Impact of Cr^{3+} pollution on microbial characteristics in purple paddy soil

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Abstract: Impact of Cr^{3+} pollution on soil microbial quantity, enzyme activity and biological activity in purple paddy soil were studied under incubation conditions. The results showed that amounts of all tested microbes and enzyme activities in soil were inhibited by low Cr^{3+} concentration (200mg/kg). After 7-day incubation, sulfate-reducing activity, methanogen activity, denitrifying activity and anaerobic nitrogen-fixing activity in soil were reduced by 34%, 66%, 98% and 65% respectively. Amounts of soil microbes were remarkably inhibited with medium Cr^{3+} concentration (400mg/kg), all with reduction of more than 50%; and all tested soil biological activity was almost recovered in the fourth week except soil denitrifying activity. Activities of urease, invertase, neutral phosphatase and catalase were decreased by 60%, 21%, 59% and 42%, respectively. With high Cr^{3+} concentration (1600mg/kg), amounts and activities of tested microbes had only about 1% of that with control. As calculated from the regression equation, the ED_{50} (ecological dose) values of activities of soil urease, invertase and catalase were around 800mg/kg; the ED_{50} values of soil sulfate-reducing activity, methanogen activity and anaerobic nitrogen-fixing activity were also around 800mg/kg with an exception of soil denitrifying activity which ranged 35 to 39 mg/kg. According to the Standards of National Soil Environmental Quality in China and their sensitivities to 400mg/kg Cr^{3+} concentration, quantity of denitrifying bacteria, urease activity and denitrifying activity could be selected as indicators of early warning for Cr^{3+} pollution in purple paddy soil.

Keywords: Cr^{3+} pollution; soil microbe; soil biological activity; indicator of early warning.

INTRODUCTION

Chromium (Cr) is one of the frequently used heavy metal contaminants and is considered to be one of the top 20 contaminants on the Superfund priority list of hazardous substances for the past 15 years. As an environment pollutant, Cr^{3+} has been concerned due to its build up to toxic levels in environment recently, and this result could be connected with various industrial and agricultural activities (Dhala *et al*., 2013; Zayed and Terry, 2003). Heavy metal contaminants can be widely spread and accumulated in environment due to some inappropriate actions. In urban, the sources of Cr include traffic emission, industrial emission, atmospheric deposited and so on; in agricultural system, the sources of Cr include industrial slag, sewage sludge, pesticides, fertilizers application and so on. These sources of chromium resulted in higher concentrations of Cr^{3+} in croplands than the background value of soil (Wei and Yang, 2010; Kavamura and Esposito, 2010; He *et al.*, 2005).

Chromium, as a kind of heavy metals, even in traces can cause serious influence to all organisms, and chromium will be highly dangerous to human health when it accumulated in the food chain. In agricultural regions, the soil-crop system has become the primary pathway for the environmental Cr^{3+} influence human health. More importantly, such kind of pollution is covert, long-term and nonreversible (Islam *et al.*, 2007; Chen, 2003).

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As any other ecological system, the soil provides a great habitat for thousands of organisms, which are associated. Among the microscopic world, different types of bacteria and variety fungi, actinomycetes can exist harmoniously. Heavy metals such as chromium can affect the microbiota directly, particularly high concentration of heavy metal contaminants in the soil can alter the population, activity and diversity of the microbiota. It is known that excessive heavy metal contaminants exerted a negative influence on microbiota, resulting in a decrease in the activity and diversity of microbiota. Heavy metals play some important roles in some essential biochemical reactions of the growth of microorganisms, animals and plants. However, the high concentration they can cause toxic effects. In addition, soil enzyme activity and biological activity to a certain extent, can represent the soil fertility status and pollution level (Chen, 2003; Wei *et al.*, 2011).

Due to its unique anaerobic environment, heavy metals effects on paddy soil would be remarkably different with that in dry land ecosystems. Researchers are mainly focused on the impact of different heavy metal contaminants on the microbial communities in dry land systems, but integrated study of anaerobic micro-organisms in the flooded soil is rarely reported. Purple paddy soil, derived mainly from sandstone with different extent of weathering, is the predominated soil type in Chongqing and Sichuan regions in China. Incubation experiments were therefore conducted with application of different Cr^{3+}concentrations to explore the response of microbial communities, enzyme activity and biological activity to Cr pollution in purple paddy soil. By doing so, we aimed to explore the type and activity indicators of sensitive anaerobic microorganisms, which could reflect levels of Cr^{3+} pollution, providing basis for determining paddy soil heavy metal environmental quality indicators and forecasting paddy soil heavy metal pollution by anaerobic micro-organisms.

MATERIALS AND METHODS

Soil characteristics

Samples of soil were collected from plow layer (0-20 cm) of purple paddy soil. Collection sites was located on the college of Southwest University, Chongqing, China (106° 26′E, 30°26′N), which was one of sites of the National Monitoring Stations of Soil Fertility and Fertilizer Efficiency. The annual mean temperature of this station was 18.3°C and annual mean precipitation of this station was 1115 mm. It is a typical neutral Purpli-Udic Cambosols, which were the primary types of all purple soil in Sichuan Basin. Physico-chemical properties of the soils were as follows: pH, 7.3 (water: soil ratio of 2.5:1); soil total nitrogen, 1.52g/kg; soil organic matter, 32.1g/kg; soil alkali-hydrolyzable nitrogen, 83.1mg/kg; soil exchangeable K, 88.2mg/kg; Olsen-P was extracted using 0.5mol/L $NaHCO_3$, 4.3mg/kg.

Experimental design

Soil samples were air-dried. Gravels, concretions and organic residues of plants and animals were removed from sample soil. Certain copies of soil (equivalent to kilogram of dry soil, DS) were placed in several 1300 ml clean plastic box, and water was added to keep the soil waterlogged. The soil was pre-cultured in 28°C for 2 weeks, which was in the purpose of microbial rejuvenation, especially for anaerobic microbes. Cr was added to soil samples as an aqueous mixture of the salt $Cr_2(SO_4)_3$ at following rates (mg/kg soil): 0, 200, 400 and 1600, respectively. And then soil samples were sufficiently mixed and cultured at 28°C. After 0, 7, 14, 21 and 28 days, the population of microbial communities and their activity of samples (3 boxes) were tested. Throughout the culture period, water level was always kept above 1-2 cm of the soil to keep the anaerobic environment, which was similar to paddy field.

Determination 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 (Garcia-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 578nm, with unit expressed as milligrams of NH_4-N per 100g soil. Unit of neutral phosphatase activity was expressed as milligrams of phenol per 100g soil. Catalase activity and invertase activity were determined by titration method (Zheng, 1986), which expressed as milliliter of 0.1mol/L $Na_2S_2O_3$ per gram soil, and milliliter of 0.1mol/L $KMnO_4$ per gram soil, respectively. The method of determination and counting of purple paddy soil methanogenic activity were referred to previous method (Pankhurst, 1997). The method of determination and counting of purple paddy soil sulfate-reducing activity were referred to past literature (Pennanen *et al.,* 1996). The method of measurement and counting of denitrification activity were referred to previous literature (Garland and Mills, 1991). Measurement and counting of paddy soil anaerobic nitrogen fixation activities were referred to literature (Kelly and Tate, 1998).

Data Analysis

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

RESULTS

Effects of Cr^{3+} on soil microbial community

Growth of all tested soil microbes were inhibited after application of low Cr^{3+} concentration (200mg/kg dry soil) in the first week (table 1 and table 2). Compared with control after 7 days of incubation, the amounts of bacteria, fungi, actinomyces, hydrogen-producing lactogenic bacteria (HPAB) and methanogen bacteria (MB), denitrifying bacteria (DB), sulfate-reducing bacteria (SRB), anaerobic nitrogen-fixing bacteria (ANFB), anaerobic fermenting bacteria (AFB), anaerobic cellulose decomposing bacteria (ACDB) was decreased by 7%, 49%, 15%, 19%, 16%, 24%, 60%, 26%, 21% and 12%, respectively. Application of medium Cr^{3+} concentration (400mg/kg) resulted in more serious inhibition effect than that of control or low Cr^{3+} concentration. Compared with control, amounts of bacteria, fungi ,actinomyces, HPAB, MB, DB, SRB, ANFB, AFB and ACDB were decreased by 58%, 63%, 50%, 68%, 63%, 53%, 86%, 47%, 50% and 51% after 7-day incubation. Application of high Cr^{3+} concentration (1600 mg/kg) suppressed nearly all of tested soil microbes, the amounts of which were only about 1% of that in control.

Linear regression of Cr^{3+} concentrations and soil microbial quantities in purple paddy soil showed that quantities of all tested soil microbes were significantly and negatively correlated with Cr^{3+} concentrations, with an exception of DB (table 2). This indicated that Cr^{3+} pollution could be characterized by soil microbial quantity to some extent. Calculated from the fitting equation in the first week, LD_{50} values (lethal dose, namely the content of heavy metals in soil when the amount of microbial decreased 50%) of soil bacteria, fungi, actinomycetes, hydrogen-producing acetogenic bacteria and methanogen, denitrifying bacteria, sulfate-reducing bacteria, anaerobic nitrogen-fixing bacteria, anaerobic cellulolytic bacteria were 773, 793, 858, 768, 773, 794, 844, 800, 837 and 868 mg/kg, respectively.

Effects of Cr^{3+} on soil enzyme activity in purple paddy soil

Compared with control, Cr^{3+} applications inhibited the activities of urease, invertase, neutral phosphatase and catalase (fig. 1). However the inhibiting effects of Cr with low concentration on these enzymes was disappeared at the end of incubation. Similarly, such inhibiting effects of medium Cr concentrations on these enzymes were partly restored. In converse, high Cr concentration resulted in strong inhibition of all tested enzymes, without recover at the end of incubation Linear regression of Cr^{3+} concentrations and soil enzymes activity showed that the four tested activities of soil enzymes were negatively correlated with Cr concentrations. Determination coefficient (R^2) of the fitted equations ranged from 0.80 to 0.99, all of which were significant at P<0.01 level (n=12). This indicated that such regression equations could be used to calculate ED_{50} value (ecological dose, namely the content of heavy metals in soil when the enzyme activity decreased 50%). The ranges of ED_{50} values of urease, invertase, neutral phosphatase and catalase activity at different culture times were 838-849, 819-833, 825-857 and 802-806 mg/kg, respectively.

Effects of Cr^{3+}on biological activity in purple paddy soil

Compared with control, Cr^{3+} applications inhibited the biological activities of sulfate reduction, denitrification, methanogenesis, and anaerobic nitrogen fixation (fig. 2). However the inhibiting effects of Cr with low concentration on these enzymes was disappeared at the end of incubation except the biological activity of denitrification, which was strongly inhibited. Similarly, such inhibiting effects of medium Cr concentration on these biological activities were recovered (SRA and MA) or partly restored (ANFA). In converse, high Cr concentration resulted in nearly lethal inhibition of all tested biological activities without recover at the end of incubation.

Linear regression of Cr^{3+} concentrations and soil biological activity showed that the four tested activities of soil biological processes were negatively correlated with Cr concentrations (fig. 2). Determination coefficient (R^2) of the fitted equations ranged from 0.82 to 0.99, all of which were significant at P<0.01 level (n=12). This indicated that such regression equations could be used to calculate ED_{50} value. The ranges of ED_{50} values of activities of sulfate reducing, denitrification, methanogenesis and anaerobic nitrogen fixing at different culture times were 813-863, 35-39, 773-807 and 776-800 mg/kg, respectively. Except for denitrification activity, all of these ED_{50} values were around 800 mg/kg, which was much higher than 400 mg/kg that is the National Standard of Soil Environmental Quality in China. In purpose of early prediction, biological activities of sulfate reducing, methanogenesis and anaerobic nitrogen fixing may be not ideal as predictor of early warning for Cr pollution in purple paddy soil.

The value of early warning of purple paddy soil to Cr^{3+} 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 Cr^{3+} on microbial amounts and biological activity, and enzyme activity.

In accordance with the Grade III criterion of soil environment quality established by Chinese Soil Environment Quality, critical value of Cr^{3+} concentration in soil is 400 mg/kg. According to this criterion, the critical value of Cr^{3+} contamination in purple paddy soil supposed to be 400mg/kg. Under this condition, we evaluated the inhibition ratio of Cr^{3+} to the selected microorganisms, activities of enzymes and biological activity. Then sensitive microorganisms to Cr^{3+} contamination were chosen as the predictors of early warning. Table 3 showed the inhibition rates of selected parameters with a range from 21%-98%. This showed that the amounts of denitrifying bacteria, urease activity and soil denitrifying activity decreased by 86%, 60% and 98%, respectively.

Table 1: Populations of major microbes affected by varied treatment and culture time. Values are mean of three replicates.

Microbial Populations	Culture Time (week)	Cr^{3+} treatments concentration (mg/kg)				Determination coefficient (R^2)	LD_{50}
		0	200	400	1600		
Bacteria (10^6 cfu/g)	1st	381	353	160	0.3	0.81	773
	2nd	594	476	246	0.2	0.87	778
	3rd	580	563	365	0.8	0.95	788
	4th	607	583	483	1.6	0.99	800
Actinomyces (10^4 cfu/g)	1st	75.1	38.3	28.0	0.0	0.98	793
	2nd	58.3	24.3	19.4	0.0	0.99	797
	3rd	32.4	20.0	9.4	0.0	0.83	775
	4th	27.8	12.8	1.1	0.0	0.45	737
Fungi (10^4 cfu/g)	1st	29.1	24.6	14.6	2.2	0.90	858
	2nd	15.8	13.6	10.8	2.0	0.99	921
	3rd	7.8	7.1	6.7	2.0	0.99	1068
	4th	4.2	5.5	4.0	1.0	0.96	962

Table 2: Populations of anaerobic bacteria affected by varied treatments and culture time. Values are mean of three replicates.

Microbial Populations	Culture Time (week)	Cr^{3+} treatments concentration (mg/kg)				Determination coefficient (R^2)	LD_{50}
		0	200	400	1600		
AFB (10^6 cfu/g)	1st	127	102	39.9	0.1	0.76	768
	2nd	238	193	89.9	0.1	0.83	774
	3rd	205	217	116	0.1	0.88	779
	4th	213	236	126	0.2	0.88	779
HPAB (10^5 cfu/g)	1st	275	231	101	0.6	0.80	773
	2nd	424	304	216	0.8	0.98	793
	3rd	555	509	240	4.4	0.83	781
	4th	513	516	297	10.4	0.91	798
Methanogen (10^5 cfu/g)	1st	32.6	24.6	15.3	0.3	0.94	795
	2nd	91.6	54.1	40.0	0.7	0.98	803
	3rd	102	91.8	52.2	1.1	0.90	790
	4th	96.1	101	82.7	1.5	0.99	809
DB (10^4 cfu/g)	1st	59.0	23.6	8.3	2.0	0.66	844
	2nd	158	80.8	53.9	3.5	0.95	822
	3rd	150	153	65.8	5.6	0.77	801
	4th	145	145	106	5.2	0.98	820
SRB (10^4 cfu/g)	1st	98.5	72.8	52.3	0.9	0.98	801
	2nd	128	90.4	63.8	1.9	0.97	806
	3rd	119	101	90.5	2.3	0.99	818
	4th	122	128	109	5.0	0.99	828
ANFB (10^4 cfu/g)	1st	38.6	30.5	19.2	1.9	0.93	837
	2nd	65.1	59.9	39.7	1.1	0.96	802
	3rd	65.3	66.4	45.8	2.3	0.97	816
	4th	62.3	65.0	59.9	3.0	0.99	837
ACDB (10^4 cfu/g)	1st	15.3	13.5	7.5	1.4	0.86	868
	2nd	36.8	37.9	18.8	1.4	0.83	807
	3rd	41.5	41.9	29.9	2.9	0.97	845
	4th	36.0	37.2	33.6	3.8	0.99	880

Note: AFB-Anaerobic Fermentation Bacteria; HPAB-Hydrogen Producing Acetogenic Bacteria; DB- Denitrifying Bacteria; SRB-Sulfate-reducing Bacteria; ANFB-Anaerobic Nitrogen Fixing Bacteria; ACDB-anaerobic cellulose decomposing bacteria

Table 3: Early-warning indicator of purple paddy soil to Cr^{3+} pollution. The values are the decreased percentage (%) of initial values by 400 mg/kg Cr^{3+} treatment after 7-day incubation

Soil microorganisms quantity				Soil enzyme activity		Soil biological activity	
Species	Values	Species	Values	Species	Values	Species	Values
DB	86	SRB	53	Urease	60	DA	98
HPAB	68	ACDB	51	Catalase	43	MA	66
Actinomyces	63	AFB	50	Invertase	21	ANFA	66
Methanogen	63	Fungi	50	Neutral phosphatase	21	SRA	34
Bacteria	58	ANFB	47				

Note: HPAB-Hydrogen Producing acetogenic Bacteria; SRB-Sulfate Reducing Bacteria; MB-methanogen bacteria; AFB-Anaerobic Fermentation Bacteria; ANFB-Anaerobic Nitrogen Fixing Bacteria; DB-Denitrifying Bacteria; ACDB-Anaerobic Cellulose Decomposing Bacteria; MA-methanogen activity; SRA-Sulfate Reducing Activity; ANFA-Anaerobic Nitrogen Fixing Activity; DA-Denitrifying Activity.

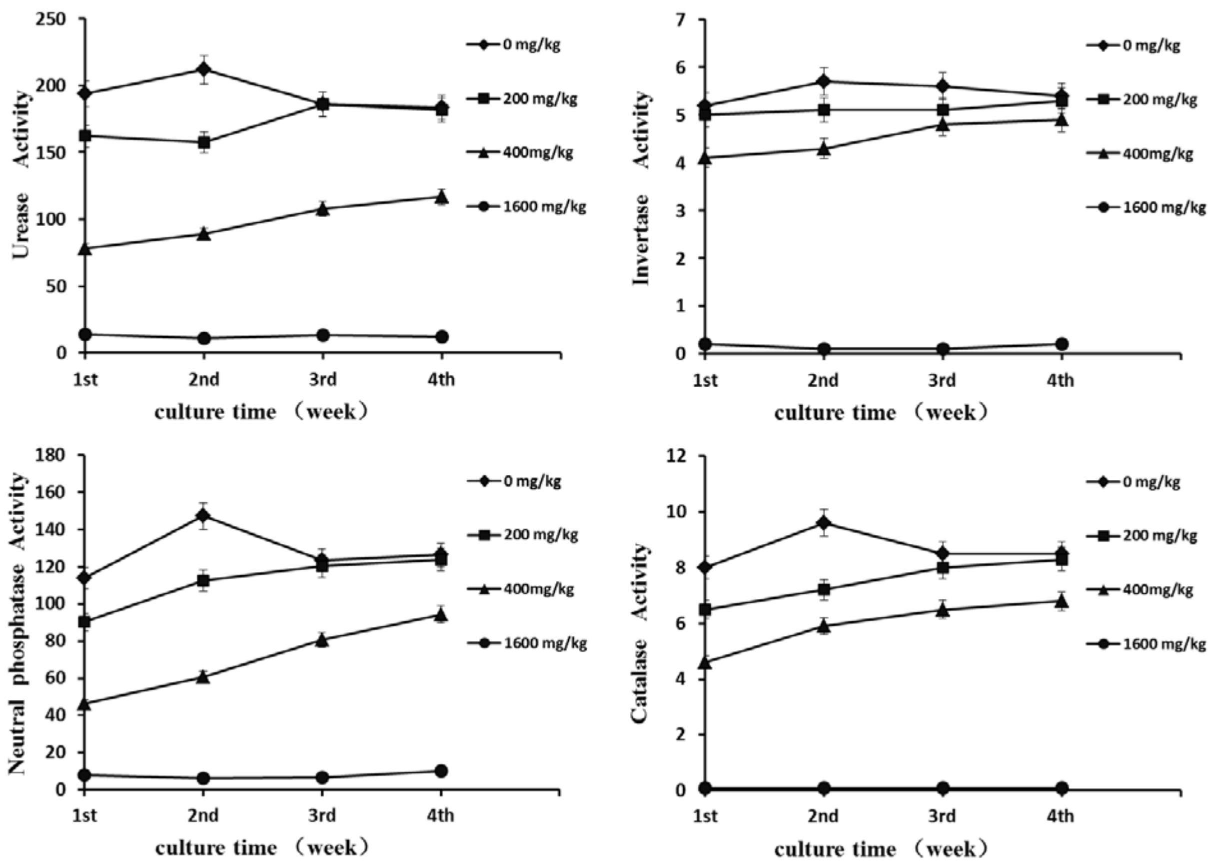

Fig. 1: The effects of Cr^{3+} on enzymatic activity in purple paddy soil during the different incubation periods. Units of urease activity, neutral phosphatase activity, catalase activity and invertase activity were expressed as NH_4-N μg/d/g, Phenol μg/d/g, 0.1mol/L $KMnO_4$ ml/30 min/g and 0.1 mol/L $Na_2S_2O_3$ ml/d/g, respectively.

DISCUSSION

As the result of the effects of Cr^{3+} on soil microbial community, SRB was seriously inhibited at low concentration of Cr^{3+}, but the inhibiting effect was gradually weakened with the increase of culture time. The tests of medium and high concentrations Cr^{3+} indicated that all of tested soil microbes were sensitive to Cr^{3+} pollution in purple paddy soil. Linear regression of Cr^{3+} concentrations and soil microbial quantities in purple paddy soil showed that the LD_{50} values of all tested soil microbial communities were similar, but were much higher than 400 mg/kg which is the National Standard of Soil Environmental Quality in China. In microbial aspect, population of soil microbes may be not suitable as predictor of early warning for Cr pollution in purple paddy soil.

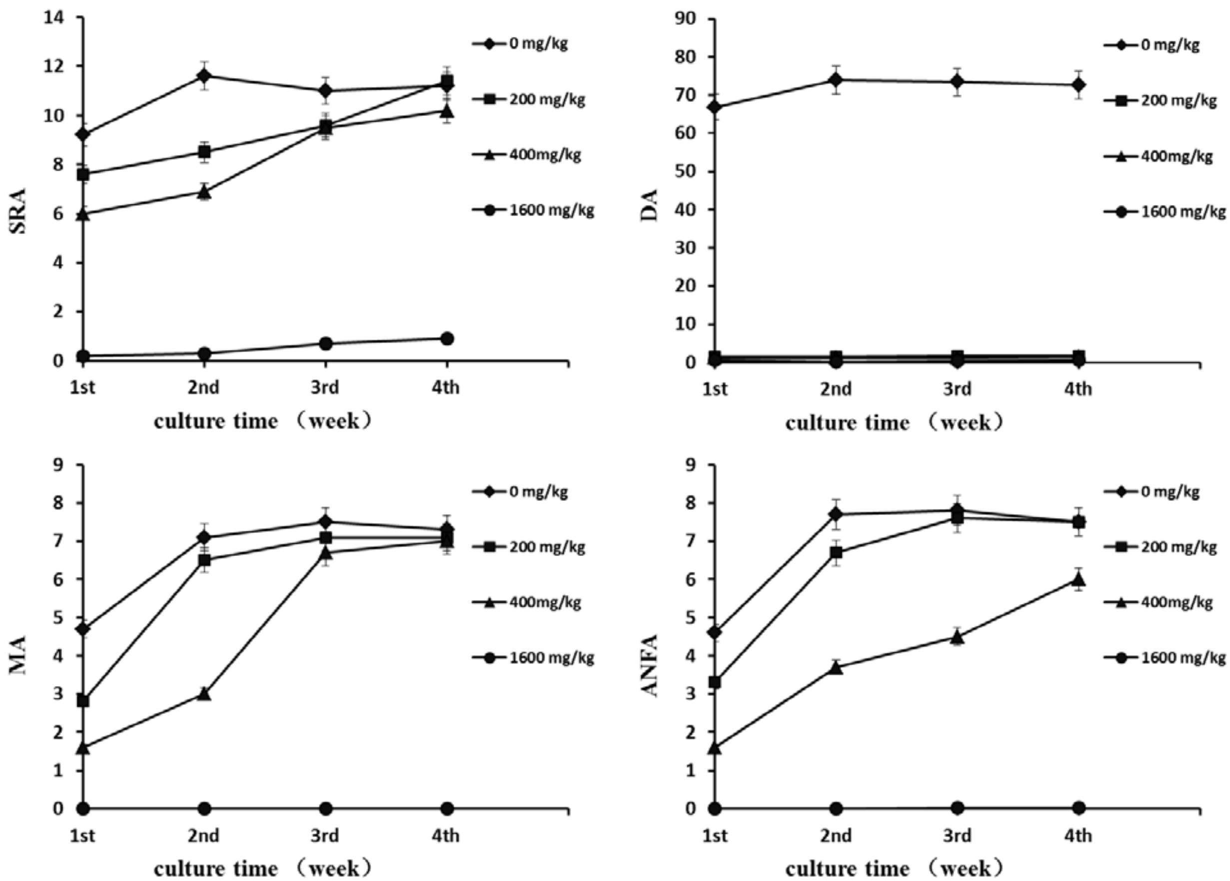

Fig. 2: The effects of Cr^{3+} on biological activity in purple paddy soil during the different incubation periods. Units of SRA (Sulfate Reducing Activity), DA (Denitrifying Activity), ANFA (Anaerobic Nitrogen Fixing Activity) and MA-Methanogen Activity were expressed as S^{2-} μg/d/g, %, 10-7 mol C_2H_2/d/g and 10-6 mol CH_4/d/g, respectively.

These findings which described in the effects of Cr^{3+} on soil enzyme activity in purple paddy soil were well consistent with previous study (Wei *et al*., 2011; Min *et al*., 2003).The result of linear regression of Cr^{3+} concentrations and soil enzymes activity indicated that all of these ED_{50} values were around 800mg/kg, which was much higher than 400 mg/kg that is the National Standard of Soil Environmental Quality in China. No similar studies could be comparable. However, in purpose of prediction, soil enzyme activities may be not ideal as predictor of early warning for Cr pollution in purple paddy soil.

As described in the effects of Cr^{3+}on biological activity in purple paddy soil, denitrofication activity in soil was most sensitive to Cr^{3+} pollution compared with the other three biologial activities in soil (fig. 2). Together with amouts of denitrifying bacteria (table 2), this results indicated the activity of denitrifying bacteria was seriously suppressed rather their quantity. Such phenomenon was well consistent with previous study (Leta, 2004). Unfortunately, the reason is still unclear, further research is needed.

We also studied the value of early warning of purple paddy soil to Cr^{3+} pollution. The result indicated that denitrifying bacteria, urease activity, and soil denitrifying activity were most sensitive to Cr^{3+} pollution for amounts of soil microbes, soil enzymes and soil biological activity, respectively. Therefore, they can be chosen as the predictor of Cr^{3+} pollution in purple paddy soil.

CONCLUSION

Chromium exists mainly as the form of Cr^{3+} in paddy soil due to the anaerobic reductive environment. With the development of modern agriculture and industry, cropland including purple paddy soil accumulated gradually a large amount of Cr, which resulted in significant impact on the soil microbes, enzyme activity and biological activity. The study confirmed that amounts of tested soil microbes were obviously inhibited by Cr^{3+} application when compared with control. Their amounts could be fully or partly restored with low or medium Cr^{3+} concentration; whereas they are lethally suppressed with high Cr^{3+} concentration, without final recover. Even so, different kinds of soil microbes reflected differently to Cr treatment with varied

LD_{50} values. Similar finding could be also found with enzyme activity and biological activity in purple paddy soil. The fitting equation illuminated that microorganism quantities, soil enzyme activity and soil biological activity were negatively correlated with Cr^{3+} concentrations. All of LD_{50} values of soil microbes, and ED_{50} values of enzyme activity and biological activity in soil were around 800mg/kg with an exception of denitrification activity whose ED_{50} value was only about 35 mg/kg. The result of early-warning value suggested that denitrifying bacteria, urease activity, and soil denitrifying activity were most sensitive to Cr^{3+} pollution for amounts of soil microbes, soil enzymes and soil biological activity, respectively. Therefore, they can be chosen as the predictor of Cr^{3+} pollution in purple paddy soil.

ACKNOWLEDGEMENTS

This study was financially supported by the Project of Special Fund for Agriculture Profession (201203030, 201003016), the National Key Technology Research and Development Program (2012BAD05B03-7), the National Natural Science Foundation of China (No. 31101610; 31372141), and the China Postdoctoral Science Foundation (2013M542244).

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