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Effect of heavy metals on soil microbial activity and diversity in a reclaimed mining wasteland of red soil area

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Abstract: The microbial biomass, basal respiration and substrate utilization pattern in copper mining wasteland of red soil area, southern China, were investigated. The results indicated that soil microflora were obviously different compared with that of the non-mine soil. Microbial biomass and basal respiration were negatively affected by the elevated heavy metal levels. Two important microbial ecophysiological parameters, namely, the ratio of microbial biomass C(C_{mic})/organic C(C_{org}) and metabolic quotient(qCO_2) were closely correlated to heavy metal stress. There was a significant decrease in the C_{mic}/C_{org} ratio and an increase in the metabolic quotient with increasing metal concentration. Multivariate analysis of Biolog data for sole carbon source utilization pattern demonstrated that heavy metal pollution had a significant impact on microbial community structure and functional diversity. All the results showed that soil microbiological parameters had great potential to become the early sensitive, effective and liable indicators of the stresses or perturbations in soils of mining ecosystems.

Keywords: reclaimed mining wasteland; soil microorganism; soil microbial diversity

Introduction

Heavy metals are inherent components of soils, but today's great concern is related to their accumulation due to anthropogenic activities. Heavy metal pollution not only could result in adverse effects on various parameters relating to plant quality and yield, but also causes changes in the size, composition and activity of the microbial community (Giller, 1998). Abiotic stress caused by heavy metals, in inorganic and organic forms, affects the growth, morphology and metabolism of the microorganisms in soils. Numerous studies have demonstrated the adverse effect of different heavy metals on soil microbial biomass and its activity (Doelman, 1985; Duxbury, 1985).

With the decline of many ecosystems in the world and the lack of knowledge of soil microbial communities, increasing awareness concerning the importance of soil microorganisms in terrestrial ecosystems has emerged (Yao, 2003). Soil microorganisms constitute a large dynamic source and sink of nutrients in all ecosystems, play a major role in plant litter decomposition and nutrient cycling, soil structure, nitrogen fixation, mycorhizal associations, reduction in plant pathogens and other alternations in soil properties influencing plant growth (Kennedy, 1995). Moreover, soil microorganisms are very sensitive to environmental changes and not only does it directly influence soil fertility levels (Insam, 1996), but also influence the microbial viability, microbial biomass turnover and microbial utilization efficiency of organic carbon, which are important indicators of soil environmental quality (Bardgett, 1994).

A number of soil microbiological parameters, notable microbial biomass, basal respiration and microbial community structure (Doran, 1994; Sparling, 1997), have been suggested as the possible indicators of soil environmental quality, and have been employed in national and international monitoring programs (Yao, 2000). Soil microbial biomass, which plays an important role in nutrient cycling and ecosystem sustainability, has been found to be sensitive to increase heavy metal concentrations in soils (Giller, 1998; Huang, 1998). Carbon dioxide evolution, the major product of aerobic catabolic processes in the carbon cycle, is also commonly measured and indicates the total carbon turnover. The metabolic quotient, i.e. the ratio of basal respiration to microbial biomass, is inversely related to the efficiency with which the microbial biomass uses the indigenous substrates (Anderson, 1990) and can be a sensitive indicator to reveal heavy metal toxicity under natural conditions (Wardle, 1995). More recently, microbial community structure has also been recommended as a biological indicator of heavy metal stress (Doelman, 1994). The assay is based on the biology system using 95 different carbon sources to produce a metabolic profile of microorganisms (Garland, 1991). It has been used to detect differences between microbial communities in soil and the rhizosphere, heavy metal polluted and unpolluted soil (Knight, 1997; Baath, 1998).

Recently, more and more attention has been paid to the situation of heavy metal contamination in tailings and there is a growing need to reclaim such sites to increase environmental quality after mining operations being ended. Ecological restoration and mine reclamation have become important parts of the sustainable development strategy of many countries. But most of studies focused on the process of vegetation restoration and engineering technology of mine soil ecological system, rather than underground soil microbes rehabilitation, evolvement and effects on the ecological system of mine area (Tordoff, 2000). However, an increasing body of evidence suggests that microorganisms are far more sensitive to heavy metal stress than soil animals or plants growing on the same soils (Giller, 1998). Thus, measures of the fate of the microbial community following the initiation of reclamation efforts or the microbial responding would therefore, serve as an indicator of restoration progress (Harris, 1991) and may give insights into potential ways to accelerate a restoration.

In this study, we measured a range of microbiological parameters in reclaimed mining wasteland of red soil area, southern China. The aim of this study was to investigate the negative effects of heavy metal contamination on soil microorganism and to assist in providing theoretical arguments on quality evaluation and bioremediation of polluted soil in the mining area.

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1 Materials and methods

1.1 Sampling sites

The present study was constructed in copper mining wasteland, located at 29°43′23″N latitude and 147°59′09″E longitude, of Zhuji City, Zhejiang Province, southeast China. The copper mine was exploited about 20 years. The total area of the mine wasteland is 0.8 km^2. The topography is a hill with elevations ranging from 147 to 350 m above sea level. There is a humid subtropical climate with an average annual temperature of 16.2℃, a mean annual rainfall of 1335.9 mm and an annual nonfrost period of up to 249 d.

1.2 Soil sampling and processing

The soil samples were collected at the depth of 0—20 cm from three sample sites(S1, S2 and S3), according to the distance away from copper mining wasteland and pollution degree, namely, heavy pollution soil, medium pollution soil and non-mine soil. The distance of S1, S2 and S3 away from mining center was 20, 150 and 400 m, respectively. The three replicate samples were taken randomly from each sampling site and were kept in sealed plastic bags before transfering to the laboratory. The composite soil samples were sieved through a 2 mm screen, homogenized, a portion of which were air-dried and ground to pass through an 1 mm sieve, adjusting to 45% of water holding capacity(WHC), stored in polythene bags at 4℃ prior to soil microbial parameters analysis, and a portion of composite soil samples sieved through a 2 mm screen were air-dried for physical and chemical analysis. Some physical and chemical properties of the soil were measured with the routine analytical methods (ACSSSC, 1983), listed in Table 1. The total metals(Pb, Zn, Cu and Cd) were determined by the atomic adsorption spectrophotometry (AAS) after digestion with a mixture of HNO_3-HCl(Soon, 1993). The available metals(Pb, Zn, Cu and Cd) were extracted with EDTA solution and analyzed by AAS(Khas, 1998), showed in Table 2.

Table 1 Basic physical and chemical properties of soil samples tested

Soil No.	Pollution degree	pH (H_2O)	Organic C, g/kg	Total N, g/kg	Available N, mg/kg	CEC, cmol/kg	Size composition, %		
							2—0.02 mm	0.02—0.002 mm	<0.002 mm
S1	Heavy	4.79	8.21	1.76	153.52	13.50	51.94	29.66	18.40
S2	Medium	5.02	8.66	1.54	156.75	10.50	49.28	27.95	22.78
S3	Non-mine	5.76	9.03	1.92	174.53	10.25	48.74	30.71	20.55

Table 2 Heavy metal contents of soil samples collected in the copper mining wasteland, mg/kg

Soil No.	Pollution degree	Total metal				Available metal			
		Cu	Zn	Pb	Cd	Cu	Zn	Pb	Cd
S1	Heavy	1626.75	11060.38	2534.25	15.13	282.27	644.75	487.17	1.16
S2	Medium	158.67	1275.60	441.95	5.16	32.03	143.77	56.09	0.19
S3	Non-mine	41.57	161.75	37.29	0.63	8.49	58.24	7.86	0.03

Note: Data is means of three repeats in the table

1.3 Soil microbial biomass and basal respiration

Microbial Biomass C was determined by the chloroform-fumigation-extraction procedure in which C is extracted by 0.5 mol/L K_2SO_4 before and after fumigation(Vance, 1987). Organic C in the extracts was determined colorimetrically on a TRAACS auto analyzer. Microbial biomass C was calculated as follows: Microbial biomass C = E_C/k_{EC}, where E_C = (organic C extracted from fumigated soils) - (organic C extracted from non-fumigated soils) and k_{EC} = 0.45 (Wu, 1990). The total N in soil extracts was measured by Kjeldahl digestion-distillation procedure and microbial biomass N was calculated by a K_N of 0.54 (He, 1997). Basal respiration (CO_2 evolution) was measured by incubating fresh soil equivalent to 20 g dw at 28℃ in 500 ml airtight jars for 30 d, adjusted to 60% of water holding capacity. Respired CO_2 was trapped in 10 ml of 1 mol/L NaOH solution, the CO_3^{2-} was precipitated with $BaCl_2$ and the excess OH^- was titrated with HCl using a phenolphthalein indicator.

1.4 Sole carbon source utilization pattern

Sole carbon source utilization tests were performed as described by Campbell *et al*.(Campbell, 1997) and Yao *et al*.(Yao, 2003) using Biolog method. Biolog(Biolog, Inc., Hayward, CA) is a method analyzing the potential soil microbial community diversity. Briefly, fresh soil(10 g) was added to 100 ml of distilled water in a 250 ml flask and shaken on a wrist action shaker at full speed for 10 min. Ten-fold serial dilutions were made and 10^{-3} dilution was used to inoculate the Biolog gram-negative(GN) plates. Plates were incubated at 25℃ for 7 d and colour development was measured as absorbance(A) 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). Plates were read twice daily and ANOVA of the average well colour development(AWCD) over time was used to select comparable time points to avoid confounding effects of inoculum density differences between treatments in the multivariate analysis(Garland, 1996). The average well colour development(AWCD) for all C sources was calculated as a measure of total activity.

1.5 Statistical analyses

All experimental data were processed by Microsoft Excel 2000. The linear regression and the stepwise linear regression were conducted by using the statistical software packages of SPSS(V10.1) and SAS(V6.12). The least significant difference(*LSD*) at the 5% level was used to test the significance between means by a one way ANOVA. For multivariate analysis of the Biolog data the absorbance values were first divided by the AWCD to avoid bias between samples with different inoculum density(Garland, 1997; Campbell, 1997) and were then analyzed by canonical variate analysis.

2 Results and discussion

2.1 Contents of heavy metals in the copper mining wasteland

For the last decades, Cu, Zn, Pb and Cd have been the

dominant sources of pollution in this area. Heavy metal contents of soil samples collected in the copper mining wasteland are listed in Table 2. Results clearly show that the three soils varied greatly in heavy metal concentrations due to their distances from the copper mining center, total and available Cu, Zn, Pb and Cd markedly decreased with the site of soil sample away from mining center. This may be explained by leaching, translocation, accumulation of heavy metals released from the mining into the soils. The contents of heavy metals had an order: Zn > Pb > Cu > Cd. In comparison with the non-mine soil(S3), the average content (S1 and S2) of total Cu, Zn, Pb and Cd increased by 20.45, 37.15, 38.95 and 15.1 fold, respectively, indicating the mining wasteland was polluted by combined heavy metals severely and might cause ecosystem problems and impacts on human health through food chain.

2.2 Effect of heavy metals on soil microbial biomass and respiratory activity

2.2.1 Effect of heavy metals on soil microbial biomass

Microbial biomass and respiration of the soil samples tested are illustrated in Table 3. As expected, in the present study, microbial biomass carbon (C_{mic}) from the non-mine soil(S3) was nearly 0.36, 1.61 times more than those from mediumly polluted soil(S2) and heavily polluted soil(S1), respectively. Soil C_{mic} was negatively correlated with toal Cu ($r = -0.74$), available Cu($r = -0.75$), total Zn($r = -0.81$), available Zn ($r = -0.82$), total Pb ($r = -0.75$), available Pb($r = -0.77$), total Cd($r = -0.81$) and available Cd($r = -0.85$)(Table 4). The C_{mic}/C_{org} ratio decreased with increasing heavy metal concentrations and was negatively correlated with soil microbial biomass and organic C(Table 4 and Table 5), suggesting the detrimental influence of heavy metals on microbial biomass. Heavy metals caused less incorporation of organic carbon into microbial cells. Similar to C_{mic}, there was a strong decrease in the microbial biomass N with increasing heavy metal concentrations. In a word, there is a consistent decrease in the microbial biomass C and N with an increase of heavy metal contamination, indicating that heavy metals had an inhibitory effect on soil microbial biomass. Meanwhile, the microbial biomass C/N ratio decreased with increasing heavy metal concentration, suggesting a change in the microbial community structure affected by heavy metals.

Soil microbial biomass, which plays an important role in nutrient cycling and ecosystem sustainability, has been found to be sensitive to increase heavy metal concentrations in soils (Giller, 1998; Vig, 2003). There is considerable evidence documenting a decrease in the soil microbial biomass C and N as a result of long-term exposure to heavy metal contamination (Speir, 1995; Knight, 1997). Which was due to microorganisms in soil under heavy metal stress divert energy from growth to cell maintenance functions(Killham, 1985). Usually, a reasonably close, linear, and positive relationship exists between the organic C and biomass C contents in uncontaminated soils, but such a relationship did not exist in soils containing high metal contents (Brookes, 1984). Therefore, the ratio of the biomass C to organic C can be used as an indicator of soil quality and soil pollution (Jenkinson, 1981). Generally, C_{mic}/C_{org} ratio comprises of 1% to 4%(Smith, 1990). A lower ratio value in soil with higher heavy metal concentration may be explained the reduced substrate utilization efficiency by the microorganisms, as more substrate is diverted towards catabolic processes at the expense of anabolic processes leading to reduced microbial biomass in the long run (Sparling, 1992). Similar kind of finding was reported by Chander and Brookes (Chander, 1991a). Several investigators have reported that heavy metal stress can induce changes in the microbial biomass C/N ratio. Khas *et al*. (Khas, 1998) found an increase in the microbial biomass C/N ratio due to metal application in an incubation experiment. They concluded that the change of the ratio might result from an increased fungal biomass. Contrarily, our results showed the microbial biomass C/Nratio decreased with increasing heavy metal concentrations, while, Yao *et al*.(Yao, 2003) found there was no systematic change in the microbial biomass C/N ratio, consequently, this parameter should be interpreted carefully as a change in the microbial community structure is not always accompanied by a change of the microbial biomass C/N ratio(Yao, 2003).

Table 3 Microbial biomass and respiration rate of the soil samples tested

Soil No.	Microbial biomass C, mg/kg	Microbial biomass N, mg/kg	C_{mic}/N_{mic}	C_{mic}/C_{org}	Respiration rate, CO_2-C mg/(kg·h)	qCO_2, h^{-1}
S1	82.6	15.3	5.39	1.01	0.235	0.0034
S2	158.4	20.7	7.65	1.83	0.274	0.0021
S3	215.5	26.9	8.00	2.38	0.382	0.0015
$LSD_{0.05}$	16.8	4.3	0.2	0.108	0.085	0.0002

Note: Data are means of three repeats in the table

Table 4 Correlation coefficients(r) among microbial parameters and soil chemical properties

	Microbial biomass-C	Microbial biomass-N	Respiration rate	qCO_2, h^{-1}	N_{mic}/C_{mic}	C_{mic}/C_{org}
Organic C	0.51	0.52	0.81**	−0.62*	−0.12	−0.82**
Total N	0.45	0.48	0.63*	−0.57	0.21	0.37
Total Cu	−0.74**	−0.82**	−0.61*	0.92**	−0.34	−0.85**
Available Cu	−0.75**	−0.84**	−0.62*	0.93**	−0.32	−0.86**
Total Zn	−0.81**	−0.86**	−0.63*	0.88**	−0.35	−0.78**
Available Zn	−0.82**	−0.85**	−0.63*	0.89**	−0.45	−0.77**
Total Pb	−0.75**	−0.91**	−0.65*	0.87**	−0.46	−0.82**
Available Pb	−0.77**	−0.93**	−0.66*	0.91**	−0.51	−0.82**
Total Cd	−0.81**	−0.78**	−0.71*	0.92**	−0.25	−0.83**
Available Cd	−0.85**	−0.76**	−0.68*	0.93**	−0.26	−0.84**

Notes: * Significance level($P < 0.05$); ** significance level($n = 9$)($P < 0.01$)

Table 5 Correlation coefficients (r) among microbial parameters under different pollution degrees

Soil No.	Pollution degree	C_{mic}/C_{org} and SMB	qCO_2 and SMB	Respiration rate and SMB	C_{mic}/C_{org} and organic C
S1	Heavy	−0.625	−0.795	0.664	−0.772
S2	Medium	−0.210	−0.650	0.832	−0.826
S3	Non-mine	−0.071	0.084	0.912	−0.951

Note: SMB = soil microbial biomass

2.2.2 Effect of heavy metals on soil basal respiration

Soil basal respiration is shown in Fig.1. The results illustrated that the soil basal respiration had an order: S3 > S2 > S1 and was correlated to organic carbon and C_{mic}(Table 4 and Table 5). The variation trend of the soil basal respiration was that first decreasing to the minimum-increasing gradually-keeping unchangeably.

Mineralization of organic carbon to CO_2 commonly known

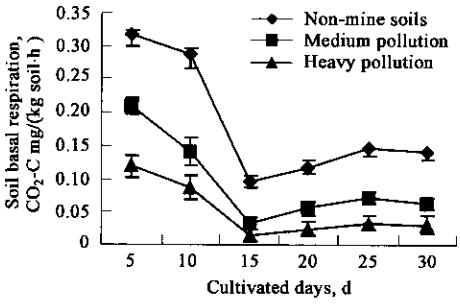

Fig.1 Soil basal respiration

as "soil respiration" is a good index of total activity of microflora involved in organic matter decomposition (Anderson, 1982). Therefore, soil respiration has been the most studied parameter on the effects of metals on microbial activities in soil(Baath, 1989). The basal respiration(Rb), apart from reflecting the rate of mineralization of soil organic carbon, reflects the respiratory activity of its microorganisms which biodegrade organic compounds in the soil(Anderson, 1978) and is closely related to soil environmental quality (Yeates, 1994). Heavy metals may reduce the substrate availability for soil respiration by forming complexes with the substrates or by killing the microorganisms(Landi, 2000).

2.2.3 Effect of heavy metals on soil microbial metabolic quotient(qCO_2)

The metabolic quotient, i. e. the ratio of basal respiration to microbial biomass, indicating how efficiently the microbial biomass is utilizing available carbon for biosynthesis, is a measure of microbial response to disturbance and has been considered a sensitive ecophysiological indicator of heavy metal induced stress in soil (Anderson, 1990; Wardle, 1995). Our results demonstrated that qCO_2 increased markedly with increasing heavy metal concentration (Table 3) and was negatively correlated with both soil microbial biomass and heavy metal concentrations(Table 4 and Table 5), indicating shifting of energy from growth to maintenance in an ecosystem.

Chander and Brookes (Chander, 1991b) reported less biomass formation from labeled substrate and higher qCO_2 values in heavy metal contaminated soils compared to normal soils. Biomass synthesis is less efficient under heavy metal stress and biomass reduction in heavy metal contaminated soils is mainly due to inefficient biomass synthesis. Although a plausible explanation, a high microbial qCO_2 in metal-contaminated soil is in itself no proof of either a higher maintenance-energy requirement or a lower substrate-utilization efficiency. The microbial maintenance-energy requirement is defined as the energy required for other functions than growth and is most accurately determined in energy-limited chemostat cultures from the dependence of the yield on the specific growth rate(Giller, 1998). Therefore qCO_2 can serve as an important indicator of soil quality and be closely related to soil pollution.

2.3 Effect of heavy metal on soil microbial community structure and functional diversity

The average utilization(AWCD) of the C sources for the three soil samples using the Biolog GN plates generally followed the same pattern with incubation time(Fig.2). After 24 h incubation, the rate of substrate utilisation (color development) at each site, was typically rapid for heavy pollution, medium pollution and non-mine soil. Although the utilisation of non-mine soil was initially as fast as the heavy pollution and medium pollution soil, the overall color development was much lower with an indication of longer lag times. The color development over time was generally sigmoid for all samples, but for the more slowly utilized carbon sources longer incubation times would be required to examine the full extent of carbon sourcce utilisation. Canonical variate analysis (CVA; Fig. 3), using all 95 carbon sources, revealed a separation of all the soil samples, indicating that they had different patterns of potential C utilization and different microbial communities. The community level physiological profile(CLPP) of the non-mine soil was clearly separated from the mine soils. The scores of the first canonical variate appeared to reflect the pollution level of the soils, indicating that there was a systematic change in substrate utilization pattern associated with different heavy metal contents. Correlation and analysis of the loadings of the most influential C sources on the first canonical variate indicated that microbial communities from the most polluted soil had increased utilization of C sources. The CVA graph (Fig. 3) also demonstrates that the variability in the metabolic profiles was the highest in heavy pollution soil(S1) and the lowest in the non-mine soil(S3). Therefore, the soil microorganism metabolic activity became stronger and could use C sources, indicating the soil microbial community structure and functional diversity were changed obviously in the reclaimed mining wasteland.

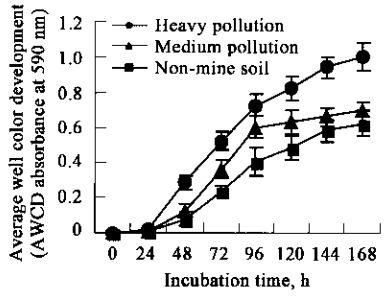

Fig.2 Mean absorbance of the Biolog plates at 590 nm for tested soils

Sole C source utilization tests using Biolog method have previously proved to be a satisfactory method of characterising microbial communities, based on their metabolic profile (Garland, 1991). It is often assumed that the difference in AWCD could be explained partly by lower biomass and activity(Garland, 1997; Grayston, 1998). In our study, AWCD values in the Biolog plates were correlated with both microbial biomass and heavy metal concentrations, however, Yao *et al*. (Yao, 2000) pointed that AWCD provides information about differences in community structure but they are not always clearly related to microbial biomass. With high concentrations of C sources in Biolog plates, some bacterial species which can use these C sources, will grow and reproduce quickly, and the number of inoculating microorganisms may not be the major factor on AWCD if the samples have a similar community structure. Colour development seems to mainly reflect species metabolic activity and the ability of the bacterial community to respond to

substrates.

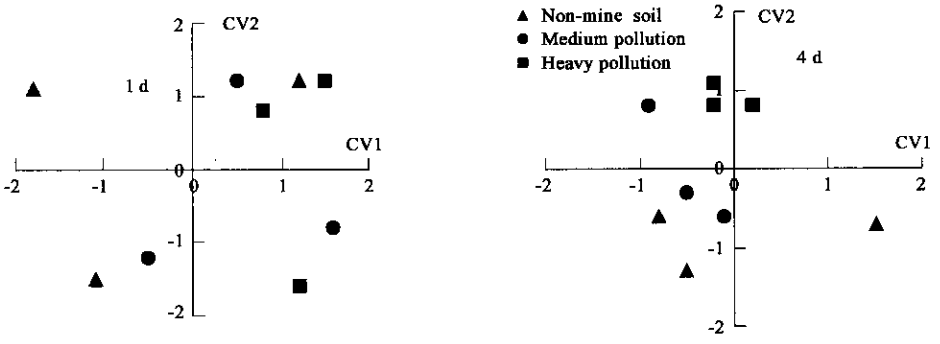

Fig.3 Plot of ordination of canonical variates (CV) CV1 against CV2 generated by canonical variate analysis of sole carbon source tests after 1 d and 4 d for 95 carbon sources in Biolog GN plates showing discrimination among the three samples

Generally, two microbial aspects of environments contaminated with heavy metals are a reduction in the numbers and species diversity of the biota and the development of metal resistant microbial populations (Yao, 2003). Kelly and Tate (Kelly, 1998) found that elevated metal loadings resulted in changes in the structure of the soil microbial communities, as indicated by changes in their metabolic profiles. Knight *et al*. (Knight, 1997) reported that both the metal concentration and reduced pH values showed significant effects on the Biolog pattern of soil microbial communities. In our study, the Biolog method also showed that there were qualitative differences in the soil microbial communities among different heavy metal levels, which was reason to explain the changes of the soil microbial biomass and soil microbial metabolic quotient among different heavy metal levels(Table 3). These supported other research results(Yao, 2003). This kind of information could be useful in evaluating soil quality and support efforts for the recovery of the reclaimed mining soil(Yao, 2003).

3 Conclusions

Heavy metals are known to affect the growth, morphology and metabolism of microorganisms in soils(Giller, 1998) as they cause protein denaturation or the destruction of the integrity of cell membranes (Leita, 1995). Moreover, heavy metal pollution can not only result in adverse effects on various parameters relating to plant quality and yield, but also cause changes in the size, composition and activity of the microbial community(Giller, 1998). In our present study, soil microbial biomass and basal respiration were negatively affected by heavy metals. Two important microbial ecophysiological parameters, namely, the microbial biomass C (C_{mic})/organic C (C_{org}) ratio and metabolic quotient (qCO_2) were closely correlated to heavy metal stress. There was a significant decrease in the C_{mic}/C_{org} ratio and an increase in the qCO_2 with increasing metal concentration. Biolog data showed that mine soil microbial community structure was changed obviously, the speed and quantity of carbon consuming were increased significantly and the kinds of carbon sources which soil microorganism used were changed, led to consume much more energies for maintaining the normal needs of its life, but the utilization efficiency was lower compared with the control.

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