Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Microbial activity and community diversity in a variable charge soil as affected by cadmium exposure levels and time*

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Received Dec. 24, 2007; revision accepted Jan. 17, 2008

Abstract: Effects of cadmium (Cd) on microbial biomass, activity and community diversity were assessed in a representative variable charge soil (Typic Aquult) using an incubation study. Cadmium was added as $Cd(NO_3)_2$ to reach a concentration range of 0~16 mg Cd/kg soil. Soil extractable Cd generally increased with Cd loading rate, but decreased with incubation time. Soil microbial biomass was enhanced at low Cd levels (0.5~1 mg/kg), but was inhibited consistently with increasing Cd rate. The ratio of microbial biomass C/N varied with Cd treatment levels, decreasing at low Cd rate (<0.7 mg/kg available Cd), but increasing progressively with Cd loading. Soil respiration was restrained at low Cd loading (<1 mg/kg), and enhanced at higher Cd levels. Soil microbial metabolic quotient (MMQ) was generally greater at high Cd loading (1~16 mg/kg). However, the MMQ is also affected by other factors. Cd contamination reduces species diversity of soil microbial communities and their ability to metabolize different C substrates. Soils with higher levels of Cd contamination showed decreases in indicator phospholipids fatty acids (PLFAs) for Gram-negative bacteria and actinomycetes, while the indicator PLFAs for Gram-positive bacteria and fungi increased with increasing levels of Cd contamination.

Key words: Cadmium (Cd), Microbial activity, Microbial community, Soil
doi:10.1631/jzus.B0710630 **Document code:** A **CLC number:** X503

INTRODUCTION

Soil biota is a significant component of soil quality as microorganisms play a vital role in soil ecosystem functioning related to soil fertility and primary production through organic matter decomposition and nutrient cycling. In recent decades, heavy metals have been continuously brought into the environment by various kinds of human activities (Ma and Rao, 1997). Heavy metals affect the growth, morphology and metabolism of microorganisms in soil, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita *et al*., 1995). A number of studies have been conducted to examine the response of soil microorganisms to heavy metal contamination (Brookes, 1995; Chander and Brookes, 1993; Ghosh *et al*., 2004; Renella and Mench, 2004). Heavy metal toxicity reduces the energy utilization efficiency of microbial metabolic processes, which then require a greater amount of carbon (C) for maintenance, thus reducing consequently the quantity of C incorporated into the microbial biomass (Valsecchi and Gigliotti, 1995). The microbial metabolic quotient (MMQ) was also found to significantly increase with increasing concentrations of heavy metals, whereas the ratio of microbial biomass C to organic C (C_{mic}/C_{org}) decreased in the soils contaminated with heavy metals (Valsecchi and Gigliotti, 1995), indicating a state of mi-

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* Project supported by the National Basic Research Program (973) of China (No. 2002CB410804), the National Natural Science Foundation of China (No. 20577044) and the Program for Changjiang Scholars and Innovative Research Team in Higher Education, China

crobial stress due to heavy metal toxicity. Chander and Brookes (1993) implied that microbial quotient (MQ) is generally more sensitive to increased heavy metal concentrations than the C_{mic} itself. Former studies also indicated that the ratio of biomass C to N (C/N) increased along a gradient of increased heavy metal contamination, suggesting that the fungal component in microbial biomass increases as contamination increases (Dai and Thierry, 2004; Hiroki, 1992). An increase in C/N ratio in microbial biomass following an increase in fungal communities seems to be a specific indicator of soil contamination by heavy metals. However, soil microbial biomass and activities, such as basal respiration, endo- or extracellular enzyme activities, C and N mineralization, show considerable differences associated with their sensitivity to heavy metal toxicity (Giller *et al*., 1998). These differences are based on many factors including methodology, heavy metal inputs and soil environmental factors. Pollution of soils with heavy metals is becoming a serious environmental problem, but limited information is available regarding the heavy metal-induced changes in the microhabitats of soil microorganisms (Ranjard *et al*., 2000).

Heavy metal pollution can cause shifts in the composition of microbial community (Li *et al*., 2006). DNA, phospholipids fatty acid (PLFA), and Biolog analyses are three commonly used analytical procedures for the evaluation of soil biological characteristics, and most of previous studies either used only one of the procedures to study the effects of Cd on microbial community structure (Muhammad *et al*., 2005; Renella and Mench, 2004), or focused on multiple heavy metal pollution (Yang *et al*., 2004). The difference in soil microbiological characteristics measured with only one method may not be reliable since the use of the other methods often leads to different interpretation of relative soil similarities (Widmer *et al*., 2001). In addition, most of previous studies used high Cd concentrations (>20 mg/kg) that were far above those found in soils or the environment (<1 mg/kg). There was minimal information available on the effects of Cd pollution on soil microbial community at low concentration range evaluated by using two or three abovementioned methods.

Compared with the correlation between chemically mobile Cd pools and the content in plants, the relationship between these pools and the responses of soil microflora is still unclear. Soil microbiological activity is generally regarded as an early and sensitive indicator of soil pollution by heavy metals (Brookes, 1995) on the consideration that soil microbes, being habitants and evenly distributed in soil, respond quickly to stressful factors. However, microbiological activity is a complex metabolic process, which cannot be evaluated by measuring a single parameter, and therefore, a set of related properties should be simultaneously determined, and the most sensitive parameter is then selected for application purpose. Most of previous studies used total Cd rather than available content in soil to relate microbial activities. The relationship is often poor or none as metal toxicity depends on its bioavailability. Most of heavy metals are bound to solid phase in soil, with a very small portion being water soluble or bioavailable (van Straalen, 2002).

In this study, the response of soil microbial biomass, activities and community to soil contamination was evaluated by using an incubation study with a relatively low concentration range of Cd. Different methods were used to measure microbial community changes as a result of Cd contamination. Microbial response to loaded Cd was then correlated with extractable Cd in the soil, which changes with incubation time. The objective of this study is to determine the most sensitive and reliable microbial parameters that can be applied to predict Cd contamination in variable charge soils.

MATERIALS AND METHODS

Soil sample collection and preparation

A representative variable charge soil (Typic Aquult) was collected at 0~20 cm depth from Deqing County, Zhejiang Province, southeastern China. Composite samples of the soils were hand-picked to remove large pieces of plant materials, grit, earthworms, etc., then air-dried, ground and passed through a 2-mm sieve prior to use. The basic properties of the soil were: pH 5.12, total organic C 22.3 g/kg, total N 2.76 g/kg, total Cd 0.40 mg/kg, total P 345 mg/kg, cation exchange capacity (CEC) 12.47 cmol/kg, and particle size composition 2~0.02 mm 22.5%, 0.002~0.02 mm 57%, <0.002 mm 20.5%.

Pretreated soil samples (each 1000 g) were placed into a series of 1000-ml plastic beakers. The soil samples were moistened to 40% of water-holding capacity by adding distilled water and pre-incubated for 14 d at 25 °C to stabilize microbial activity. Then, the soil was treated with Cd as $Cd(NO_3)_2$ in an aqueous solution at the rates of 0, 0.5, 1, 2, 4 and 16 mg/kg soil, and more deionized water was added to adjust soil moisture to 70% of its water-holding capacity. $NaNO_3$ was added to counterpoise NO_3^- in different treatments. Throughout the incubation period, soil moisture was kept constant by adding distilled water at regular intervals. All treatments were carried out in triplicates and the beakers with soil were randomly arranged in an incubator at 25 °C. Subsamples of the soils were taken at the intervals of 15, 30 and 80 d after incubation, and analyzed for available Cd (ammonium acetate extractable), C_{mic}, microbial biomass N (N_{mic}), basal respiration rate (BRR), MMQ, MQ and microbial community diversity.

Chemical analyses

Soil available Cd was determined following an ammonium acetate extraction procedure (van Ranst *et al*., 1999). Briefly, fresh soils in portion equivalent to 6 g oven-dry weight was shaken with 30 ml of ammonium acetate solution (pH 7.0) for 2 h (200 r/min) at 25 °C, then the suspension was filtered, and the filtrate was analyzed for Cd concentration using atomic absorption spectroscopy (AA6800, Shimadzu, Japan).

Soil organic C was measured following the Walkley-Black potassium dichromate sulfuric acid oxidation procedure (Nelson and Sommers, 1982). Total Cd in soils was analyzed by digestion with a mixture of concentrated HCl:HNO_3 (v/v, 4:1) and determining Cd concentration in the digester using inductively coupled plasma-mass spectrometer (ICP-MS, 7500a, Agilent, Germany).

Microbial parameters analyses

1. Microbial biomass C (C_{mic})

The fumigation-extraction (FE) method was used for measuring C_{mic} (Wu and Joergensen, 1990). Briefly, subsamples of fresh soil (each 20 g) were weighed into 25-ml beakers, and fumigated for 24 h in a vacuum desiccator containing 50 ml alcohol-free chloroform ($CHCl_3$). Both fumigated and unfumigated soil samples were extracted with 0.5 mol/L K_2SO_4 (soil to solution ratio of 1:2.5) by shaking for 30 min on an end-to-end shaker. The extracts were filtered through a 0.45 μm glass fibre filter and stored at −20 °C prior to analysis. Soluble organic C in the extracts was measured using a Shimadzu TOC-500 analyzer after the samples were defrozen. C_{mic} was calculated as the difference in soluble organic C between the fumigated and unfimigated samples using a k_{EC} factor of 0.45 to account for the non-extractable C_{mic}.

2. Microbial biomass nitrogen (N_{mic})

N_{mic} was determined by analyzing total N in the 0.5 mol/L K_2SO_4 extracts obtained from the above fumigation-extraction (Brookes *et al*., 1985). Twenty ml K_2SO_4 extract was transferred into digesting tubes, and 5 ml digesting solution (sulfuric acid-mercuric sulfate potassium sulfate solution) was added to each tube. The mixture in the tubes was digested on a hot block at 160 °C for 1 h and then 380 °C for 2.5 h. After cooling, the sample was diluted to 25 ml with ammonia free water (James, 1993) and the concentration of NH_4^+-N in the digester was determined using a discrete auto-analyzer (Astoria-Pacific Inc., Clackamas, USA). The N_{mic} was calculated using the equation: $N_{mic}=(N_f-N_{nf})/k_{EN}$, where N_f is the total N in fumigated soil, N_{nf} is the total N in non-fumigated soil, and k_{EN} is 0.54, a factor used to convert total N flush from the fumigation to N_{mic}.

3. Basal respiration rate

Basal respiration (BR) was measured by a procedure modified by Öhlingerhy (1995). Briefly, 20 g fresh soil was placed into a 500-ml jar containing a small vessel with 7 ml of 0.1 mol/L NaOH at 25 °C in the dark for 24 h, and the CO_2 trapped by NaOH was determined by titration with 0.05 mol/L HCl with carbonates being precipitated with 0.5 mol/L $BaCl_2$. Each sample was measured in triplicate, and all the samples were corrected for CO_2 content of blanks. Data were expressed as mg CO_2-C/(kg soil·24 h).

4. Soil microbial community structure measured by Biolog method

Biolog ECO plates were used to determine the substrate utilization pattern of soil microbial communities. Briefly, fresh soil (10 g) was added to 90 ml of distilled water in a 250-ml flask, and the suspension was shaken at 200 r/min for 30 min. Ten-fold

serial dilutions were made and 1000-fold dilution was used to inoculate the Biolog ECO plates. Plates were incubated at 25 °C for 7 d, and color development was measured as absorbance using an automated plate reader (VMAX, Molecular Devices, Crawley, UK) at 590 nm and the data were collected using Microlog 4.01 software (Biolog, Hayward, CA, USA).

5. Soil microbial community structure measured by PLFA analysis

The PLFAs of frozen-dried soil samples were extracted with chloroform-methanol-citrate (v:v:v, 1:2:0.8) buffer (0.15 mol/L, pH 4.0) modified after Frostegård *et al*.(1993a). Pooled supernatant solution samples (two repeated extractions) were split into two phases by adding chloroform and above extracting buffer. The lipid-containing phase was transferred to burned glass tubes, dried under N_2, dissolved in 600 μl of chloroform, and transferred to a silica gel cartridge (500 mg, 3 ml; Supelco, Bellofonte, PA, USA). Following the elution of neutral lipids and glycolipids with 10 ml chloroform and 10 ml acetone, respectively, phospholipids were eluted with 8 ml methanol and dried under N_2. Methyl myristate fatty acid (14:0) was added as internal standard, and PLFAs were subsequently derived by mild-alkali methanolysis (Yao *et al*., 2000). The resulting fatty acid methyl esters were then separated and identified by Agilent 6890N gas chromatography (Agilent, Wilmington, DE, USA) fitted with a MIDI Sherlocks Microbial Identification System (Version 4.5, MIDI, Newark, NJ, USA).

Statistical analyses

All soil microbial measurements were triplicated and the data were expressed as mean±*SD*. All the data among the different treatments were subjected to one-way analysis of variance (ANOVA) using the statistical package of SPSS (Version 11.0).

RESULTS AND DISCUSSION

Changes in Cd extractability

External loading of Cd resulted in a significant increase in ammonium acetate extractable Cd in the soil (Fig.1). There was a close relationship between extractable Cd and total Cd in the soil. The increase in extractable Cd was minimal at low loading rate (<1 mg/kg) probably because of Cd being adsorbed onto soil surface with high binding energy, and became significant with increasing Cd loading rate as Cd adsorption occurred on low-energy sites. For example, at the loading rates of <1 mg/kg, <25% of the added Cd was extractable, and the extractability was increased to >50% at the highest rate (16 mg/kg) (Fig.1). Ammonium acetate (pH 7) is a neutral solution with a large buffering capacity; In addition to water soluble Cd, it can extract Cd^{2+} in soil by electrostatic forces and by forming Cd-OAc complexes.

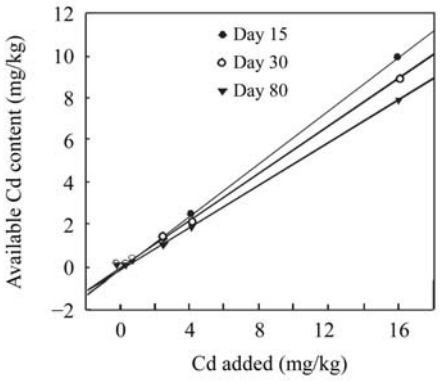

Fig.1 Relationship between soil extractable Cd^{2+} and initial Cd loading rate

However, the extractability of added Cd generally decreased with incubation time. For instance, at the Cd loading rate of 4 mg/kg, ammonium acetate extractable Cd were 64%, 57% and 41% of total added Cd, respectively at the 15th, 30th and 80th days of incubation. The results agreed with previous findings by Rajaie and Karimian (2006) that Cd availability in soil was affected by external Cd loading and time. As time went by, some of the surface adsorbed Cd could diffuse into soil aggregate particles or be occluded by newly formed oxides, thus lose its extractability.

Changes in microbial biomass C and N

In general, soil (C_{mic}) decreased with incubation time regardless of Cd treatment rate (Fig.2), which was likely caused by the depletion of available C source in the soil. After 15 d of incubation, C_{mic} increased at low Cd loading rates (0.5 mg/kg, with extractable Cd of 0.42 mg/kg). Similar results were obtained from plant growth and metabolic activity response to Cd loading of 5×10^{-8} mol/L (Nyitrai *et al.*,

2003) by solution culture. This stimulating effect of Cd on soil microbial biomass has not been reported in literature, likely because of high Cd loading rates (>20 mg/kg) being used in previous studies (José *et al.*, 2002; Muhammad *et al*., 2005). The biological response to Cd at low loading rate may be more meaningful as it is closer to field conditions. At higher Cd loading rates (2~16 mg/kg), C_{mic} consistently decreased with increasing Cd loading rate or extractable Cd in the soil (Fig.2). At the Cd loading of 16 mg/kg, C_{mic} decreased by 8.7% and 8.3% of the control, respectively at the 30th and 80th days of incubation.

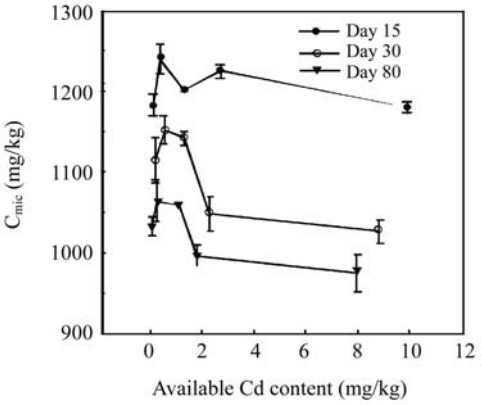

Fig.2 Effect of Cd external loading on the dynamics of soil microbial biomass C (C_{mic})
The error bar is the standard error of the means (*n*=4)

As shown previously by Renella and Mench (2004), C_{mic} was generally lower in Cd contaminated soil than that in normal soil, but the differences were often not significant. They pointed out that microbial physiological adaptation induced by Cd stress relied on cellular mechanisms, which were energy-demanding. Cellular mechanisms would increase the maintenance energy and reduce the conversion of substrate into new microbial biomass and other processes. In our experiment, the lowest C_{mic} occurred in the most severely polluted soil as measured at the 30th and 80th days of incubation, which was in accordance with previous studies.

Soil N_{mic} increased at low Cd loading rates (<1 mg/kg), due to some stimulating effect discussed in the previous section, but decreased consistently with increasing Cd concentration in the soil (Fig.3). N_{mic} in the controlled soil at the 15th, 30th and 80th days of incubation time were 56.7, 53.7 and 82.8 mg/kg, respectively, and the corresponding values at the Cd loading rate of 0.5 mg/kg were 81.7, 135 and 153 mg/kg, respectively. Compared with the control, the N_{mic} in the treatment of 16 mg/kg decreased by 49.3%, 9.0% and 48.6%, respectively at the 15th, 30th and 80th days of incubation. These results indicate that Cd contamination could significantly affect soil microbial biomass. Similar results were reported by Ghosh *et al*.(2004) for the effect of arsenic contamination on N_{mic}. Microbial biomass synthesis may be less efficient under heavy metal stress and biomass reduction in heavy metal contaminated soils was mainly due to inefficient biomass synthesis.

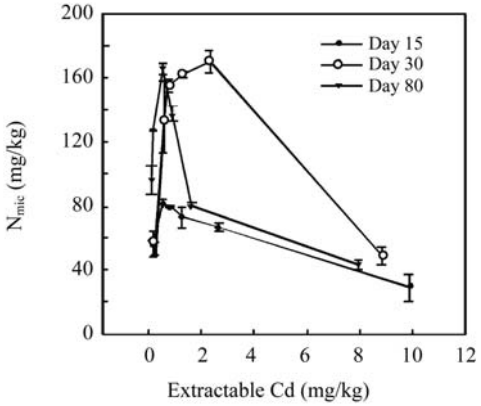

Fig.3 Effect of Cd external loading on the dynamics of soil microbial biomass N (N_{mic})
The error bar is the standard error of the means (*n*=4)

The value of MQ decreased generally with increasing available Cd concentration, although the lowest value was not observed at the highest Cd levels (Table 1). The adverse effect of Cd on MQ was greatest at 1~2 mg/kg of extractable soil Cd. Similar trend was reported by Barajas Aceves *et al*.(1999) on C_{mic} change in Zn polluted soil. Although MQ was decreased by Cd loading, its values were still within the 1%~5% range reported by Lavelle and Spain (2001). The MQ constitutes an "internal standard" and indicates a detrimental influence of Cd on soil microbial biomass. A reduction in this ratio as a result of metal pollution was reported from other studies (Dai and Thierry, 2004; Fliessbach and Reber, 1992). Anderson (1990) attributed the difference in MQ to the quality of the organic matter and to its availability for microorganisms. Cd contamination may cause a lesser incorporation of organic carbon into microbial cells. The decrease in MQ additionally explains the

reduced substrate utilization efficiency by the microorganisms because more substrate is used for catabolic processes at the expense of anabolic processes, and in the long run, it will lead to the reduction of microbial biomass in the heavy metal-contaminated soils (Ghosh *et al*., 2004).

Table 1 Effects of Cd on microbial quotient (MQ) and microbial metabolic quotient (MMQ)

Cd (mg/kg)	MQ (%)			MMQ (%/24 h)		
	Day 15	Day 30	Day 80	Day 15	Day 30	Day 80
0	4.71^{a}	3.78^{a}	3.65^{ab}	2.11^{a}	2.15^{a}	2.01^{a}
0.5	4.21^{bc}	3.57^{ab}	3.53^{bc}	2.08^{b}	1.95^{ab}	1.71^{c}
1	3.98^{cd}	3.76^{a}	3.57^{c}	1.75^{d}	1.54^{d}	1.49^{d}
2	3.73^{d}	3.65^{ab}	3.49^{bc}	1.96^{c}	1.70^{c}	1.42^{d}
4	4.31^{c}	3.56^{b}	3.33^{a}	2.09^{ab}	1.85^{bc}	1.70^{c}
16	4.49^{ab}	3.51^{ab}	3.45^{c}	2.10^{ab}	1.91^{b}	1.82^{b}

The different superscripts in the same column indicate the significant difference at 5% lever according to LSD test

In order to better evaluate the impact of Cd on soil microbes, the C/N ratio of soil microbial biomass was calculated (Fig.4). According to Wardle (1992), a high microbial biomass C/N ratio may indicate stressful conditions. As shown in Fig.4, the ratio of microbial biomass C/N varied with the levels of Cd contamination. The ratio decreased in the slightly contaminated soils (0~0.7 mg/kg available Cd), and increased progressively in the highly contaminated soils. For instance, at the 80th day of incubation, this ratio was around 7 in the slightly contaminated soils (Cd loading rate <2 mg/kg), but increased to 23 in the highly contaminated soil (16 mg/kg). According to previous investigations, fungi appear to be more tolerant to heavy metals than bacteria and actinomycetes (Hiroki, 1992). Paul and Clear (1996) indicated that bacteria had a C/N ratio as low as 3.5, and the ratio was 10 to 15 for fungi. These results suggested that increased microbial biomass C/N ratio is caused by increased fungal to microbial biomass ratio. Our results also suggested that soil microbial biomass varied with the Cd gradient with dominance of fungi in highly polluted soils and bacteria in the slightly contaminated soils, as bacteria are more sensitive to Cd than fungi. Fungi have high tolerance to heavy metals, and the reduction of bacteria and actinomycetes would lead to the weakening of their antagonistic action to fungi.

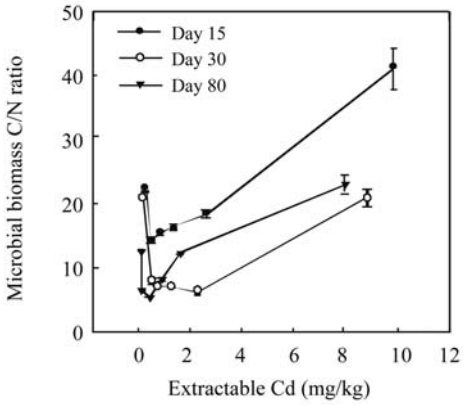

Fig.4 Effect of Cd external loading on the dynamics of soil microbial biomass C/N ratio
The error bar is the standard error of the means (n=4)

The variation in the C/N ratio was not directly related to soil incubation time, suggesting the occurrence of succession in the microbial community according to their greater or smaller specificity for the decomposition of different organic matter forms (Fernandes *et al*., 2005).

Changes in microbial activity

Soil respiration was restrained at low Cd loading rates (<1 mg/kg), with the lowest respiration occurring at the Cd rate of 1 mg/kg at the 15th day of incubation, but was enhanced at higher Cd loading rates (>1 mg/kg). Our findings agreed with the results of Landi *et al*.(2000). As a whole, the variation in respiration in Cd contaminated soils was small. In addition, soil respiration decreased over time irrespective of treatments (Fig.5).

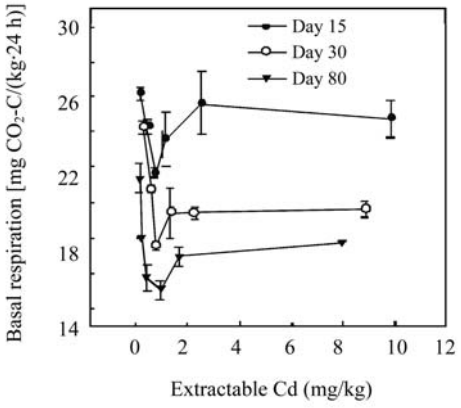

Fig.5 Soil basal respiration in relation to Cd external loading rate
The error bar is the standard error of the means (n=4)

Cd may reduce substrate availability for respiration by forming complexes with the substrate or by killing microorganisms. Heavy metals like Cd can produce a shift from a prokaryote toward a eukaryote dominated population and it is well known that growth and reproductive rate of eukaryotes are lower than those of prokaryotes, and eukaryotes have more C used for biosynthesis. Increasing concentrations of heavy metals often increases soil basal respiration. This means that heavy metal toxicity reduces the energy utilization efficiency of microbial metabolic processes, which then require greater amounts of C for maintenance, thus reducing the quantity of C incorporated into the microbial biomass (Valsecchi and Gigliotti, 1995). In addition, higher respiration rates in the Cd contaminated soils may be also due to the mineralization of killed microbial cells by surviving microorganisms.

Soil is a complicated system, and has a buffering capacity for the external input of pollutants. Various processes, directly or indirectly involved in soil respiration, can be affected by heavy metals. Therefore, the response of soil respiration to Cd contamination could be complicated.

The MMQ indicates how efficiently the microbial biomass is utilizing available C for biosynthesis, and has been suggested as a physiological characteristic of the microbial communities that is sensitive to environmental change (Anderson, 1994). In this study, a decrease in MMQ was observed in most of the Cd-loaded soils, especially at low levels, and this trend was maintained until the end of the incubation experiment (Table 1). In the soil with 0.7~0.9 mg/kg available Cd, the MMQ declined by 28.4% and 29.4%, respectively compared to the control at the 30th and 80th days of incubation. Furthermore, the high levels of Cd treatment resulted in a higher MMQ value, but the changes in MMQ were not specific to Cd stress, and therefore, it cannot be used as sole criterion for assessing the risk of heavy metal contamination.

The MMQ has been considered as a good index of heavy metal-induced stress in soil because high MMQ values were found in heavy metal contaminated soils (Ghosh *et al*., 2004; Renella and Mench, 2004; Fernandes *et al*., 2005). However, our study show that the MMQ was lower in slightly contaminated soils than the control. A decrease in MMQ, especially in Cd-polluted soils, was observed under both laboratory and field conditions (Palmborg and Nordgren, 1996). These results suggested that Cd pollution causes the changes in microbial communities and the newly developed microbes utilize different substrates from the original ones (Insam and Hutchinson, 1996).

We also found that the high levels of external Cd loading had higher MMQ values than the low Cd treatments. The increased MMQ was likely attributed to increased organic C metabolized for the maintenance of soil microorganisms, followed by a lower incorporation of organic C into the microbial biomass (Landi *et al*., 2000). These results indicate that faster respiration, reduced efficiency of substrate incorporation into new soil microbial biomass and increased microbial turnover may occur in Cd contaminated soils. However, microbial activities are influenced by many factors. Heavy metal loading can kill those sensitive microorganisms, thus causing a flush of decomposition of dead microbial cells by the surviving microorganisms (Giller *et al*., 1998) and subsequently increased MMQ.

Changes in microbial community diversity

The rate of color intensity on the Biolog plates over time was determined by calculating the average well color development (AWCD) on each plate at each reading time, the effect of Cd loading on the activity of soil microbial communities was reflected by decreased AWCD with increasing Cd loading rate, but the differences in AWCD between Cd treatments were not significant up to 120 h of incubation (Fig.6). It was manifested that Cd had detrimental effects on the substrate versatility of the soil bacterial community. Similar results were obtained by Wang *et al.*(2004), who examined the ability of a soil microbial community to metabolize 31 C substrates using Biolog ECO plates amended with Cd over a range of concentrations, and found that the substrate utilization rates were reduced by Cd toxicity. Cd affects bacterial species diversity by causing cell death and inhibiting the growth of Cd-sensitive bacteria. Biolog profiles for different treatments were compared using principal component analysis (PCA). The results indicate that the patterns of potential C utilization and microbial communities were changed in the Cd treated soils after 80 d of incubation, as evidenced by

the scores of the principal component 1 (PC1) relative to Cd loading rates (Figs.6~8).

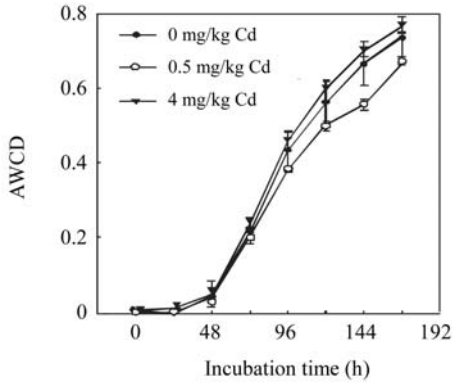

Fig.6 Effect of Cd external loading on the functional activity of soil microbial communities as indicated by average well color development (AWCD) at 590 nm
The error bar is the standard error of the means (n=3)

The microbial community in Cd treated soil generally has less ability for C substrate utilization, and might have developed tolerance to Cd during the short term of incubation. In many studies, the tolerance of microbial communities has been considered to be developed under heavy metal exposure (Kelly *et al*., 1999; Rasmussen and Sørensen, 2001). The PCA showed a change in C substrate utilization patterns in high Cd concentration treatments (Fig.7), suggesting that the active members of the microbial community under Cd stress were different from those in the control soil (without external Cd loading). However, no significant changes in microbial community structure occurred in heavy metal contaminated soils as reported by Yang *et al*.(2004). The major reason they provided was the strong binding between heavy metal and soil components, which decreased the bioavailability and toxicity of the added heavy metals. It is likely that the effects of heavy metals on microbial community structures are closely related to the type and chemical form of metals and the physical-chemical properties of soils.

An advantage of using the Biolog system is that Cd bioavailability can be determined precisely (Wang *et al*., 2004). This approach provides a sensitive and rapid measurement of functional diversity of soil bacterial communities. However, the culture-based approach used here also has limitations: (1) It is culture dependent, and reproducible results can be obtained only if replicates contain identical community profiles and are of similar inoculation density; (2) Changes in the microbial community can occur during the incubation; (3) The contribution of fungi is not measured because of their slow growth. Despite the shortcomings of the technique, it can still provide some useful information on the functional diversity of microbial communities, though this method could not evaluate whole microbial functional diversity.

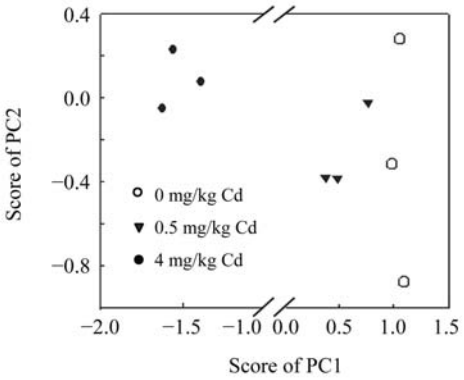

Fig.7 Effects of Cd loading on the pattern of substrate utilization by soil microbial communities after 120 h of incubation
Principal component 1 (PC1) and Principal component 2 (PC2) accounted for 28.0% and 23.4% of the total variance in the data, respectively

The quantitative description of microbial diversity in soils has become a topic of profound interest. Most soil microorganisms cannot be characterized by conventional cultivation techniques. According to the assessments by Amann *et al*.(1995), about 80%~99% of species of microorganisms have not yet been cultured. To overcome the problem of selective culture, the examination of microbial populations using ribosomal RNA and PLFA analyses has been suggested (Findlay, 1996). Overall, PLFA analysis is better suitable for studies of viable organisms and provides a more reliable basis for inferences about community composition. Cellular lipids, both in membranes and storage molecules, reflect the physiological status of the organisms. Many lipids are associated with taxonomic or functional groups of microorganisms and can provide insight into the types of organisms present in an environmental sample.

i15:0 and a15:0 are indicators of Gram-positive bacteria (Frostegård *et al*., 1993b; Konopka *et al*., 1999), which have positive correlation with PC1, with the correlation coefficients (r) of 0.582 and 0.576,

respectively. Their relative contents were higher in the Cd treated samples than the control. The relative content of cy17:0, an indicator of Gram-negative bacteria (Zogg *et al.*, 1997), decreased with increasing Cd loading, with a correlation coefficient (*r*) of −0.862 between the relative content of cy17:0 and PC1 (Table 2 and Fig.8). Soils with high Cd loading tended to have lower levels of indicator fatty acids for Gram-positive bacteria than the low Cd-treated soil, and thus Gram-negative bacteria appeared to be more sensitive to Cd pollution. These results are comparable with previous findings by John *et al*.(2003).

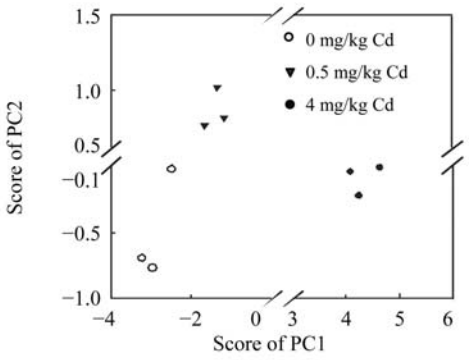

Fig.8 Principal component analysis of phospholipid fatty acid (PLFA) profiles in soils treated with different levels of Cd

PC1 and PC2 accounted for 84.9% and 4.63% of the total variance in the data, respectively

Table 2 Phospholipid fatty acid contents in soils treated with different levels of Cd

PLFAs	PLFA composition (%)		
	0 mg/kg Cd	0.5 mg/kg Cd	4 mg/kg Cd
14:0	15.17±0.12	15.85±0.18	20.66±0.15
i14:0	0	0.55±0.05	0.77±0.02
i15:0	4.79±0.02	4.90±0.03	4.88±0.05
a15:0	3.02±0.01	3.16±0.01	3.15±0.04
i14:0 3OH	0	0.62±0.01	0.56±0.04
i16:0	5.41±0.06	4.90±0.06	4.56±0.11
a16:0	0	0.61±0.01	0.86±0.01
16:0	18.67±0.07	17.46±0.11	15.51±0.07
i15:0 3OH	0.67±0.10	0.73±0.03	0.91±0.04
16:0 10Me	3.29±0.04	3.13±0.04	2.84±0.04
17:0 10Me	1.31±0.04	1.14±0.06	1.01±0.04
18:0 10Me	3.87±0.06	3.61±0.07	3.16±0.10
i17:0	4.64±0.08	4.39±0.06	3.72±0.05
a17:0	2.99±0.07	2.75±0.04	3.04±0.02
cy17:0	1.54±0.01	1.51±0.04	1.40±0.06
17:0	1.75±0.01	1.79±0.00	1.29±0.04
16:1 2OH	3.04±0.08	2.75±0.08	2.01±0.08
18:3ω6c	4.93±0.09	5.36±0.08	5.48±0.06
18:0	16.59±0.15	15..38±0.05	15.55±0.04
cy19:0ω8c	6.97±0.25	6.77±0.11	4.71±0.07
20:0	1.35±0.04	1.65±0.07	1.38±0.05
Total PLFA	100	98.71	97.45

i15:0, a15:0, i17:0 and 17:0 are ubiquitous fatty acids in bacteria, and the total percents of the four fatty acids were 14.2%, 13.9% and 13.0%, respectively in the 0 mg/kg, 0.5 mg/kg, 4 mg/kg Cd-treated soils (Table 2), whereas the relative content of 18:3ω6c, an indicator of fungi, increased with increasing Cd level, indicating that soil microbial community structure changes towards more fungi and less bacteria under Cd stress. This finding supported the previous suggestion that fungi were more resistant to heavy metals than bacteria (Bååth *et al*., 2005; John *et al*., 2003; Kelly *et al*., 1999).

16:0 10Me, 17:0 10Me and 18:0 10Me are considered the indicators of actinomycetes (Frostegård *et al*., 1993b). The total percents of these three fatty acids were 8.47%, 7.88% and 7.01%, respectively in the 0, 0.5 and 4 mg/kg Cd-treated soils (Table 2). The decrease in indicator fatty acids for nocardioform actinomycetes demonstrated that actinomycetes were less resistant to heavy metals than fungi. This result agreed with the previous observation in Pb and Zn contaminated soils (John *et al.*, 2003; Konopka *et al.*, 1999).

PCA of the PLFA profiles obtained from the soils at the 80th days of incubation indicated that there were differences in PLFA profiles among the soils with different Cd loading rates (Fig.8). External loading of Cd resulted in shifts in the structure of soil microbial communities as evidenced by the separation of sites on the PCA graphs (Fig.8). Soil samples on PC1 were related to Cd level in the soils. Soils with the highest Cd loading had a trend towards higher scores on PC1 in the PCA (Fig.9). The scores on PC1 were well correlated (R^2=0.987) with the levels of Cd loading in these soils (Fig.9), showing that heavy metal level was the most important factor determining the microbial community structure, as the first component in the PC plots explained majority of the variation in the PLFA data. Similar changes resulting from other metal pollution in different soils have been previously reported by Bååth *et al*.(2005) and Ekelund *et al*.(2003).

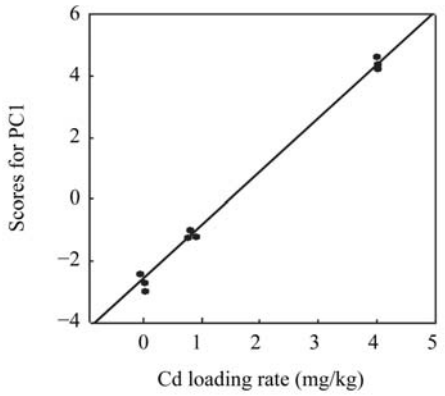

Fig.9 Correlation between Cd concentrations and scores for PC1 in Cd-treated soils (R^2=0.987)

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