Microbial characteristics of purple paddy soil in response to Pb pollution

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Abstract: The study focused on the change of microbial characteristics affected by Plumbum pollution with purple paddy soil in an incubation experiment. The results showed that low concentration of Plumbum had little effect on most of microbial amounts, biological activity and enzymatic activity. However, denitrifying activity was inhibited severely, and inhibition rate was up to 98%. Medium and high concentration of Plumbum significantly reduced the amounts and activity of all microorganisms and enzymatic activity, which increased with incubation time. Negative correlations were found between Plumbum concentrations and microbial amounts, biological activity and enzymatic activities except fungi and actinomyces. Thus they can be used to indicate the Plumbum pollution levels to some extent. LD_{50} of denitrifying bacteria (DB) and ED_{50} of denitrifying activity were 852mg/kg and 33.5mg/kg. Across all test soil microbes, denitrifying bacteria was most sensitive to Plumbum pollution in purple paddy soil. Value of early warning showed that anaerobic cellulose-decomposing bacteria (ACDB) and actinomyces were also sensitive to Plumbum pollution. We concluded that denitrifying activity, actinomyces, ACDB or DB can be chosen as predictor of Plumbum contamination in purple paddy soil.

Keywords: Plumbum; microbial community; soil biological activity; purple paddy soil.

INTRODUCTION

Plumbum (Pb) is a toxic heavy metal which causes a serious threat to humans and the environment as a result of wastewater irrigation, sludge applications, solid waste disposal, automobile exhaust, and industrial waste dumping (Khan *et al*., 2008; Cheng *et al*., 2002). Pb pollution in the atmosphere in the city mainly comes from leaded petroleum and most of the Pb from vehicle emissions was deposited to the soil. It has been demonstrated repeatedly that heavy metals could have toxic effects on soil biological processes and long-term adverse impacts on the health of soil ecosystems (Bhattacharyya *et al*., 2008). Heavy metals have sensitive influence on microbial community structure in soil, which ultimately lead to the changes of microbial amounts microbial activities including enzymes (Wang *et al*., 2008). Soil enzyme activities are known as sensors because of its sensitive to any natural and anthropogenic disturbance occurring in the soil ecosystem. The soil biological characteristics are more dynamic and more sensitive than physicochemical properties, so they are recognized as bio-indicators of soil quality (Khan *et al*., 2010). Assessment of heavy metal polluted soil quality by microbiology indicators has become a hot spot in current soil biology field (Pan and Yu, 2011).

Purple paddy soil, derived mainly from sandstone with different degrees of weathering, is the predominated soil type in Chongqing and Sichuan regions in China (Huang *et al*., 2006). However, there have been few studies concerning the effect of Pb Pollution on the microorganism characteristics of Purple Paddy Soil. Incubation experiment was conducted with application of different Pb concentrations. It was used to investigate Pb effects on the amounts of purple paddy soil microbial communities, enzymes activity and biological activity in this study. The aims were to select the type and biological indicators of sensitive anaerobic microorganisms which can reflect levels of Pb pollution, providing information for determining purple paddy soil heavy metal environmental quality indicators and forecasting paddy soil heavy metal pollution by anaerobic microorganisms.

MATERIAL AND METHODS

Soil characteristics

Soil samples (0-20 cm, purple paddy soil) were collected from a cultivated farm in National Purple Soil Fertility and Fertilizer Effective Long-term Location Experiment Farm, Southwest University. Soil samples removed visible gravels and remained organic residues. Physicochemical properties of the soils were tested according to standard procedures. Soil organic matter content was 32.1g/kg; soil total N was 1.52g/kg; soil available P and exchangeable K were 4.3mg/kg and 88.2 mg/kg, respectively. Soil pH was 7.27 determined in H_2O.

Experimental design

Certain copies of soil (equivalent to kilogram of dry soil, DS) were placed in 1300ml clean plastic box, and water was added to maintain the soil waterlogged, and it was incubated for 2 weeks at 28^{o}C for rejuvenation of

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anaerobic microbes. The soil samples were added with Pb using was $Pb(CH_3COO)_2$ solution, at following rates (mg/kg soil): 0, 200, 400 and 1600, recording as Pb0, Pb200, Pb400 and Pb1600, respectively. And then soil samples were sufficiently mixed at 28°C. Samples (3 boxes) were collected at 0, 7, 14, 21 and 28 days for the population of microbial communities and their activity. During the time of culture, water level was always kept 1-2 cm above the soil for simulating the anaerobic environment of paddy field.

Incubation and counting of microbial communities

Anaerobic microorganisms were cultured their mediums under anaerobic conditions, the methods of roll tube count or MPN count were referred to Hungate anaerobic technology (Frankenberger and Dick, 1983). Culturing at 28 °C, incubating time for denitrifying bacteria (DB) and anaerobic nitrogen fixing bacteria (ANFB) were 7 days, while 30 days for methanogenic bacteria (MB), about 3 days for sulfate-reducing bacteria. Denitrifying bacteria was counted by the bubble in Du-canaliculus; the growth indicator of methanogenic bacteria was the visible turbidity, supplemented by 102G gas chromatograph testing H_2, CH_4, counted by MPN; anaerobic nitrogen fixing bacteria was counted directly by vitro colony (García-Gil *et al*., 2000), sulfate-reducing bacteria was counted by the amount of black colonies in anaerobic tube (Doran, 1980). Colony forming units (cfu) were counted and the amounts of soil microbes in dry soil sample were calculated with a unit of cfu/g.

Determination of enzyme activity and biological activity

Activities of soil urease and neutral phosphatase were determined by colorimetric method (Zheng, 1986). The NH_4^+ released by urease enzymatic hydrolysis of urea was determined colorimetrically at 578 nm, with unit expressed as milligrams of NH_4-N per 100 g soil. Unit of neutral phosphatase activity was expressed as milligrams of phenol per 100 g soil. Catalase activity and invertase activity were determined by titration method (Zheng, 1986), which expressed as milliliter of 0.1 mol/L $Na_2S_2O_3$ per gram soil and milliliter of 0.1 mol/L $KMnO_4$ per gram soil. The measurement method of paddy soil methanogenic activity was referred to previous literature (Pankhurst, 1997). Determination and counting of paddy soil sulfate-reducing activity were according to the method (Pennanen *et al*., 1996). Measurement and counting of denitrifying activity were referred to literature (Garland and Mills, 1991). Measurement and counting of anaerobic nitrogen fixation activities with paddy soil were referred to previous method (Kelly *et al*., 1998).

Data Analysis

All data were the means of three repeated tests, and data analyses were determined by using SPSS 19.0 for windows software (IBM, New York, USA).

RESULTS

Effects of Pb on soil microbial community

Table 1 and table 2 showed the influence of Pb pollution on populations of soil microbes. Compared with control, application of Pb at low concentration (Pb200 treatment) stimulated growth of anaerobic fermenting bacteria and methanogen bacteria. Seven days later, the amounts of anaerobic fermenting bacteria (AFB) and methanogenic bacteria (MB) increased by 28% and 13%, respectively. However, the amounts of actinomyces and anaerobic cellulose decomposing bacteria (ACDB) decreased by 18% and 19%, respectively. It also showed that Pb with low concentration inhibited the growth of actinomyces. While application of medium (Pb400) or high (Pb1600) Pb concentrations decreased the amounts of all soil microbial communities, which were obviously lower than that with the control. By Pb400 treatment two weeks later, the amounts of bacteria, fungi, actinomycetes, hydrogen-producing acetogenic bacteria (HPAB) and methanogen bacteria (MB), denitrifying bacteria (DB), sulfate-reducing bacteria (SRB), anaerobic nitrogen-fixing bacteria (ANFB), anaerobic fermenting bacteria (AFB) and anaerobic cellulose-decomposing bacteria (ACDB) reduced by 24%, 44%, 31%, 7%, 23%, 21%, 33%, 29%, 26% and 41%, when compared with control; while inhibition effect were weakened with the elongation of incubating time. It was showed that fungi and anaerobic cellulose decomposing bacteria were most sensitive to Pb pollution (tables 1 and 2).

Table 1: Major microbial population influenced in different treatments and incubation time

Microbial Populations	Time (week)	Treatment				LD_{50}
		Pb0	Pb200	Pb400	Pb1600	
Bacteria (10^6cfu/g)	1st	381	369	290	187	1594
	2nd	594	571	408	209	1242
	3rd	580	586	554	214	1198
	4th	607	580	586	224	1221
Actinomyces (10^4cfu/g)	1st	75	62	42	30	1587
	2nd	58	58	32	29	1583
	3rd	32	41	27	21	1690
	4th	28	29	20	16	1790
Fungi (10^4cfu/g)	1st	29	31	20	19	2277
	2nd	16	15	10	9.9	2762
	3rd	7.8	9.2	6.4	5.6	2182
	4th	4.2	6.8	4.9	3.5	1702

Values are mean of three replicates.

Linear regression of Pb concentration and their amounts of ten tested microbes showed that microbial quantities in purple paddy soil was significantly negative correlated with Pb concentration except fungi and actinomyces. This indicated that Pb^{2+} pollution can be characterized by the quantity of some kinds of soil microbes. In the first week, LD_{50} (lethal dose, namely the content of heavy metals in soil when the amounts of microbial decreased 50%) for

soil bacteria of AFB, HPAB, MB, DB, SRB, ANFB and ACDB were 1594, 1163, 1372, 1685, 852, 1263, 1137 and 1206 mg/kg, respectively. This showed that LD_{50} for all of these microorganisms were high, with a range of 852-1594 mg/kg.

Table 2: The population of anaerobic microbe influenced in different treatments and incubation time

Microbial Populations	Time (week)	Treatment				LD_{50}
		Pb0	Pb200	Pb400	Pb1600	
AFB (10^6cfu/g)	1st	127	163	119	43	1163
	2nd	238	233	200	102	1359
	3rd	205	245	217	118	1473
	4th	213	213	199	117	1662
HPAB (10^5cfu/g)	1st	275	290	212	123	1372
	2nd	424	458	284	199	1445
	3rd	555	579	345	224	1322
	4th	513	531	351	258	1601
MB (10^5cfu/g)	1st	33	37	26	19	1685
	2nd	92	104	70	21	985
	3rd	102	117	82	34	1108
	4th	96	112	89	42	1235
DB (10^4cfu/g)	1st	59	58	40	4.5	853
	2nd	158	154	97	39	1032
	3rd	150	145	113	38	1055
	4th	145	154	103	28	971
SRB (10^4cfu/g)	1st	99	102	70	38	1263
	2nd	128	134	83	43	1174
	3rd	119	123	92	48	1292
	4th	122	119	96	53	1390
ANFB (10^4cfu/g)	1st	39	37	29	12	1137
	2nd	65	60	49	21	1192
	3rd	65	70	59	25	1202
	4th	62	65	64	32	1461
ACDB (10^4cfu/g)	1st	15	13	9.0	4.4	1206
	2nd	37	31	19	8.3	1077
	3rd	42	43	32	8.7	982
	4th	36	44	41	10	1013

Values are mean of three replicates
AFB-Anaerobic Fermentation Bacteria; HPAB-Hydrogen Producing Acetogenic Bacteria; MB-methanogen bacteria; DB-Denitrifying Bacteria; SRB-Sulfate-reducing Bacteria; ANFB-Anaerobic Nitrogen Fixing Bacteria; ACDB- Anaerobic Cellulose Decomposing Bacteria

Effects of Pb on soil enzyme activity

Fig. 1 showed that four kinds of soil enzymatic activity were fluctuated with incubation time under control or Pb200 treatments. Low Pb concentration had no effect on activities of soil urease, invertase, neutral phosphatase and catalase, which were similar with the control. Pb400 and Pb1600 treatments inhibited significantly the activities of these enzymes than control. After 7 days with Pb400 treatment, the activities of urease, invertase, neutral phosphatase and catalase decreased by 21%, 8%, 25% and 18%, comparing with control. With the increase of Pb concentration, enzyme activities were gradually decreased.

Performance of four kinds of soil enzymatic activity varied with Pb levels. The activities were similar between the control and Pb200 treatment, while they reached the maximum in the second week of incubation and decreased thereafter except for catalase which reached the maximum in the third week with Pb200 treatment. In Pb400 treatment, urease and neutral phosphatase activity reached the maximum in the second and third week, whereas activities of invertase and catalase increased with time. In Pb1600 treatment, activities of invertase and catalase reached the maximum in the fourth week, but activities of urease and neutral phosphatase reached the maximum in the third week and then decreased. Compared with control, Pb application, range of all activities of soil enzyme in purple soil reached maximum in the second week under Pb1600 treatment.

Linear regression of Pb concentrations and soil enzymes activity showed that the four tested activities of soil enzymes were significantly and negatively correlated with Pb concentrations. Calculated from the fitted equation, the ranges of ED_{50} (ecological dose, namely the content of heavy metals in soil when the enzyme activity decreased 50%) of urease, invertase, neutral phosphatase and catalase activity were 1730-1998, 2924-4163, 1690-2061 and 2454-2909 mg/kg, respectively.

Effects of Pb on soil Biological activity

Fig 2 showed the performance of four kinds of soil biological activity varied with Pb levels; low Pb concentration had no effect on sulfate reduction activity (SRA), which reached maximum in the second week with control. Sulfate reduction activity was inhibited most obviously under medium or high Pb concentration, and it was growing with time. Denitrifying activity (DA) was inhibited significantly with Pb, which were lower significantly than control. It decreased with the increasing Pb concentrations; denitrifying activity had a decrease of 97.6% in low Pb concentration and 98% in medium Pb concentration and 99.0% in high Pb concentration. It showed denitrifying activity was weakened after adding Pb to Purple Paddy Soil, which was very sensitive to Pb contamination.

Methanogens activity was promoted slightly under low Pb concentration (fig. 2). It dropped by 11.0% under Pb medium concentration and 53.4% high Pb concentration after four weeks, compared to control. This inhibitory effect decreased with the incubation proceeded. Low Pb concentration had no effect on anaerobic nitrogen fixation activities of purple paddy soil, which were similar with control. However, it was inhibited in medium and high Pb concentration, and which increased with the incubation proceeded. The inhibitory effect decreased with incubation time and increased with the increasing of Pb concentrations.

Table 3: Early-warning indicator of Purple paddy soil at 400mg/kg Pb concentration

Parameter	The percentage of microorganisms suppression after 7 days				
Microbial Quantity	HPAB	SRB	MB	Bacteria	AFB
	7	21	23	24	26
	ANFB	Fungi	DB	ACDB	Actinomyces
	29	31	33	41	44
Enzyme Activity	Invertase	Catalase	Urease	Neutral phosphatase	
	9	17	21	25	
Biological Activity	MA	SRA	ANFA	DA	
	1	21	34	98	

AFB- Anaerobic Fermentation Bacteria; HPAB- Hydrogen Producing Acetogenic Bacteria; MB- methanogen bacteria; DB- Denitrifying Bacteria; SRB- Sulfate-reducing Bacteria; ANFB-Anaerobic Nitrogen Fixing Bacteria; ACDB- Anaerobic Cellulose Decomposing Bacteria; MA- Methanogen Activity; SRA- Sulfate Reducing Activity; ANFA- Anaerobic Nitrogen Fixing Activity; DA- Denitrifying Activity

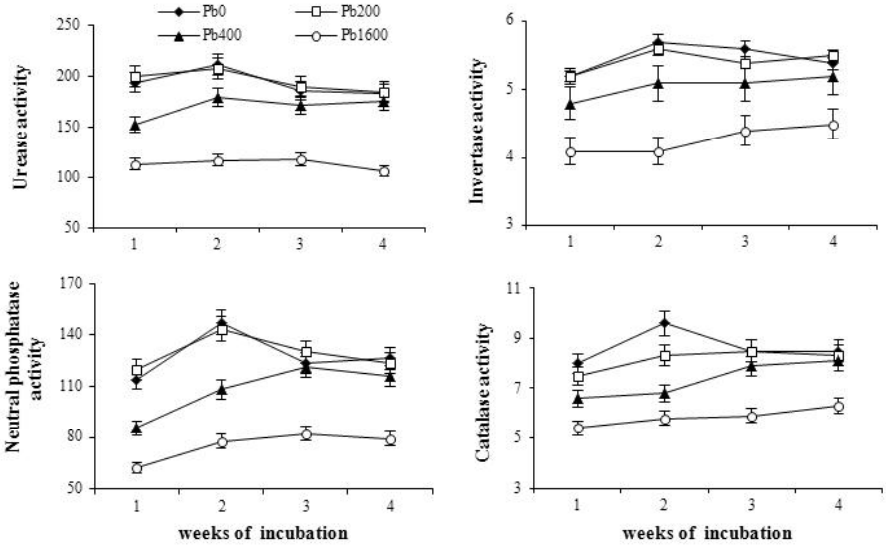

Fig. 1: The effects of Pb on enzymatic activity in purple paddy soil during the different incubation periods. Units of urease activity, neutral phosphatase activity, catalase activity and invertase activity were expressed as NH4-N μg/d/g, Phenol μg/d/g, 0.1 mol/L $KMnO_4$ ml/30 min/g and 0.1 mol/L $Na_2S_2O_3$ ml/d/g, respectively.

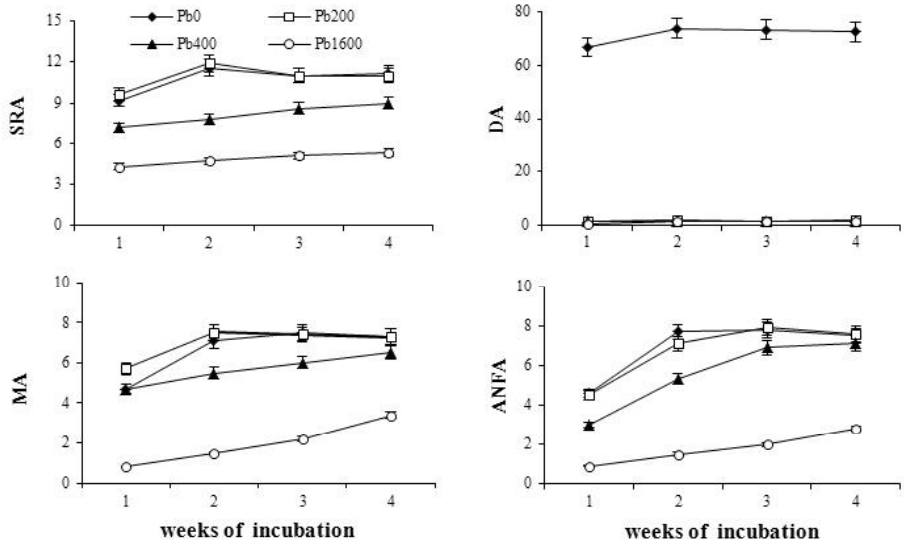

Fig. 2: The effects of Pb on biological activity in purple paddy soil during the different incubation periods. Units of SRA (Sulfate Reducing Activity), DA (Denitrifying Activity), ANFB (Anaerobic Nitrogen Fixing Activity) and MA-Methanogen Activity were expressed as S^{2-} μg/d/g, %, 10^{-7} mol C_2H_2/d/g and 10^{-6} mol CH_4/d/g, respectively.

Linear regression of Pb concentrations and soil biological activity showed that the four tested biological activities were significantly and negatively correlated with Pb concentrations. Hence, to some extent, soil biological activity can be used to characterize the extent of Pb contamination in purple paddy soil. Calculated from the fitted equation, the ranges of ED_{50} of SRA, DA, MA, ANFA were 1333-1484, 33.5-36.4, 908-1411 and 986-1209 mg/kg, respectively. Thus, denitrifying activity was most sensitive to Pb contamination in purple paddy soil

The value of early warning of purple paddy Soil to Pb pollution

The amounts of soil microorganisms, enzyme activity and biological activity can be used to indicate soil heavy metals pollution because of their sensitivity and relevance to heavy metals. The tested results showed that the correlation between pollutants concentration and amounts (activities) of soil microorganisms was significantly negative. Therefore, the percentage of microorganism suppression can be used to reflect the heavy metal pollution levels. The suppression of heavy metals on different microorganisms varied. According to the National Standard of Soil Environmental Quality, this study determined the early-warning value of heavy metals based on the different suppression rate of Pb on microbial amount and activity, and enzyme activity.

In accordance with the three-scale criterion of soil environment quality established by Chinese Soil Environment Quality, critical value of Pb concentration in soil is 500mg/kg. According to this criterion, the critical value of Pb contamination in purple paddy soil supposed to be 400 mg/kg. Under this condition, we evaluated the inhibition ratio of Pb to the selected microorganisms, activities of enzymes and biological activity. Then sensitive microorganisms to Pb contamination were chosen as the predictors of early warning. table 3 showed the inhibition ratio of selected parameters with a range from 1%~98%. This showed that the amounts of denitrifying bacteria (DB) and denitrifying activity decreased by 33% and 98%, respectively. It indicated that denitrifying bacteria (DB) and denitrifying activity were most sensitive to Pb contamination. Therefore, they can be chosen as the predictor of Pb contamination in purple paddy soil. In addition, anaerobic cellulose-decomposing bacteria (ACDB) and actinomyces were also sensitive to Pb pollution with inhibition ratio of 41% and 44%, respectively.

DISCUSSION

In the soil environment, heavy metals have toxicological effects on soil microbes, whose contamination may result in the decrease of their amounts and activities. In general, heavy metals would reduce the synthesis and secretion of microbial enzymes by restraining the growth and reproduction of the soil microorganisms, leading to decline of activities of soil enzyme activity and soil biological function. This study showed that hazardous effects of Pb on soil microbial community structure and enzymatic activities depended mostly on its concentration rather than incubation time (figs. 1 and 2). Low Pb concentration in soil stimulated slightly the growth of AFB and MB, but inhibited the growth of actinomyces and ACDB, while had little effect on the amounts and activities of other tested microorganisms. This result was agreed with the finding of previous study (Khan *et al*., 2007); whereas medium or high Pb concentrations reduced the amounts and activities of all tested microorganisms, enzyme activities. Among the parameters, actinomyces, DB, ACDB and denitrifying activity declined most than the control, particularly under high Pb condition. The negative impact of Pb on enzyme activities was weakened with time. The reason may be the resistance of microbes increased with time and then their growth recovered. Microbial amounts, activity and enzyme activities were significantly and negatively correlated with Pb concentration, with exception of fungi and actinomyces. Enzyme activities and microbial populations were decreased with increasing Pb concentration in purple paddy soil, which was similar with other study (Pan and Yu, 2011). Therefore, to some extent, the amounts and activity of most of soil microbes can reflect the pollution levels of Pb in purple soil. However the sensitivities of different microorganisms to Pb pollution were different.

In this study, LD_{50} of denitrifying bacteria was 852 mg/kg, which was lowest among tested soil microbes. It indicated that denitrifying bacteria was most sensitive to Pb pollution among these microorganisms. ED_{50} of soil enzyme activities were much higher than 500mg/kg, which was not suitable as indicator of early warning for Pb pollution. ED_{50} of denitrifying activity was lowest among tested parameters, indicating an excellent indicator of early-warning for Pb pollution. The early-warning value of purple paddy soil showed that ACDB and actinomyces were also sensitive to Pb pollution. Therefore, denitrifying activity, actinomyces, ACDB and DB can be used as the predictor of purple paddy for Pb contamination according to their sensitivity. However, those results in this study were based on laboratory incubation, and many factors such as temperature and tillage may limit its application in fields. Further studies therefore are needed to verify the conclusion.

CONCLUSION

Low Pb treatment had little impact on the amounts and activity of most of tested microbes. Excessive Pb concentration reduced both the amounts and activities of all microbes, biological activities. Significantly negative correlations were found between Pb concentration and

microbial amounts, enzymatic activities and biological activity with exception for fungi and actinomyces. Hence, to some extent, these parameters can be used to characterize the extent of Pb contamination in purple paddy soil. However, LD_{50} of most of microorganisms and ED_{50} of all enzyme activities were high. LD_{50} of denitrifying bacteria was relatively lower than other microorganisms, and ED_{50} of denitrifying activity was lowest among tested parameters. The result of early-warning value showed that ACDB and actinomyces were also sensitive to Pb pollution in purple paddy soil. Therefore, denitrifying activity, actinomyces, ACDB and DB can be used as the predictor of early warning of purple paddy soil for Pb contamination according to their sensitivity.

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