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Combined effects of cadmium and butachlor on soil enzyme activities and microbial community structure

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Abstract The combined effects of cadmium (Cd, 10 mg/kg of soil) and butachlor (5, 10 and 50 mg/kg of soil) on enzyme activities and microbial community structure were assessed in phaeozem soil. The result showed that phosphatase activities were decreased in soils with Cd (10 mg/kg of soil) alone whereas urease activities were unaffected by Cd. Urease and phosphatase activities were significantly reduced by high butachlor concentration (50 mg/kg of soil). When Cd and butachlor concentrations in soils were added at milligram ratio of 2:1 or 1:2, urease and phosphatase activities were decreased, while enzyme activities were greatly improved at the ratio of 1:5. This study indicates that the combined effects of Cd and butachlor on soil urease and phosphatase activities depend largely on the addition concentration ratios to soils. The random amplified polymorphic DNA (RAPD) analysis

showed that the changes occurring in RAPD profiles of different treated samples included variation in loss of normal bands and appearance of new bands compared with the control soil. The RAPD fingerprints showed substantial differences between the control and treated soil samples, with apparent changes in the number and size of amplified DNA fragments. The results showed that the addition of high concentration butachlor and the combined applied Cd and butachlor significantly affected the diversity of microbial community. The present results suggest that RAPD analysis in conjunction with other biomarkers such as soil enzyme parameter etc. would prove a powerful ecotoxicological tool.

Keywords Cadmium (Cd) · Butachlor · Urease · Phosphatase · Combined effect · DNA polymorphism change

Introduction

Cadmium (Cd) pollution in soil is of major environmental concern on a world scale and in China in particular with the rapid development of industry and agriculture (Bååth 1989; Shen et al. 2005). Cd is considered to be the metal having adverse effects on soil enzyme activity and microbial community structure in heavy metal contaminated soils (Ciecko et al. 2001; Vig et al. 2003; Renellaa et al. 2004), but there were few

reports on the combination toxicity of Cd and pesticides. The herbicide butachlor, *N*-(butoxymethyl)-2-chloro-2',6'-diethyl acetanilide, is applied in agricultural fields to control weeds. It is now one of top three herbicides applied widely in China (Yu et al. 2003). Butachlor is a persistent pollutant in agricultural soil, posing potential threat to the agro-ecosystem and human health through food chains (Sapna et al. 1995; Debnath et al. 2002). Su et al. (2005) reported the interaction between Cd and atrazine on rice seedlings. Due to the ubiquity of soil Cd

contamination and the wide use of butachlor in China, there is a need to assess their combined toxicity to soil microorganism.

Soil enzymes are the catalysts of important metabolic processes including the decomposition of organic pollutants and the detoxification of xenobiotics (Margesin et al. 2000b). Enzyme activities are considered to be sensitive to pollution and have the further advantage of being easy to determine without expensive, sophisticated instruments. So, soil enzyme activities have been proposed as indicators for measuring the degree of soil pollution. Soil urease, which catalyses the hydrolysis of urea to carbon dioxide and ammonia, greatly affects the fate and performance of an important fertilizer (urea). Soil phosphatase plays a major role in the mineralization of organic P in the soil, namely, catalysis of the hydrolytic cleavage of ester-phosphate bond, and thus its activity might be an indicator of the bioavailability of organic P (Zheng et al. 1999). Therefore, urease and phosphatase are frequently used for determining the influence of the various pollutants (heavy metals, pesticide, crude oil, etc.) on the microbiological quality of soil (Margesin et al. 2000a, b).

Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis in the field of genotoxicology. Random amplified polymorphic DNA (RAPD) has been widely used in species classification and phylogenetic analysis, resistance gene identification, and genetic analysis of populations (Martin et al. 1991; Dweikat et al. 1993; Atienzar et al. 2002), as it is quick, simple and inexpensive. In fact, RAPD analysis has become one of the most popular DNA-based methods for assessing genetic diversity in plants (Liu et al. 2005) and has been used in DNA analysis of soil microbial community (Yang et al. 2000). RAPD fragments are detected after agarose gel electrophoresis and ethidium bromide (EB) staining by visualizing band shifts, missing bands or the appearance of new bands in a DNA gel electrophoresis. Detection of genotoxic effect using RAPD involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA. Using multiple primers also helps ensure that a sufficiently large region of the target DNA is scanned when an estimate of overall variance between samples is desired (Ogram and Feng 1997).

This study aimed to assess the combined effects of Cd and butachlor on microbial activity in phaeozem soils.

Traditional assays such as soil enzyme activities were combined with RAPD technique to determine the changes of microbial activities in soils treated with different Cd and butachlor concentrations in laboratory condition.

Materials and methods

Experimental soils

Tested phaeozem samples (0–20 cm in depth) used for this study was collected from a field that had not been planted for more than 10 years in the Hailun Agro-ecological Trial Station (47°26'N, 126°38'E), Hailun Country, Heilongjiang Province, China, which is located in the continental temperate monsoon zone, with a dry and cold winter and a warm and wet summer. After transportation to the lab, the soil was air-dried, ground, passed through a 3 mm mesh and stored as the stock sample for this study. The basic soil properties were determined using standard methods recommended by the Chinese Society of Soil Science (Lu 1999). The soils had the following basic properties: pH 6.58, organic matter 37.83%, cation exchange capacity (CEC) 32.92 cmol/kg, total N 2.56 g/kg and total P 0.61 g/kg, sand 51.49%, silt 39.6%, clay 26.6%.

Experimental treatments

The samples were adjusted to 50% of water-holding capacity and preincubated at 25°C for 7 days. Cadmium was added to soil as an aqueous mixture of salt: CdSO₄. Butachlor was applied to soil as a CH₃OH solution. The concentrations of Cd were 10 mg/kg of dry weight soil, and the concentrations of butachlor were 5, 10 and 50 mg/kg of dry weight soil, respectively. The details are shown in Table 1. Sck means the control soil sample without any artificial contaminant received the same amounts of distilled water and CH₃OH. S1 means the soil samples exposed to Cd alone. S2, S3 and S4 mean the soil samples exposed to butachlor alone. S5, S6 and S7 mean the soil samples exposed to Cd in combination with butachlor (at the same Cd level). Three separate soil samples were prepared for each tested dose. After thorough mixing, the treated soils were incubated at 25°C retaining the same moisture content for four weeks before the analyses.

Table 1 Experimental factors and levels (mg/kg soil)

Factor	Level							
	Sck	S1	S2	S3	S4	S5	S6	S7
Cd	0	10	0	0	0	10	10	10
Butachlor	0	0	5	10	50	5	10	50

Determination of urease and phosphatase activity

Soil urease activity was determined by the method of Chang (1988). Briefly, 5 g of air-dried soil was mixed with 1.5 ml methylbenzene, 10 ml buffer with pH 6.7 and 5 ml 10% (w/v) urea solution in a reaction flask and incubation at 37°C for 24 hours. This method based on the determination of the NH_4^+ released and expressed as mg $\text{NH}_4\text{-N/kg/h}$. In this study, acid phosphatase activity was determined by the method of Tabatabai and Bremner (1969). The *p*-nitrophenol (PNP) in the filtrate was determined colorimetrically at 410 nm after 1 h incubation with *p*-nitrophenyl phosphate.

DNA extraction and RAPD procedures

Microbial community DNA in soil was extracted by the SDS-based method described by Zhou et al. (1996) with some modifications. Soil samples of 5 g were mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 μl of proteinase K (10 mg/ml) in centrifuge tubes by horizontal shaking at 225 rpm for 30 min at 37°C. After the shaking treatment, 1.5 ml of 20% SDS was added, and the samples were incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15–20 min. The supernatants were collected after centrifugation at $6,000 \times g$ for 10 min at room temperature and transferred into 50 ml centrifuge tubes. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroformisoamyl alcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h or overnight. The pellet of crude nucleic acids was obtained by centrifugation at $16,000 \times g$ for 20 min, resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) to give a final volume of 500 μl . DNA was then purified by the low-melting-point agarose gel recovery method (Zhou et al. 1996).

PCR amplification reaction in a 20 μl total volume, containing 2 μl $10 \times$ Taq buffer, 2 mM MgCl_2 , 1 unit Taq DNA polymerase (supplied by Promega Co.), 0.25 mM dNTP (supplied by Gibco BRL Co., Ltd.), 25 pmol primer (Table 2), and 10 ng soil DNA. DNA amplification was carried out in a MJ research PT-200 thermocycler with the following procedure: an initial denaturing step at 94°C for 3 min; 40 cycles for 30 s at 94°C (denature), 45 s at 36°C (annealing), 90 s at 72°C (extension), and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.8% agarose gel and after which the gels were stained with ethidium bromide (EB) solution (0.015%) in distilled water and photographed. The standard DNA samples (1 kb DNA ladder marker) were used as molecular size marker (supplied by Promega Co.).

RAPD fingerprints profiles

The photographic plates were scanned into computer and analyzed using a computer image analysis system (CAIS). We used 10 random primers to amplify the microbial community DNA from the treated soils. Since primer sequences were random and non-selective to DNA samples, amplification for one primer was equal to one random sampling from the whole microbial DNA sequences. The number of RAPD fragments was considered to represent the RAPD fragment richness (S) of the whole DNA sequences. Polymorphism observed in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison to control RAPD profiles (Atienzar et al. 1999; Luceri et al. 2000). The presence and absence of amplified fragments were scored.

Coefficient of DNA sequence similarity was defined following the formula of Nei and Li (1979) as follows: $S_{xy} = 2N_{xy}/(N_x + N_y)$, where S_{xy} is the coefficient of DNA sequence similarity between DNA samples *x* and *y*; N_{xy} represents the numbers of RAPD fragments shared between DNA samples *x* and *y*; N_x and N_y are the numbers of RAPD fragments from DNA samples *x* and *y*, respectively. Coefficient of DNA sequence similarity can reflect the difference in the DNA sequences between soil microbial communities.

Statistics

Analysis of variance by the Tukey–Kramer test was used to assess the significance of differences ($P < 0.05$) of the means ($n = 3$) using the SAS software.

Results and discussion

Effects of Cd and butachlor on soil enzyme activities

The effects of single element and compound pollution of butachlor and cadmium on urease and phosphatase

Table 2 Sequences of ten primers used in this experiment

No. of primers	Sequences of primers	Percentage of GC
Primer 1	ACTTCGCCAC	60
Primer 2	GAGGCCCGTT	70
Primer 3	GTCGCCGTCA	70
Primer 4	TCCGATGCTG	60
Primer 5	AGGACTGCCA	60
Primer 6	ACGGACGTCA	60
Primer 7	GCGTCGAGGG	80
Primer 8	GGGTTTGCA	60
Primer 9	TGGTCTGGC	70
Primer 10	TAGGCGGCGG	80

activities are showed in Fig. 1. The results were expressed as percentage of control (activity of control = 100). Urease and phosphatase activities varied in the different treated soil samples. The activities of urease ranged from 75.7 to 120% and phosphatase activities ranged from 41.2 to 136.4%. Phosphatase activities were decreased in soils with Cd (10 mg/kg of soil) alone whereas urease activities were unaffected by Cd. The higher butachlor concentration (50 mg/kg of soil) in soils significantly reduced the enzyme activities (S4) and urease and phosphatase activities were dropped to 75.7 and 41.2% of the control, respectively. Moreover, phosphatase seemed more sensitive than urease in both single and combined polluted soil samples. When Cd and butachlor concentrations in soils were added at milligram ratio of 1:5 (S7), urease and phosphatase activities were greatly improved. The enzyme activities of urease and phosphatase were 120 and 136% of the control, respectively. However, the same results didn't find with other ratios, such as 2:1 or 1:2. This study indicated that the combined effects of Cd and butachlor on soil urease and phosphatase activities depended largely on the addition concentration ratios to soils.

Many studies have demonstrated that phosphatase activity of microorganisms is among most sensitive parameters for evaluation of toxicity. Landi et al. (2000) demonstrated that the acid phosphatase activity was more sensitive than dehydrogenase activity to the presence of Cd. Renellaa et al. (2004) also reported that soil phosphatase activity was significantly affected by Cd, while urease activity was unaffected. The similar results observed in our work. Both in single and in combined polluted soils, phosphatase seemed more sensitive than urease. We cannot explain with the present approach why phosphatase was more sensitive to the presence of

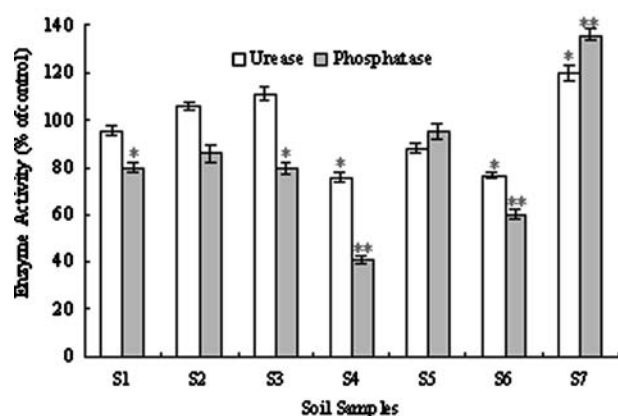


Fig. 1 Effect of Cd and butachlor on the activities of soil enzyme (percentage of control). Each point is the mean of three replicates. Error bars represent standard error (SE). Single asterisk and double asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$ level, respectively compared to respective control soils

pollutants. Pollutants can reduce enzyme activity by interacting with the enzyme-substrate complex, denaturing the enzyme protein or interacting with the protein-active groups. And an indirect effect is also possible because changes in the community structure can modify the enzyme activity (Nannipieri 1994). In addition, in soils with Cd + butachlor, the enzyme activities were decreased when Cd and butachlor concentrations in soils were added at mg ratio of 1:5 (S7), while increased when the ratios were of 2:1 (S5) or 1:2 (S6). It can be assumed that the combined effect of butachlor and Cd depend on the concentration ratios in soils. Su et al. (2005) reported that when atrazine and Cd^{2+} concentrations in solution were maintained at mole ratio of 1:1, the complex between atrazine and Cd^{2+} reduced the individual toxicities and the accumulation of atrazine by seedlings was less and the seedling biomass was greater than found with other ratios, such as 1:2 or 2:1, which was similar to our results on soil enzyme activities. The sensitivity of soils to contamination correlated reasonably with organic matter (Bååth 1989; Shen et al. 2005). The bioavailability of heavy metals and PAH in soils are largely affected by organic matter through the decrease of the solution concentration of contaminants.

DNA extraction and RAPD profile

DNA extraction

The suitability of the CTAB extraction methods for DNA extraction was used to extract DNA from the soil samples. The integrity of the crude DNA extracted was shown in Fig. 2. The electrophoresis indicated that the size of DNA obtained from the soil samples was about 23.1 kb (the first band of the marker was 23.1 kb) and the DNA yields was about $15.8 \pm 1.5 \mu\text{g/g}$ of soil. Concentration and purity of DNA extracted are usually measured at OD260 and by 260/280 nm absorbance ratio. The purity grade of DNA extracted from the control and the treated soils was in the range of 1.65–1.77, and the concentration obtained was approximately 180 ng/ μl . These results indicated that CTAB method was suitable for DNA extraction and yielded high quality DNA samples, which is crucial for good RAPD analysis (Williams et al. 1990).

RAPD profile

The 10 primers tested gave specific and stable results (Fig. 3). The reproducibility of RAPD fragments can be improved by careful laboratory operation and strict control of reaction condition (Hadrys et al. 1992). The RAPD fingerprints showed substantial differences between the control and treated soil samples, with apparent changes in the number and size of amplified DNA

fragments. For instance, there were significant differences of the fingerprints between the control soil and the treated soil by using primer 5 (Fig. 3). Labra et al. (2003) reported that RAPD was more sensitive than classic genotoxic tests i.e. the comet and micronucleus assay since RAPD analysis was capable of detecting temporary DNA changes that may not finally manifest themselves as mutations (Savva 1998). Recently, RAPD technique has been successfully utilized to detect various types of DNA damage and mutation in animals, bacteria and plants induced by pollutants (Conte et al. 1998; Atienzar et al. 2002; Rong and Yin 2004). Other studies have indicated that changes in RAPD (and related markers) banding patterns may be associated with heritable mutations, chromosomal rearrangements, or other DNA lesions (Theodorakis 2001; Atienzar et al. 2002).

The number of disappearing RAPD bands was greater than that of the appearing bands (Table 3) and the greatest number of disappearing bands was 26 (S4, S5 and S6). The number of denoting polymorphic bands was greater in combined polluted soils (S5, S6 and S7) and the value of polymorphisms was P (%) = 50.6%. In all cases, polymorphisms were due to the loss and gain of amplified bands in the treated samples compared with the control. These results indicated that pollutants might decrease the richness of soil microbial community DNA sequence but the diversity at DNA sequence could still stay at high level. It seemed that both changes in the balance of microbial populations and genetic recombina-

tion contributed to the increased diversity (Bej et al. 1992).

In addition, the molecular size [base pair (bp)] of the appearance and disappearance of the RAPD bands was calculated by using Software quantity one 4.2.3. The denoting polymorphic bands of molecular size varied from approximately 250–2,050 bp. These results indicated that genomic template stability in soil was significantly affected by the addition of Cd and butachlor. Changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations and DNA damage in the primer binding sites (because the binding site is only ten base long whereas genomic rearrangements occur in much longer fragments, e.g. several kilobytes), which could act to block or reduce polymerization of DNA in the PCR reaction (Nelson et al. 1996). Pollutants could induce DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA-protein cross-links, oxidized bases and even bulky adducts etc. in organisms (Aust and Eveleigh 1999; Waisberg et al. 2003; Ates et al. 2004), which may also induce important structural changes that can significantly affect the kinetics of PCR events (Bowditch et al. 1993). Appearance of new PCR products occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events), and/or large dele-

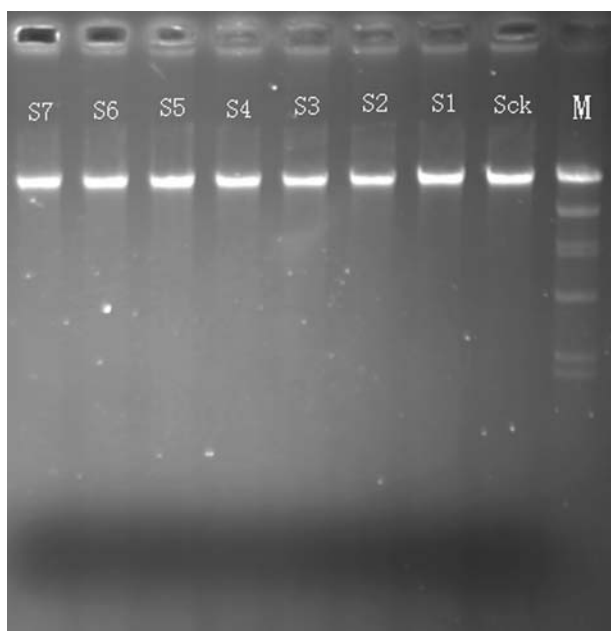


Fig. 2 Genomic DNA extracted by CTAB in the control soil (Sck) and the treated samples (S1–S7). M the DNA marker and the bands from top to bottom: 23,130, 9,416, 6,557, 4,361, 2,322 and 2,027 bp

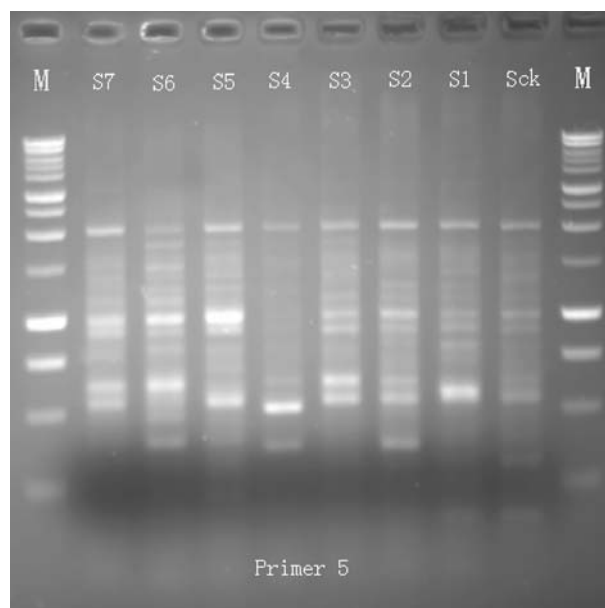


Fig. 3 The RAPD fingerprints of genomic DNA from the control soil (Sck) and the treated samples (S1–S7). M: the DNA ladder consisting of 13 blunt-ended fragments with sizes ranging from 250 to 10,000 bp

tions (bringing two pre-existing annealing sites closer), and/or homologous recombination (juxtaposing two sequences that match the sequence of the primer) (Atienzar et al. 1999). In the present study, the disappearance of normal bands and the appearance of extra bands occurred at treated soil and the mechanism needs to be further investigated.

Comparison of RAPD profiles and soil enzyme activity in different treated soil samples

It was demonstrated that changes in RAPD profiles induced by toxic pollution exposure could also be regarded as modifications in genomic DNA template stability and that this qualitative measure of genotoxic effect could be directly compared with alterations in other parameters (Atienzar et al. 1999; Liu et al. 2005). In the present study, Sck had smaller coefficient of DNA sequence similarity to S4, S5, S6 and S7 than the other samples (Table 4). And the coefficients of DNA sequence similarity were only 0.4098, 0.4356, 0.4266 and 0.4071, respectively. This result showed that high concentration butachlor and the

combined pollution of Cd and butachlor significantly affected the diversity of the microbial communities. Moreover, this result was similar to the enzyme activity in the control samples and the treated samples (Fig. 1). Liu et al. (2005) reported that example, a significant reduction in root growth of barley seedlings correlated with a significant change in RAPD profiles, which was similar to our study. In addition, S5 had the highest coefficient of DNA sequence similarity to S6 and S7 implied that combined pollution had similarity in the number of the RAPD bands (Table 4). In this experiment, genomic DNA template stability was more sensitive than enzyme activity (Fig. 1; Table 3). Similar evidence was reported by Liu et al. (2005). RAPD technique was used to study diversity of the microbial communities in soils at DNA level, which provided the information for the effects of pollutants and a clue to possible changes of microbes in the soil (Yang et al. 2000). The simultaneous use of more than one biomarker could enhance the detection of toxic effects since different biomarker responses are induced at different stages of the organism's health status curve (Depledge 1994).

Table 3 Changes of total bands in control, and of polymorphic bands and varied bands in treatments

No. of Primers	Sck	S1		S2		S3		S4		S5		S6		S7	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b
Primer 1	11	2	0	0	2	2	1	1	3	2	2	1	3	1	1
Primer 2	8	1	4	1	3	2	3	2	3	2	2	2	4	0	4
Primer 3	10	1	2	3	3	3	3	2	1	2	3	2	1	3	0
Primer 4	10	0	2	1	2	1	1	2	3	1	3	1	4	1	1
Primer 5	6	3	2	3	1	2	2	1	3	0	1	2	2	2	0
Primer 6	8	0	0	1	3	3	3	2	3	2	3	2	2	1	4
Primer 7	6	2	1	2	1	2	2	1	2	1	4	3	3	3	3
Primer 8	9	1	2	2	3	0	3	1	3	2	4	2	2	2	4
Primer 9	9	1	2	1	1	1	1	2	1	2	4	2	2	2	4
Primer 10	6	1	3	1	2	0	4	0	4	2	0	1	1	2	2
Total bands	83	12	18	15	21	16	23	14	26	16	26	18	24	17	23
a + b		30		36		39		40		42		42		40	
P (%)		36.1		43.4		47.0		48.2		50.6		50.6		48.2	

a appearance of new bands, *b* disappearance of normal bands, *a* + *b* polymorphic bands, *P* value of polymorphism and the loss and gain of amplified bands in the treated samples compared with the control

Table 4 Coefficients of microbial community DNA sequence similarity of different soil samples

Soil sample	Sck	S1	S2	S3	S4	S5	S6
S1	0.6111						
S2	0.5108	0.7017					
S3	0.5168	0.5663	0.5844				
S4	0.4098	0.4854	0.5656	0.5501			
S5	0.4356	0.5336	0.6332	0.5788	0.6755		
S6	0.4266	0.5567	0.4824	0.5435	0.5046	0.6917	
S7	0.4071	0.5788	0.5033	0.5998	0.4874	0.6867	0.7848

Conclusion

This paper presented that the combined effects of Cd and butachlor on soil urease and phosphatase activities depend largely on the addition concentration ratios to soils. In addition, the RAPD analysis indicated that the addition of high concentration butachlor and the combined applied Cd and butachlor significantly affected the diversity of microbial community. The present results suggest that RAPD analysis in conjunction with other

biomarkers such as soil enzyme parameter etc. would prove a powerful ecotoxicological tool. Furthermore, more experiments should be considered to better understand the relationship of different biomarkers.

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