Parameters for the Gompertz Model[a] and EC_{25} and EC_{50}

pore water Cu	biosensor	parameters				standard error	R^2_{adj}	EC_{25}	EC_{50}
		a	*b*	x_0	y_0				
soluble Cu	*E. coli*	723.5	−8.07	−5.95	3.74	14.0	0.76	1.09	2.43
concn (mg L^{-1})	*P. fluorescens*	110.3	−0.37	0.92	0.28	16.4	0.79	0.56	0.83
free Cu^{2+}	*E. coli*	1661	1.30	0.20	−1564	14.0	0.79	5.69	4.78
activity pCu^{2+}	*P. fluorescens*	111.6	0.81	4.77	−11.5	12.6	0.87	5.88	5.19

[a] $y = y_0 + a\,(\exp(-\exp(-(x - x_0)/b)))$.

at $p < 0.001$).

The coefficients for pH and soil total Cu in the above equation were similar to those reported by Sauvé et al. (*4*), although the constant was far smaller in this case. Relationships between pore water pCu^{2+} and total Cu concentration and soil bulk pH are presented in Figure 1, panels b and c, respectively.

The percentage of total soluble Cu in soil pore waters present as free Cu^{2+} varied from 0.02 to 95.5% (Table 2). Figure 1d shows the strong influence of pore water pH on Cu speciation. In most pore water samples with pH >6, free Cu^{2+} accounted for ≤1% of the total soluble Cu. High proportions of free Cu^{2+} were found in the samples of low pH (<5.5), particularly when soluble Cu concentration was high. DOC was another important factor influencing the percentage of free Cu^{2+} in soil pore water. This can be seen by comparing the Prescot soil no. 4 with Cachapoal Basin soil no. 1 (Table 2). Both had similar pore water pH but differed greatly in DOC. The low DOC sample (Prescot no. 4) had 58% of the soluble Cu present as free Cu^{2+} as compared to a percentage of only 2.4% in the high DOC sample (Cachapoal Basin no. 1).

Prediction of Free Cu^{2+} Using WHAM Model VI. Previously, Christensen and Christensen (*34*) showed that the WHAM model V gave reasonable estimates of Cd and Ni complexation by DOC in groundwater samples. Figure 2 shows excellent agreement ($R^2 = 0.96$, $p < 0.001$) between measured pCu^{2+} and those calculated by the WHAM model VI in the soil pore water samples, across 6.5 orders of magnitude in the free Cu^{2+} activity. The difference between measured and calculated values was <0.5 pCu^{2+} unit in 82% of the samples. In applying the model, default parameters for proton and metal binding by humic substances were used (*20*), and it was assumed that the active proton and metal binding compound was fulvic acid (50% C). The model was optimized by adjusting the fraction of the DOC due to fulvic acid until the sum of the squared differences between observed and calculated pCu^{2+} was minimized. The optimized fraction was 0.69, i.e., the best agreement is obtained on the assumption that 69% of the DOC is due to fulvic acid, the remainder being inert with respect to ion binding. This value represents an overall average for the 22 soils from different regions, suggesting quite consistent behavior of DOC among soils. Model calculations showed that Cu speciation

was insensitive to Fe within the concentration range of 5−53 μg of Fe L^{-1} in the soil pore water samples. Variation of pCO_2 from 1 to 10 times atmospheric concentration also had little effect on Cu speciation in the pore water samples.

Impacts of Pore Water Cu on Bioluminescence of Bacterial Biosensors. Soil pore water Cu was considered to be the main factor affecting the bioluminescence of the biosensors used in this study. Concentrations of Zn and Cd in most samples were not high enough to cause a significant impact on the biosensors (*12*, *18*), except perhaps the Prescot soils 6 and 7. However, the extremely high concentrations of Cu in the pore waters from these two soils were likely to be the main factor determining the response of the biosensors. A previous study also showed that the bioluminescence response of the biosensors was stable across the pH range 4.5−7 (*17*).

Figure 3 shows the relationships between bioluminescence of the two biosensors and the concentration of soluble Cu or free Cu^{2+} activity in the pore water. The fitted values for the parameters used in the Gompertz model and the threshold values causing 25% (EC_{25}) and 50% (EC_{50}) reduction in bioluminescence are presented in Table 3. In all cases, the model explained >76% of the variation (R^2_{adj}). Judging from R^2_{adj} and standard error of estimate (Table 3), free Cu^{2+} activity appeared to give a better fit than total soluble Cu concentration for *P. fluorescens*. This may be taken as an indication that it is the free ion activity that determines acute toxicity, as has been suggested by researchers using a range of aquatic organisms (*7*−*10*, *35*−*37*). However, it is difficult to draw an unambiguous conclusion based on the present data, partly because of the fact that in natural soils the concentration of soluble Cu and free Cu^{2+} activity are correlated. In the case of *E. coli*, free Cu^{2+} activity and soluble Cu concentration produced a similar degree of fit.

EC_{25} and EC_{50} values for soluble Cu or free Cu^{2+} activity were similar for the two biosensors (Table 3). The EC_{25} for total soluble Cu in soil pore water varied from 0.6 to 1.1 mg L^{-1}. In a previous study using the same biosensors, Chaudri et al. (*12*) found that the bioluminescence decreased by 18% and 9%, respectively, in one soil pore water sample containing 0.6 mg L^{-1} total soluble Cu.

EC_{50} values for free Cu^{2+} activity (pCu^{2+}) ranged from 4.8 to 5.2. Using metal salt solutions, Paton et al. (*18*) reported EC_{50} values of 1.9, 0.4, and 0.1 mg of Cu L^{-1} for Microtox, *Rhizobium trifolii* F6 pUCD607, and *P. fluorescens* PUCD607, respectively. They did not determine the corresponding EC_{50} values for free Cu^{2+} activity, but the estimated pCu^{2+} was likely to be in the range of 4.7−6.0, assuming that no significant complexation occurred in their test medium (0.01 M KCl and small concentrations of $Cu(NO_3)_2$). The EC_{50} values obtained with soil pore waters in this study are therefore comparable to those reported by Paton et al. (*18*). The differences in sensitivity between different biosensors are not surprising, considering that they were isolated from such contrasting environmental niches and highlights the importance of using ecologically relevant biosensors.

The EC_{50} values for the two bacterial biosensors used in this study are higher than the toxicity thresholds reported for several aquatic organisms, which are in the range of 6−12 pCu^{2+} (*7*−*10*, *34*−*36*). The differences could be due to two reasons: (i) the different levels of tolerance between aquatic organisms and the soil bacteria used in the present study or (ii) differences in methodology of toxicity tests. On one hand, there is evidence that some marine and freshwater algae are very sensitive to Cu (*7*, *9*, *36*, *38*). Toxicity thresholds of 10−12 pCu^{2+} reported for these organisms imply that they are unlikely to survive in pore water even from uncontaminated soils, unless tolerance to Cu is evolved in response to prolonged exposure. Soil microorganisms present in pore waters are therefore expected to be less sensitive to Cu toxicity than these aquatic organisms. On the other hand, our measurements demonstrate acute toxicity, whereas aquatic tests mostly involve growth of the organisms over periods of days. It is likely that chronic exposure leads to greater toxicity.

For the reasons outlined above, we have used soil bacterial biosensors for the first time to establish realistic EC_{50} values for Cu in soil pore waters using direct methods of Cu speciation in well-equilibrated soils. These are therefore environmentally relevant to the soil ecosystem.

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