Influences of Copper Speciation on Toxicity to Microorganisms in Soils[1]

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Objective To investigate the relationship between copper speciation and microbial features (microbial communities and copper tolerance level) in order to determine the adverse effect of different forms of Cu on microorganisms. **Methods** Tessier's sequential extraction procedure was used to qualify the different Cu forms (exchangeable, carbonate bound, Fe/Mn oxide bound, residue and organic matter bound), and the copper tolerance level (expressed as IC_{50}, influence concentration) was measured by the plate-count method. **Results** By simple correlation analysis, the IC_{50} was positively correlated with the concentration of exchangeable Cu (R^2=0.8204), while weakly correlated with other forms of Cu. **Conclusion** The bacterial community tolerance increases in the copper-contaminated soil while sensitive bacteria decrease in the copper-contaminated soils. The exchangeable Cu exerts high toxicity to microbial communities.

Key words: Copper speciation; Toxicity; Microbial community

INTRODUCTION

The trace metal copper is an essential nutrient for virtually all forms of life on earth. However, excessive copper released into the environment may cause environmental pollution. Excessive copper in the environment is toxic to organisms, resulting in disturbance of the ecosystem. In general, heavy metal contamination lessens soil respiration[1], microbial biomass[2], and microbial numbers[3]. However, these variables can be influenced not only by heavy metals, but also by different environmental factors such as organic matter content, pH, and temperature. In addition, because heavy metals in the soils are present in various speciation due to interactions with various soil properties, total heavy metal concentrations in soils cannot provide a precise evaluation of their influence upon soil microorganisms[4].

Some characteristics of soil microorganisms, such as the microbial biomass and soil enzyme activity, are often used as bioindicators for the ecotoxicity of heavy metals[5]. Diaz-Ravina *et al*.[6] have evaluated the effects of heavy metals on the soil bacteria by the tolerance level of the community. The tolerance level is indicated by IC_{50}, which is the heavy metal concentration in growth media where a bacteria colony forming unit (CFU) decreases to 50% of that in original media. Kunito *et al*.[7] reported that the microbial numbers are negatively correlated with the 0.1 mol/L $CaCl_2$-extractable copper concentrations in the copper-contaminated soil samples collected near a copper mine in Akita Prefecture, Japan.

In the present study, soil samples with different pH, organic matters, and total copper contents were collected in the area of Tianjin Electrolytical Copper Factory. The microbial characteristics, copper content, and copper speciation were investigated to clarify the relationship among them.

MATERIALS AND METHODS

Sampling Sites

The area selected was around Tianjin Electrolytical Copper Factory, Tianjin, China, whose main business is to produce pure copper plate and other nonferrous metals. The annual output of this factory is 23 000 tons of pure copper plate which is made from rough copper and its scraps.

Soil Sampling

Five soil samples were collected in different places. Samples 1 and 2, collected near the sewage

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pond of the factory, were mainly sludge taken from the pond. Sample 3 was collected from the garden of the factory while sample 4 was taken from the riverside near the factory. Sample 5 was taken from the garden in Nankai Universiy, Tianjin, China, which was used as control.

All the soil samples were collected at a depth of 0-10 cm within each plot using a hand trowel. Three soil samples of about 500 g were randomly collected in each site, and then mixed together. The soils were placed in plastic bags, sent cold to the laboratory and stored at 4℃ until sample preparation. The soils were passed through a 2-mm sieve and dispersed well. A portion of the soil samples was air-dried for chemical analysis.

Soil Analysis

The pH values (in water) and organic matter contents (Org-M) are presented in Table 1. Soil pH was measured with a ORP measurement instrument (ORP 431) using a 1:2.5 soil-to-water ratio. The amount of soil Org-M was measured by dichromate digestion and titration method[8].

TABLE 1

Chemical Properties of Soils

Soil	pH_W	Org-M (%)	Ex-Cu (mg/kg)	Carb-Cu (mg/kg)	Oxid-Cu (mg/kg)	Org-Cu (mg/kg)	Re-Cu (mg/kg)	T-Cu (mg/kg)
1	7.8	13.8	14.82	1.01×10^4	1.76×10^4	6.41×10^3	597	3.47×10^4
2	7.5	13.5	13.16	7.54×10^3	1.59×10^4	8.41×10^3	835	3.27×10^4
3	6.8	6.62	4.368	270	675	675	265	1.89×10^3
4	6.5	2.28	1.328	67.4	149	150	47	415
5	6.9	6.52	0.5216	4.89	10.2	7.98	1.71	25.3

Copper Fractionation

The sequential extraction method developed by Tessier[9] was used to estimate exchangeable carbonate, Fe/Mn oxide, and organic matter binding forms of Cu in the soil. In brief, exchangeable Cu (Ex-Cu) was extracted from 1.00 g air-dried soil using 1 mol/L $MgCl_2$ (pH=7.0) solution by shaking for 1 h at room temperature. After Ex-Cu extraction, carbonate-bound Cu (Carb-Cu) was extracted from the residue using 1 mol/L NaOAc (pH=5.0) by shaking for 3 h. The Fe/Mn oxide-bound Cu (Oxid-Cu) was extracted from the residue using 0.04 mol/L $NH_2OH{\cdot}HCl$ in 25% HOAc for 3 h at 96℃±3℃ subsequently. After that, organic matter-bound Cu (Org-Cu) was extracted from the residue using 0.02 mol/L HNO_3 in 30% H_2O_2, pH2, 3.2 mol/L NH_4OAc in 20% (v/v) HNO_3 for 5 h at 85℃±2℃. In each extraction, the mixture was centrifuged at 5000 rpm for 15 min, and the supernatant was filtered through a 0.45 μm filter membrane before analysis. Total Cu (T-Cu) content in the soil was determined according to the method described by Chino *et al.*[10]. That is, 1.00 g air-dried soil sample was digested with an acid mixture (HNO_3:$HClO_4$:H_2SO_4=10:4:1), and the residue Cu (Re-Cu) was calculated by subtracting the Cu concentrations of the previous four steps (Ex-Cu, Carb-Cu, Oxid-Cu, and Org-Cu) from the total Cu. Copper was analyzed using ICP-AES-CID (inductively coupled plasma-atom emission spectroscopy-charge injection detector) (IRIS Intrepid II XSP, Thermo Electron Corporation). These extractions were carried out in triplicate and represented their mean values.

Microorganism Community in Soils

Soil was mixed thoroughly in each bag, and then two replicates of 1 g fresh weight samples were taken. One replicate sample was used for moisture analysis, and the other was used for isolation of bacteria, actinomycetes, and mildew (the main fungi species in the soil).

The samples for microorganism isolation were then placed in 9 mL sterilized water and vortexed vigorously. Each sample was gradually diluted to 10^{-6}, and then plated on culture medium.

The culture medium for bacteria incubation was nutrient agar medium (beef extract 3 g, peptone 10 g, NaCl 5 g, agar 15 g in one liter medium, pH 7.2-7.4), the medium for actinomycete incubation was starch-agar medium (starch 20 g, KNO_3 1g, K_2HPO_4 0.5g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, agar 15 g in one liter medium, pH 7.2-7.4,) supplemented with 0.0005 g/L nystatin to reduce fungal contamination, while the medium for mildew incubation was glucose-agar medium (glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, agar 15 g in one liter medium, natural pH) supplemented with 3 mL 1% streptomycin to reduce bacteria contamination. The plates were incubated in a incubator under aerobic conditions for several days (5 days for bacteria, 7 days for actinomycetes, and 12 days for mildew) at 28℃. The microorganisms were counted after incubation and the number was recorded.

TABLE 2

The Number of Microorganism in Soils

Soil CFU(soil/g)	1	2	3	4	5
Bacteria	3.8×10^6	---	2.5×10^6	6.1×10^6	8.1×10^6
Actinomycete	---	---	8.7×10^5	1.4×10^6	1.1×10^6
Mildew	4.2×10^3	---	3.2×10^4	1.4×10^4	8.7×10^4

Note. --- No microorganism detected.

Tolerance Level of Bacterial Community

The tolerance of the bacterial community towards copper (expressed as IC_{50}) was determined to evaluate the influence of copper on bacteria in the soil. According to Diaz-Ravina *et al*.[6] and Kunito *et al*.[7], IC_{50} values were determined by the plate-count method. Because the bacterial community extracted from the soil was examined under the same conditions, this technique could determine the metal tolerance level of the soil bacterial community. Thus, this value is scarcely affected by various soil properties other than heavy metal effects[11]. The number of bacteria was estimated by a dilution plate-count technique. For a medium, a diluted agar plate (3 g beef extract, 5 g peptone, 15 g agar, 5 g NaCl in one liter medium) was supplemented with Cu concentration (IC_{50}), resulting in a decrease of 50% from the control plate (no presence of copper) in colony forming units (CFU).

In this part of the experiment, the soil samples were gradually diluted to 10^{-6} with sterilized water. The IC_{50} values for the plate counts were calculated from the slope of the decreasing linear part of the plot of the percentage of inhibition *versus* the logarithms of Cu concentration. In this study, the CFU values represented the number of colonies on agar plate copper.

TABLE 3

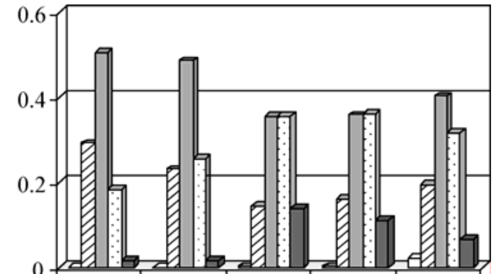
Copper Tolerance of Soil Bacterial Communities

Soil Samples	1	2	3	4	5
IC_{50} (logCu (mol/L))	-2.85	-2.90	-3.23	-3.45	-3.93

RESULTS

Copper in Each Fraction

Six Cu fractions (Ex-Cu, Carb-Cu, Oxid-Cu, Org-Cu, Re-Cu, and T-Cu) and other soil properties were determined for the soils (Table 1). It was evident that each form of Cu concentration was high in the soil samples 1 and 2. Furthermore, it could be found that copper fractions bound to Fe/Mn oxides in the soil samples 1 and 2 were higher (50.7% and 48.6% of total Cu concentration) than those in other samples (sample 3, 35.7%, sample 4, 35.9%, sample 5, 40.3%) as shown below (Fig. 1).



	1	2	3	4	5
Ex-Cu	4E-04	4E-04	0.002	0.003	0.021
Carb-Cu	0.291	0.231	0.143	0.162	0.193
Oxid-Cu	0.507	0.486	0.357	0.359	0.403
Org-Cu	0.185	0.257	0.357	0.361	0.315
Re-Cu	0.017	0.017	0.14	0.11	0.068

FIG. 1. Copper fraction.

Microorganisms in Soils

Soil microorganisms play a major part in the edaphon with respect to their abundance, diversity, and biomass. The main microorganism communities in the soil are bacterium community, actinomycete community, and fungus community.

From Table 2, it could be found that the number of microorganisms in the soil samples 1 and 2 was much lower than that in the other soil samples, suggesting that copper adversely influences microorganisms. At the same time, different species of microorganisms showed a different sensitivity to copper in the soils.

Relationship Between IC_{50} and Each Fraction

Fig. 2 shows the relationship between various fractions of copper and the tolerance level of the bacterial community (IC_{50}). There are four fractions of copper (Ex-Cu, Carb-Cu, Oxid-Cu, Org-Cu, and T-Cu); each one shows a weak relationship with IC_{50}, being not in accordance with Kunito *et al*.[7] IC_{50} tends to increase with each fraction, with the correlation coefficients of R^2=0.8204, 0.676, 0.6942, 0.6859, and 0.703, respectively. The relationship between the IC_{50} and exchangeable Cu has also been previously reported by Kunito *et al*.[7] and Saeki *et al*.[12], which has the highest correlation coefficient (R^2=0.8204).

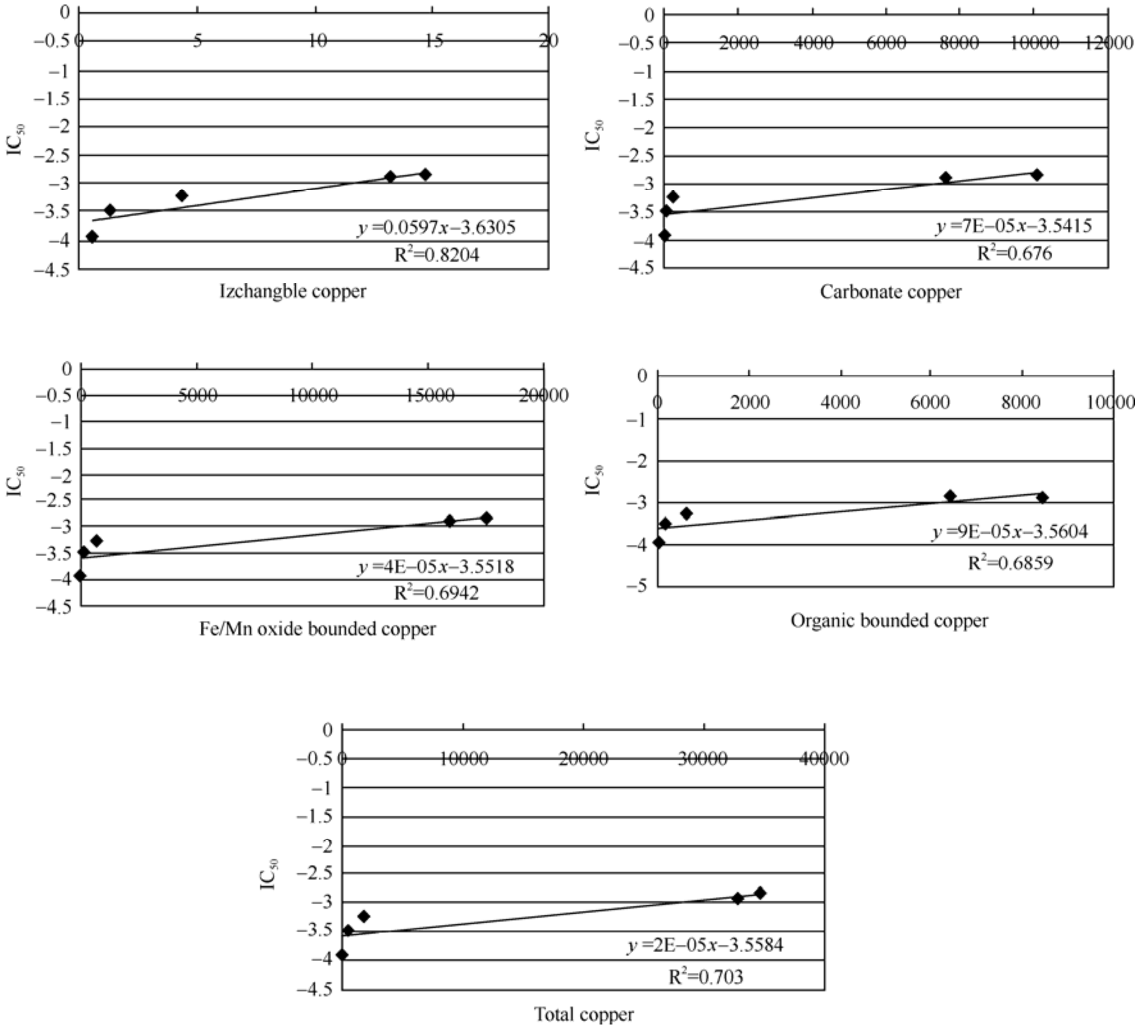


FIG. 2. Relationship between copper tolerance level of the bacteria community (IC_{50}) and Cu in various fractions in soils.

DISCUSSION

Copper fraction bounded to Fe/Mn oxides in the soil samples 1 and 2 are higher than those in other samples, the potential reason may be that the soil samples 1 and 2 were taken from the sewage sludge of Tianjin Electrolytical Copper Factory, which include other heavy metals such as Fe, Mn, and Ag besides Cu. The difference of pH in each sample was not big enough to show the influence of pH on the speciation of copper as reported by Kunito *et al*.[7].

Among the three microorganism communities detected, actinomycete community was most sensitive to copper, which could not grow in the soil samples 1 and 2 at all. However, no microbial community was found in the soil sample 2; the possible reason may be that the sludge in sample 1 were dredged up from the sewage pond for three years while the sludge in sample 2 were just dredged up from the sewage pond, suggesting that copper toxicity in the soils may decrease with elapse of time.

High Cu tolerance level (IC_{50}) was found in the soil samples 1 and 2, whereas the lowest (IC_{50}) value was found in the soil sample 5. The results indicate that tolerant bacteria increase while sensitive bacteria decrease in copper-contaminated soils, accounting for survival and adaptation to the metals in the soil, resulting in the high IC_{50} value of the soil bacterial community. The high IC_{50} means a strong effect of the metal on the soil bacterial community.

Relationships between IC_{50} and each fraction imply that exchangeable fractions of copper include the portion of metals that affect microbial communities in the soils.

CONCLUSIONS

The bacterial community tolerance, expressed as IC_{50}, increases in all the copper-contaminated soils in contrast to the uncontaminated control soil. The effect is most pronounced for tolerant bacteria and decreased sensitive bacteria in the copper-contaminated soils. The value of IC_{50}, is highly correlated with the value of exchangeable-Cu concentration (R^2=0.8204), and weakly correlated with other forms of Cu. Further studies are needed to clarify other effects of various soil properties on the copper forms.

REFERENCES

1. Hattori H (1992). Influence of heavy metals on soil microbial activities. *Soil Sci Plant Nutr* **38**, 93-100.
2. Chander K, Brooks P C, Hardings S A (1995). Microbial biomass dynamics following addition of metal-enriched sewage sludges to a sandy loam. *Soil Biol Biochem* **27**, 1409-1421.
3. Pennanen T, Frostegard A, Fritze H, *et al.* (1996). Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along two heavy metal-polluted gradients in coniferous forests. *Appl Environ Microbiol* **62**, 420-428.
4. Kunito T, Oyaizu H, Matsumoto S (1998). Ecology of soil heavy metal-resistant bacteria and perspective of bioremediation of heavy metal-contaminated soils. *Recent Res Dev Agric Biol Chem* **2**, 185-206.
5. Brookes P C (1995). The use of microbial parameters in monitoring soil pollution by heavy metals. *Biol Fertil Soils* **19**, 269-279.
6. Diaz-Ravina M, E Baath, A Frostergard (1994). Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. *Appl Environ Microbiol* **60**, 2238-2247.
7. Kunito T, Saeki K, Oyaizu, H Matsumoto S (1999). Influences of copper forms on the toxicity to microorganisms in soils. *Ecotoxicol Environ Safety* **44**, 174-181.
8. Kalembasa S J, Jenkinson D S (1973). A comparative study of titrimetric and gravimetric methods for the determination of organic carbon in soil. *Journal of the Science of Food and Agriculture* **24**, 1085-1090.
9. Tessier A, Campbell P G C, Bisson M (1979). Sequential extraction procedure for the speciation of particulate trace metals. *Anal Chem* **51,** 844-851.
10. Chino M S. Goto, K Kumazawa, N Owa, *et al.* (1992). Behavior of zinc and copper in soil with long term application of sewage sludges. *Soil Sci Plant Nutr* **38**, 159-167.
11. Baath E, Diaz-Ravina M, Frostergard A, Campell C D (1998). Effect of metal-rich sludge amendments on the soil microbial community, *Appl Environ Microbiol* **64**, 238-245.
12. Saeki K, Kunito T, Oyaizu H, Matsumoto S (2002). Relationship between bacterial tolerance levels and forms of Copper and Zinc in soils. *J Environ Qual* **31**, 1570-1575.
13. Welp Gerhard, Brummer Gerhard W (1997). Microbial toxicity of Cd and Hg in different soils related to tatal and water-soluble contents. *Ecotox Environ Safety* **38**, 200-204.
14. Diaz-Ravina M, E Baath (1996). Development of metal tolerance in soil bacterial communities exposed to experimentally increased metal levels. *Appl Environ Microbiol* **62**, 2970-2977.
15. Baath E A Frostergard, H Fritze (1995). Microbial community structure and pH response in relation to soil organic matter quality in wood ash fertilized, cleared-cut or burned coniferous forest soil. *Soil Biol Biochem* **27**, 229-240.
16. Diaz-Ravina M, Baath E (2001). Response of soil bacterial communities pre-exposed to different metals and reinoculated in an unpolluted soil. *Soil Biol Biochem* **33**, 241-248.

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