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Microbiological Response to Copper Contamination of a Paddy Soil

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Heavy metal contamination of soil is a significant environmental issue in China in recent years because of rapid industrialization and wide use of Cu-, Zn-containing fertilizers and fungicides. Due to their function and ubiquitous presence microorganisms can serve as an environmentally relevant indicator of pollution. Some features of soil microorganisms, such as the microbial biomass size, soil respiration rate, and soil enzyme activity, are often used as bioindicators for the ecotoxicity of heavy metals (Krasnova 1983; Niklińska et al. 2005). Brookes (1995) suggested that the ratio of basal respiration rate (BR) to microbial biomass C (C_{mic}), or C_{mic} as a percentage of soil organic carbon might be better indicators of soil pollution than either microbial activity or biomass measurement alone. In addition, due to the redundancy of function within soil microbial communities, some researchers have suggested that measures of microbial community structure by phospholipids fatty acid (PLFA) assay may be more sensitive to the changes in soil quality than assays which focus on general microbial processes or overall community size (Zelles et al. 1994).

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The effects of heavy metal contamination on the size and activity of soil microbial communities have been well documented (Alef and Nannipieri 1995; Freya et al. 2006). However, little is known about the toxic effect of Cu on microbial biomass, microbial activity, and the structure of microbial community in paddy soil, which is widespread in southern China and serves as an important land resource for food production in Asia. The objectives of this study were: (1) to measure microbiological responses of Cu pollution in paddy soil; (2) to evaluate the potential of using some sensitive microbial indicators for predicting Cu contamination at an early stage.

Materials and Methods

Soil samples were randomly collected at 0–20 cm depth from a paddy field in Xiasha District of Hangzhou City, Zhejiang province. The soil was silt loam soil (SLS) by local name. After removing visible plant materials, part of the soil was sieved (<2 mm) and stored in plastic bags at 4°C in a refrigerator, and the remaining sample was air dried, ground to <2 mm particle size for basic soil properties test (Table 1). Soil pH was determined using a pH meter at 1:2.5 soil: water ratio, soil particle distribution, Organic carbon, total and DTPA-extractable copper and other soil properties were measured according to Page et al. (1982).

Portions of moist soil (1 kg oven-dry basis) were each placed in a glass vessel and treated with a $CuNO_3 \cdot 3H_2O$ aqueous solution to reach the following external concentrations: 0, 50, 100, 200, 400, 800, 1,200, and 1,600 mg Cu^{2+} kg^{-1} soil. After the soil moisture was adjusted to 70% of water holding capacity, the soil samples were incubated

Table 1 Soil physical and chemical properties of SLS

Sand (%)	Silt (%)	Clay (%)	pH	O.C (g kg^{-1})	CEC (cmol kg^{-1})	Total Cu (mg kg^{-1})	Extractable Cu (mg kg^{-1})
65.0	28.8	6.2	7.80	4.52	8.42	18.25	1.20

Table 2 PLFAs used in this study as bioindicators (Zelles 1994; White et al. 1996; Steinberger et al. 1999)

Marker	Microbial group
12:0, 14:0, 15:0, 16:0, 17:0, 18;0, 19:0, 20:0	Saturated (bacteria in general)
14:0 iso, 15:0 anteiso, 15:0 iso, 15:0 iso 3OH, 16:0 iso, 17:0 anteiso, 17:0 iso	Gram-positive bacteria
15:1 iso G, 16:1 iso G, 16:1ω5c, ω7c,ω9c, 17:0 cy, 17;1 iso, 17:1ω8c, 17:1ω5c, 18:1 ω5c, 18:1ω9c, 19:0 cy ω10c, 19:0 cyω8c, 20:1ω9t or c all these may have OH groups	Gram-negative bacteria
TBSA 10 methyl 18:0, 16:0 10 methyl, 17:0 10 methyl	Actinomycete
18:2 ω6,9c, 18:3ω6, 18:3ω3	Fungi

at 25°C in the dark. Each treatment was replicated three times. At the end of 8-week incubation, subsamples of the soil were taken from the vessels for analyzing for chemical and microbiological properties in response to Cu treatments.

Invertase activity was measured following the methods of Ross (1975).

Urease activity was assayed by the buffer method of Tabatabai (1977), which involves the determination of the NH_4^+–N released when a soil sample is incubated with THAM buffer at the optimal pH (pH 9.0) with or without toluene and urea (0.2 M) at 37°C for 2 h. When chloroform fumigation was used, urease activity was assayed within 1 h after removing the chloroform by evacuation.

Acid phosphatase activity (APA) was assayed by Hoffman's method (Alef and Nannipieri 1995). Ten ml of disodium phenyl phosphate solution (25 g/L) was used as a substrate and added to 5 g of soil sample. The soil samples were incubated for 12 h at 37°C in pH 5.0 acetate buffer for APA determination.

Microbial biomass C was determined by the fumigation–extraction method (Wu et al. 1990). For each treatment soil, out of six subsamples (each containing 10 g fresh soil) three were fumigated with ethanol-free chloroform for 24 h. After removal of chloroform, the soil was extracted with 0.5 M K_2SO_4 (using a soil:solution ratio of 1:4) by shaking for 30 min. The remaining three unfumigated soil samples were subjected to 0.5 m K_2SO_4 extraction at the same time as the fumigated samples. The extracts were filtered through Whatman filter paper (No. 42). The filtrates were analyzed for dissolved organic carbon using a TOC analyzer (TOC-500, Shimadzu, Japan).

Soil respiration was measured by placing 20 g of the incubated soil in a sealed flask and incubating for 24 h at 28°C. The CO_2 evolved was trapped by 0.05 M NaOH, precipitated with 0.5 M $BaCl_2$, and then titrated with 0.05 M HCl using phenolphthalein as an indicator. The soil respiration was expressed as mg CO_2–C kg^{-1} soil per day.

PLFA profiles were determined using the method described by Zelles and Bai (1994). Lipid extracts were evaporated to dryness under a nitrogen atmosphere, redissolved in a small amount of chloroform, and separated into neutral lipids, glycolipids and phospholipids using an SPE-SI column (Varian, Harbor City, CA, USA). Fatty acid methyl esters were prepared from the phospholipid fraction with mild alkaline methanolysis using toluene:methanol:0.2 M KOH (0.5 ml:0.5 ml:5 ml). Nonadecanoic acid methyl ester was used as an internal standard. PLFAs were assayed using a gas chromatograph (GC) with a FID detector (HP6890; Hewlett Packard, Palo Alto, CA, USA) and each fatty acid was identified with the Sherlock system ver. 4.0B (Microbial ID, Inc, Newark, Del.). The PLFAs used in this analysis are presented in Table 2.

Statistical analyses were performed using the SAS statistical package and Excel 2003 program.

Results and Discussion

One of the most proposed biological tests to assess the impacts of heavy metals is the measurement of microbial biomass change. The response of microbial biomass C (C_{mic}) to external Cu input varied from positive to negative, depending on Cu loading rate (Table 3). The growth of microorganisms in the paddy soil was enhanced by low levels of Cu loadings (<200 mg Cu kg^{-1} soil) as evidenced by the increase in C_{mic}. However, C_{mic} was significantly decreased at high Cu levels (1,200 mg kg^{-1}). At the

Table 3 Microbial activities of paddy soil

Cu (mg kg^{-1})	C_{mic} (mg kg^{-1})	BR (mg $kg^{-1}day^{-1}$)	MQ (C_{mic}/C_{org}, % day^{-1})	MMQ (BR/C_{mic}, % day^{-1})
0	73.89 ± 12.12 c	42.93 ± 6.15 e	1.63 ± 0.06 c	58.1 ± 0.50 f
50	117.0 ± 11.00 a	85.85 ± 4.37 a	2.66 ± 0.02 a	73.3 ± 0.39 e
100	106.4 ± 10.41 a	79.45 ± 3.82 b	2.02 ± 0.02 b	74.7 ± 0.36 e
200	93.46 ± 9.00 b	74.34 ± 5.23 b	2.00 ± 0.07 b	79.5 ± 0.58 d
400	73.76 ± 10.86 c	62.96 ± 4.18 c	1.41 ± 0.03 d	85.4 ± 0.38 c
800	63.67 ± 6.55 c	55.64 ± 3.79 d	1.29 ± 0.02 e	87.4 ± 0.89 c
1,200	46.93 ± 6.55 d	55.44 ± 5.13 d	0.97 ± 0.02 f	118.1 ± 0.78 b
1,600	39.06 ± 5.01 d	53.22 ± 3.96 d	0.94 ± 0.02 f	136.2 ± 0.79 a

(Mean ± standard error, n = 3, letters indicate the significant statistical at $p < 0.05$)

external loading of 1,600 mg kg^{-1}, C_{mic} only accounted for 52.9% of the control. The stimulating effect of Cu on microbial biomass at low inputs may result from low availability of Cu in the original soil (Table 1) as Cu is essential for the growth of microorganisms, whereas the inhibited influence was caused by Cu toxicity to the microorganisms in the soil.

There were significant increases in CO_2 evolution from the soil caused by external Cu loading, which agrees with the results of C_{mic} measurement, although an inhibited trend was observed when Cu loadings were over 50 mg kg^{-1}.

The ratio of the C_{mic} in the total soil organic C (defined as microbial quotient-MQ) reflects the linkage and interactions between the two parameters and the quantum of carbon immobilized into the microorganisms (Insam and Domsch 1988). The MQ tended to increase at low external Cu loadings (<200 mg kg^{-1}), but decreased at higher Cu loadings (>200 mg kg^{-1}) (Table 2), indicating that Cu pollution in the soil causes a reduced amount of C being incorporated into microbial biomass and subsequently decreases soil organic matter quality. The decrease in MQ could be caused by decreased microbial biomass and/or partially disabled functions of its ability to mineralize organic matter. Since C_{mic} only accounted for a small proportion of total organic C in soil (1–5%), the change of organic C due to Cu contamination within a short term is limited, and therefore, the response of MQ to Cu loading was mainly related to the response of C_{mic}.

The percentage of respiration rate per unit microbial biomass (called as microbial metabolic quotient—MMQ) is considered as an index for evaluating the efficiency of soil microbial community for substrate utilization (Insam 1990). Insam et al. (1996) suggested that microorganisms in soils under heavily polluted stress have a lower efficiency of C utilization, resulting in more CO_2 evolved per unit of substrate. The results of present study indicated that in general the MMQ was increased with increasing Cu loading. An increased respiration rate and decreased C_{mic} caused by

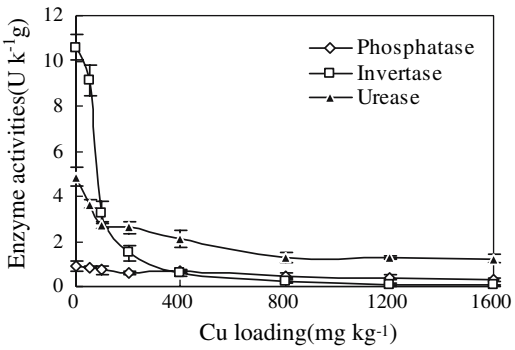

Fig. 1 Effects of Cu loading on the activities of phosphatase, invertase, and urease in soil. The units were mg phenol kg^{-1} h^{-1}, glucose mg kg^{-1} 24 h^{-1}, and NH_3–N mg kg^{-1} 24 h^{-1}, respectively. Averages of three replicates and SEM are shown

external Cu loading in the paddy soil resulted in a significant ($p < 0.05$) increase in the MMQ (Table 3). An increase in external Cu loading from 50 to 1,600 mg kg^{-1} raised the MMQ from 26.2% to 135%. The increase in the MMQ may result from Cu pollution stress that promoted microbial respiration and thus caused decline in the efficiency of substrate utilization by soil microbial community.

Enzyme activity has been suggested as an indicator of soil quality (Ascoli et al. 2006). In general, enzyme activities decreased with increasing external Cu loading (Fig. 1). The decrease was more dramatic at low to medium levels of Cu loading (<400 mg kg^{-1}) and diminished at higher levels (>400 mg kg^{-1}).

At the highest Cu loading (1,600 mg kg^{-1} Cu), the activity of phosphatase was decreased by over 60%, invertase activity by 99%, and urease activity by approximately 75%, as compared to the control (Fig. 1). It appears that the invertase and urease activity can serve as a promising indicator of Cu contamination to the paddy soil. These results agree with previous reports by Yeates et al. (1994) and Krasnova (1983).

Based on the fact that different groups of microorganisms contain different fatty acids in the phospholipid

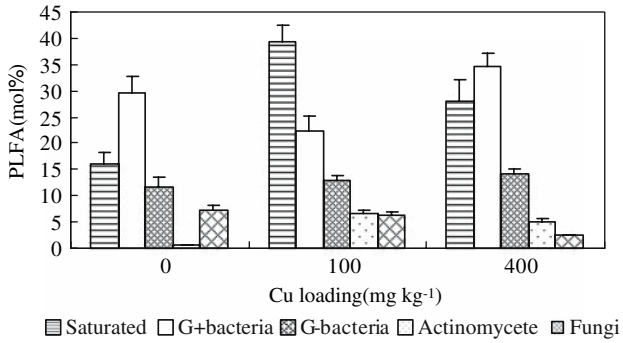

Fig. 2 Portion of fatty acids under Cu pollution. Mean of three replicates ± standard error

Table 4 Ratios of bacteria, actinomycetes and fungi PLFAs in the soil

Cu loading (mg kg^{-1})	0	100	400
Total PLFAs (mol%)	64.90 ± 5.92	87.30 ± 9.54	84.11 ± 4.96
Bacteria PLFAs (% total)	88.18 ± 4.73	85.18 ± 3.76	90.98 ± 3.58
Actinomycetes (% total)	0.86 ± 0.08	7.64 ± 0.82	6.16 ± 1.03
Fungi (% total)	10.96 ± 1.26	7.18 ± 1.11	2.87 ± 0.12
Actinomycete:bacteria (PLFAs) (%)	0.98 ± 0.13	8.97 ± 0.39	6.77 ± 0.79
Fungi:bacteria (PLFAs) (%)	12.42 ± 0.78	8.43 ± 1.24	3.15 ± 0.06

fraction of their membranes, the phospholipid fatty acid (PLFA) assay is frequently used to evaluate microbial composition and estimate the amount of microbial biomass (White et al. 1996; Steinberger et al. 1999; McKinleya et al. 2005). The measured PLFAs of the paddy soil ranged from C_{11} to C_{20} (Fig. 2). The major fatty acids in the soil were bacteria, which accounted for more than 85% of the total PLFAs (Table 4). Increasing Cu loading promoted the PLFAs, especially increasing the saturated bacteria PLFAs from 16.0 mol% to 39.2 mol% (100 mg kg^{-1}) or 27.9 mol% (400 mg kg^{-1}). The proportion of actinomycete was significantly increased but fungi were decreased especially at higher Cu loading (400 mg kg^{-1}) (Table 4).

Different Cu loadings resulted in different amounts of individual fatty acids and fatty acid ratios. As compared to the control, application of external Cu increased Gram positive bacteria, but decreased Gram negative bacteria and significant decreased fungi, whereas actinomycetes increased (Table 4; Fig. 2). Similar results were obtained from an acidic paddy soil regarding the effects of external Cu loading on C_{mic}, enzyme activities, and microbial community structure, except that the proportion of fungi increased, but that of actinomycetes decreased with external Cu loading in the acidic soil. This discrepancy could be attributed to the better adaptation of fungi in an acidic soil and of actinomycetes in a slightly alkaline soil.

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