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Short-term effects of copper, cadmium and cypermethrin on dehydrogenase activity and microbial functional diversity in soils after long-term mineral or organic fertilization

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ABSTRACT

A long-term field experiment was employed to evaluate the short-term effects of Cu, Cd, cypermethrin (C) and their combinations (C + Cu and C + Cd) on soil dehydrogenase activity (DHA) and microbial functional diversity (average well color development, substrate utilization, Shannon index and evenness), as well as their differences in different fertilized soils. Three fertilization modes are arranged: wheat straw compost (OM), fertilizer NPK (NPK) and no fertilization (CK). The soil microbial activities were impacted greatly by long-term fertilization. The highest DHA and the lowest functional diversity (average well color development, Shannon index and substrate utilization with exception of carbohydrate) appeared in OM soil. The functional diversity was highest in NPK soil. DHA in Cu treatment and functional diversities in Cd, Cu + C and Cd + C treatments were significantly decreased to the greatest extent in NPK soil. The order of the influence of contaminants on soil microbes was NPK > CK > OM. Despite the slight effect of cypermethrin on functional diversity, when applied together with heavy metals, especially with Cd, the negative effect was more pronounced. In sum, long-term compost application has benefits to promote DHA and decrease bioavailability of contaminants in soil. At the same time, with the microbial community shifting, the microbial functional diversity reduces greatly. Our findings suggest that more attentions should be paid to the microbial community shifts in organic farming system.

1. Introduction

Soil microbes respond rapidly to farm management and play important roles in many ecosystem processes such as biogeochemical cycling of nutrients, energy flow as well as transformation of contaminants (Doran and Zeiss, 2000; Sukul, 2006; Zhang et al., 2006). Thus maintenance of the biological activity in soil is generally regarded as a key feature of sustainable production to ensure ecosystem functions (Swift, 1994). Upon contaminants entering into soils, agricultural ecosystem was disturbed, and disturbance extent was affected by soil properties (Sannino and Gianfreda, 2001; Lu et al., 2004; Wang et al., 2004; Kong et al., 2006). Many studies have proved that increase of soil organic matter content by addition of organic manures can decrease the bioavailability of heavy metals and pesticides in soils (Pérez-de-Mora and Madrid, 2007; van Herwijnen et al., 2007; Sannino and Gianfreda, 2001). Long-term fertilization can result in microbial community shifts in soils (Marschner et al., 2003; Widmer et al., 2006; Chu et al., 2007), which would result in the varied sensitivity to soil pollution, because microbial community responded differently to soil contaminants (Gflier et al., 1998; Wang et al., 2004). Therefore, studies on the interaction between fertilization and contaminant bioavailability can develop approaches to eliminate or decrease the eco-environmental risks of those contaminants through disturbance of microbial communities.

Cu, Cd and pesticides, as the main soil contaminants, are entering agricultural field through waste irrigation (Hu et al., 2006), fertilization and agrochemicals application (Zheljazkov and Warman, 2003; Perkiömäki and Fritze, 2005; Wang et al., 2006).

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With the organophosphate products being phased out, the use of pyrethroid insecticides is increasing (Amweg et al., 2005). The synthetic pyrethroid cypermethrin is commonly found in rivers, sediments, soils and even foodstuffs (Allan et al., 2005; Amweg et al., 2005; Sannino et al., 2003), and thus concerns regarding its fate and effect on eco-environment are increasing. Soil dehydrogenase activity is an indicator of soil quality and microbial activity. It is the most frequently used to determining the influence of the various pollutants (heavy metals, pesticide) on the microbiological quality of soils (Brookes, 1995; Pascual et al., 2000; Sannino and Gianfreda, 2001). Determining community level substrate utilization (CLSU) patterns with the Biolog™ system is one approach to characterize microbial communities (Garland and Mills, 1991). CLSU assays have been applied in many studies in order to gain information on responses of microbial communities to fertilization (Widmer et al., 2006), heavy metals (Akmal et al., 2005) and pesticide (de Lipthay et al., 2004). To our knowledge, the effects of cypermethrin on CLSU are still not well understood. In fact, microorganisms are usually exposed simultaneously or sequentially to a variety of pollutants via multiple exposure routes (Feron and Groten, 2002). Hence, it is of great significance for us to evaluate the combined effects of heavy metals and cypermethrin in soils to ensure agroecosystem sustainability.

Changes in soil quality may develop slowly and may adjust to a new long-term steady state after a change of management or conversion to a different farming system (Widmer et al., 2006). Therefore, long-term agricultural field experiments might reveal more changes that were undetectable in short-term fertilization studies. In our present study, a 17-year field experiment was conducted to elucidate (i) the effect of long-term fertilization on soil microbial activity, and (ii) the responses of soil microbial community to Cu, Cd, cypermethrin and their combinations as well as their differences in three kinds of fertilized soils.

2. Materials and methods

2.1. Soil description and preparation

This study was carried out in a long-term field experiment site, which was established in 1989 in the Fengqiu Ecological Experimental Station (35°04′N, 113°10′E) of the Chinese Academy of Sciences, Henan Province, China. The soil in this area was derived from alluvial sediments of the Yellow River, and classified as aquic inceptisol. It has a sandy loam texture (about 9% clay, 21.8% silt). Three fertilization modes, i. e., wheat straw compost (OM), fertilizer NPK (NPK) and no fertilization (CK) were employed in this study, which were under an annual crop rotation of winter wheat and summer maize. The annual application rates of N, P and K in both OM and NPK modes were 150 kg N ha^{-1}, 32.7 kg P ha^{-1}, and 124 kg K ha^{-1} for winter wheat, and 150 kg N ha^{-1}, 26.2 kg P ha^{-1}, 1, and 124 kg K ha^{-1} for maize. Compost was applied in the OM mode, which was made of wheat straw mixed with soybean cake and cotton seed cake to enrich N content. N, P and K contents were determined before application, and then the amount needed for application was calculated based on the N content. Inorganic fertilizers complement the shortage of P and K from the applied compost. Inorganic N, P and K fertilizers were applied as urea, superphosphate and $K_2(SO_4)$, respectively. For each fertilization mode, soil samples (0–20 cm) were collected from 16 points, then mixed and sieved through a 2-mm sieve to remove the roots in April 2006. The fundamental properties of soil samples were shown in Table 1. Soil pH was measured in 1:2.5 soil–water suspension. Soil organic matter content, total N and total P were determined by dichromate oxidation, Kjeldahl digestion, and sodium carbonate fusion, respectively (Lu, 2000). Available N was extracted with 2 mol L^{-1} KCl and analyzed with a segmented flow analyzer. Available P and available K were extracted by sodium bicarbonate and ammonium acetate, respectively (Lu, 2000).

The background contents of Cu^{2+} and Cd^{2+} in three fertilized soils had no significant difference ($P > 0.05$) and were 14.2–14.7 mg kg^{-1}and 0.29–0.37 mg kg^{-1}, respectively. Pilot study showed that the critical doses of Cu and Cd which inhibit cypermethrin degradation in used soils were 100 mg Cu kg^{-1} and 5 mg Cd kg^{-1}, respectively.

2.2. Experimental design

Soil samples of each fertilization mode were amended with Cu^{2+} (100 mg kg^{-1}, $CuCl_2{\cdot}2H_2O$) and Cd^{2+} (5 mg kg^{-1}, $CdCl_2{\cdot}2.5H_2O$) dissolved in water, respectively. The contaminated soils were shaken and sieved through a 2-mm sieve again to distribute Cu^{2+} and Cd^{2+} homogeneously. The water contents in both contaminated and uncontaminated soils were adjusted to 60% of soil water-holding capacity and stored for 30 days.

Cu^{2+}, Cd^{2+} contaminated soils and uncontaminated soils were amended with cypermethrin (purity >96%, Sigma) dissolved in acetone (0.5 mL) to obtain a pesticide concentration of 10 mg kg^{-1}, which is a field residue after pesticide application in China (Xie et al., 2008). The pesticide was mixed homogeneously when acetone evaporated off. In sum, there were six treatments for each fertilization soil, i.e., uncontaminated soil as control (B), Cu^{2+} contaminated soil (Cu), Cd^{2+} contaminated soil (Cd), cypermethrin contaminated soil (C), combination of Cu^{2+} and cypermethrin (Cu + C), and combination of Cd^{2+} and cypermethrin (Cd + C). Soil samples (dry weight equivalent) of 20 g in each treatment was adjusted to 60% of soil water-holding capacity and placed in beakers, covered with perforated aluminum foil. All treatments were made in triplicate and incubated at 25 ± 2 °C for 28 days.

2.3. Soil dehydrogenase activity and Biolog Eco-plate analyses

After incubation for 0, 7, 14, 21 and 28 days respectively, soil dehydrogenase activities (DHA) were determined by the reduction of triphenyltetrazolium chloride to triphenylformazan (Casida et al., 1964).

At day 14, soil microbial functional diversity of each treatment was measured in Biolog Eco-plates (Biolog, Hayward, CA). The method was similar to the description by Waldrop et al. (2000) and Kong et al. (2006). Briefly, 10 g (dry weight equivalent) of incubated soils was suspended in 100 mL sterile saline solution

Table 1
The physicochemical properties of soils with different fertilization mode.

Fertilization mode	pH (H_2O)	SOM (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})
OM	8.03b	13.84a	0.94a	0.64a	30.57ab	45.34a	171.9a
NPK	8.26b	9.31b	0.58b	0.65a	37.20a	24.80b	147.0b
CK	8.51a	6.83c	0.44c	0.52b	12.75b	2.05c	57.1c

SOM, soil organic matter. Means ($n = 3$) in the same column followed by the same letter are not significantly differed at $P < 0.05$. Fertilization mode: CK, no fertilization; NPK, fertilization with NPK fertilizers; OM, fertilization with organic manure.

(0.85%, m/v) with 5 g of 3 mm glass beads on a rotary shaker at 300 rpm for 10 min at 25 °C. Suspensions (150 μL) from a 10^{-3} dilution were added to Biolog Eco-plates. Plates were incubated at 28 °C and read on a BIOLOG™ Microplate Reader every 24 h over 7 days. The readings at 72 h incubation were used for subsequent analysis (see below). At this reading time, the microbial substrate utilization potential (OD) had stabilized and the greatest differences in utilization pattern were observed.

2.4. Statistical analysis

Well absorbance values were adjusted by subtracting the absorbance of the control well (water only) before data analysis, and substrates with an OD < 0 were excluded from further analysis. Average well color development (AWCD), calculated as the average optical density across all wells per plate, was used as an indicator of general microbial activity. Soil microbial community structure and functional diversity were determined using Shannon index and evenness (Zak et al., 1994; Staddon et al., 1997). Shannon index was measured by $H' = -\sum pi \ln pi$, and pi was calculated by subtracting the control from each substrate absorbance and then dividing this value by the total color change recorded for all 31 substrates. Evenness was calculated as $E = H'/\ln(\text{richness})$, where richness referred to the number of substrates utilized (Zak et al., 1994). According to their chemical nature, the substrates were divided into six substrate categories, i.e., carboxylic acids, amines and amides, amino acids, polymers, and miscellaneous (Preston-Mafham et al., 2002), and total absorbance of each category was calculated. DHA, AWCD, substrate utilization of each substrate category, Shannon diversity and evenness were compared by ANOVA with the SPSS 10.0 for Windows.

3. Results

3.1. Soil dehydrogenase activities (DHA)

The changes of DHA in all treatments are shown in Fig. 1. Due to long-term different fertilization, DHA differed significantly in three fertilized soils ($P < 0.01$). The highest value was observed in OM soil, followed by NPK, and the lowest in CK soil. Cd of 5 mg kg^{-1} had little effect on DHA, while Cu of 100 mg kg^{-1} suppressed DHA significantly ($P < 0.01$). During the entire period of incubation, Cu down-regulated DHA by 52.3%, 41.4% and 25.6% in NPK soil, CK soil and OM soil, respectively. The results indicated that DHA in NPK and CK soils were more sensitive to Cu than that in OM soil. Meantime, for each fertilization soil, DHA in the treatments of C, Cu + C and Cd + C were higher than those in the treatments of B, Cu and Cd, respectively ($P < 0.05$). Therefore, cypermethrin of 10 mg kg^{-1} can significantly improve DHA, which was in accord with the report by Rangaswamy et al. (1994).

3.2. Soil microbial functional diversity

3.2.1. Microbial functional diversity of different fertilization soils

Average well color development (AWCD) was used as an indicator of microbial activity in soil (Garland and Mills, 1991). As affected by long-term different fertilization, the significant differences of AWCD among three fertilized soils were observed ($P < 0.01$, Fig. 2). Contrary to changes of DHA, the highest AWCD was in NPK soil and the lowest in OM soil. AWCD of OM soil was almost zero in the first 24 h incubation. AWCD of NPK soil was 1.38 and 1.21 times as high as that of OM soil and CK soil, respectively. Compared to CK and OM fertilization, the highest potential to utilize the six substrate categories of microorganisms appeared in NPK soil (Fig. 3). The substrate utilization potential except carbohydrate, was the least in OM soil. Carbohydrate utilization in CK soil was lower than that in NPK and OM soils ($P < 0.05$). Compared to the NPK soil, polymer degradation in CK soil decreased significantly ($P < 0.05$), but the decrease of substrate utilization for other groups did not reach the significant level.

Shannon indices of NPK and CK soils were higher than that of OM soil ($P < 0.05$), whereas Shannon evenness of CK soil was higher than that in NPK and OM soils ($P < 0.05$) (data not shown). The results indicated that fertilization had no benefit to functional evenness in the present study.

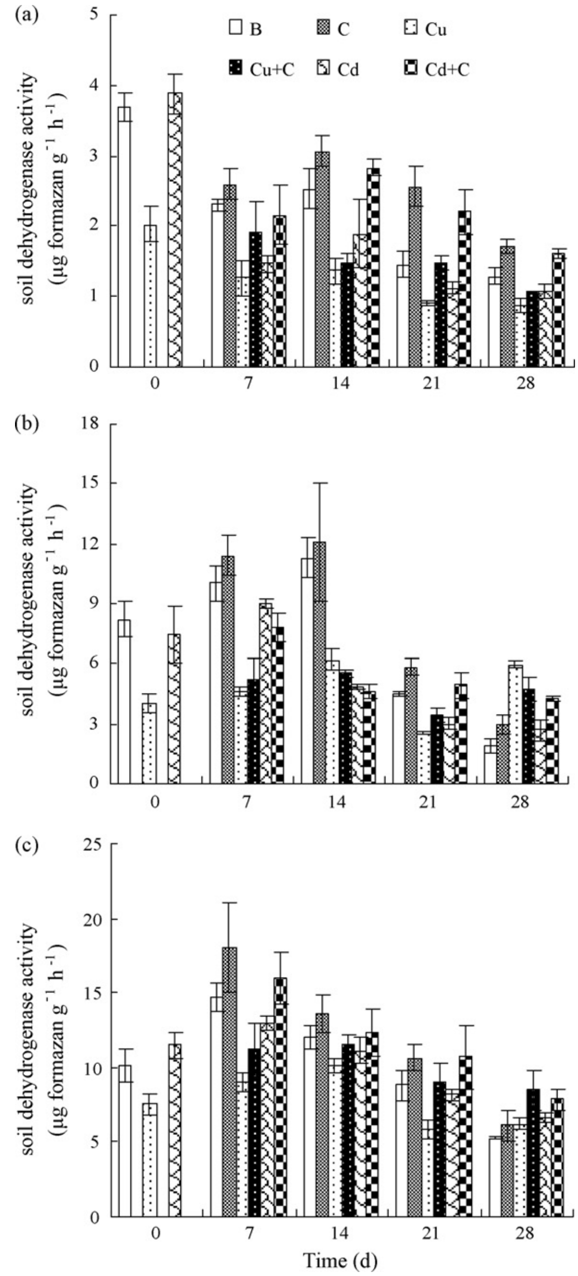

Fig. 1. Changes of soil dehydrogenase activities in three fertilized soils following addition of Cu, Cd and cypermethrin singly or in combination. Fertilization mode: (a) CK, no fertilization; (b) NPK, fertilization with NPK fertilizers; (c) OM, fertilization with organic manure. Error bars represent the standard deviation. Treatments: B, the control (without contamination); C, cypermethrin contamination; Cu, Cu^{2+} contamination; Cd, Cd^{2+} contamination; Cu + C, combination of Cu^{2+} and cypermethrin; Cd + C, combination of Cd^{2+} and cypermethrin.

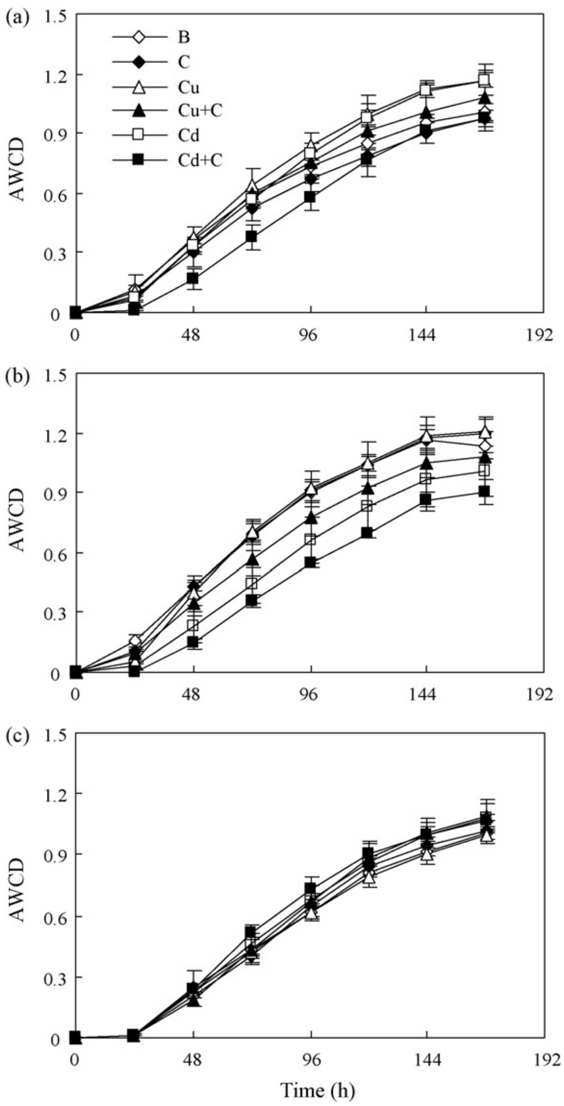

Fig. 2. AWCD of 31 carbon sources in different fertilized soils following addition of Cu, Cd and cypermethrin singly or in combination. Fertilization mode: (a) CK, no fertilization; (b) NPK, fertilization with NPK fertilizers; (c) OM, fertilization with organic manure. Error bars represent the standard deviation. Treatments: B, the control (without contamination); C, cypermethrin contamination; Cu, Cu^{2+} contamination; Cd, Cd^{2+} contamination; Cu + C, combination of Cu^{2+} and cypermethrin; Cd + C, combination of Cd^{2+} and cypermethrin.

3.2.2. Effects of Cu, Cd and cypermethrin on soil microbial functional diversity

After addition of contaminants into soils, decrease of the microbial functional diversity occurred to different extent (Figs. 2 and 3 and Table 2). The obvious separation of AWCDs among treatments was observed in CK and NPK soils, but it was unclear in OM soil (Fig. 2). Compared to the control treatment (B), combination of Cd and cypermethrin inhibited AWCD by 19% in CK soil ($P < 0.05$). AWCDs of Cu + C, Cd and Cd + C treatments decreased by 14%, 26% and 37%, respectively ($P < 0.05$), in NPK soil. Although the lowest substrate utilization potential (except carbohydrate) appeared in OM soil, little suppression of substrate utilization was found in contaminated treatments (Fig. 3 and Table 2). In NPK soil, polymer degradation was significantly inhibited by combination of Cu and cypermethrin and substrate utilization for six groups significantly decreased in the treatments of Cd and Cd + C ($P < 0.05$). Except carbohydrate, the decrease of substrate utilization for other five groups in the treatment of Cd + C also reached the significant level in CK soil ($P < 0.05$), especially amines utilization (deceased by 78.9%). Cd and combination of Cd and cypermethrin in NPK soil decreased Shannon index by almost 9% ($P < 0.05$), whereas application of contaminants had slight influence in CK and OM soils (Table 2). Evenness of all contaminated treatments decreased significantly in CK soil ($P < 0.05$). Cd and combination of Cd and cypermethrin also reduced evenness to a significant level in OM soil ($P < 0.05$). However there was no significant change of evenness in NPK soil (Table 2). Therefore, single addition of Cu or cypermethrin had little influence on AWCD, substrate utilization, Shannon index (except CK soil) and evenness. But combinations of cypermethrin and heavy metals, especially Cd, could result in the great reduction of functional diversity (Table 2).

4. Discussion

4.1. Effect of fertilization on the soil microbial activity

DHA is often used as the indicator of soil fertility and it also can denote the amount and activity of soil microbes (Casida et al., 1964; Gil-Sotres et al., 2005). Hence higher DHA in OM soil indicated that long-term application of composed straw was more beneficial to microbial biomass and activity than the application of NPK and no fertilization. However, compared to the NPK and CK soils, the substrate utilization potential of OM soil decreased significantly except carbohydrate ($P < 0.05$) in our study (Fig. 3). These results suggested that soil microbial community shifts had occurred after long-term fertilization. Soil nutrients, especial

Table 2
Significance of effects of cypermethrin, Cu, and Cd singly or in combination on substrate utilization, Shannon index and evenness in each fertilization mode determined with two-way ANOVA.

	Significance levels														
	CK					NPK					OM				
	C	Cu	Cd	C × Cu	C × Cd	C	Cu	Cd	C × Cu	C × Cd	C	Cu	Cd	C × Cu	C × Cd
Carbohydrates	–	–	–	–	–	–	–	*	–	–	–	–	–	–	–
Carboxylic acids	–	–	–	*	*	–	–	**	–	*	–	–	–	–	–
Amines	–	–	–	–	**	–	–	**	–	–	–	–	–	–	–
Amino acids	–	–	–	–	*	–	–	**	–	**	–	–	–	–	–
Polymers	–	–	–	–	–	–	*	**	*	–	–	–	–	–	–
Miscellaneous	**	–	*	*	*	–	–	*	–	–	–	–	–	–	–
Shannon index	–	–	–	–	–	–	–	*	–	–	–	–	–	–	–
Shannon evenness	*	*	*	–	–	–	–	–	–	–	–	–	*	–	–

Significance levels in same line in each fertilization mode: (–) $P \geq 0.05$; (*) $P < 0.05$; (**) $P < 0.01$. Treatments: B, the control (without contamination); C, cypermethrin contamination; Cu, Cu^{2+} contamination; Cd, Cd^{2+} contamination; C × Cu, interaction between Cu^{2+} and cypermethrin; C × Cd, interaction between Cd^{2+} and cypermethrin. Fertilization mode: CK, no fertilization; NPK, fertilization with NPK fertilizers; OM, fertilization with organic manure.

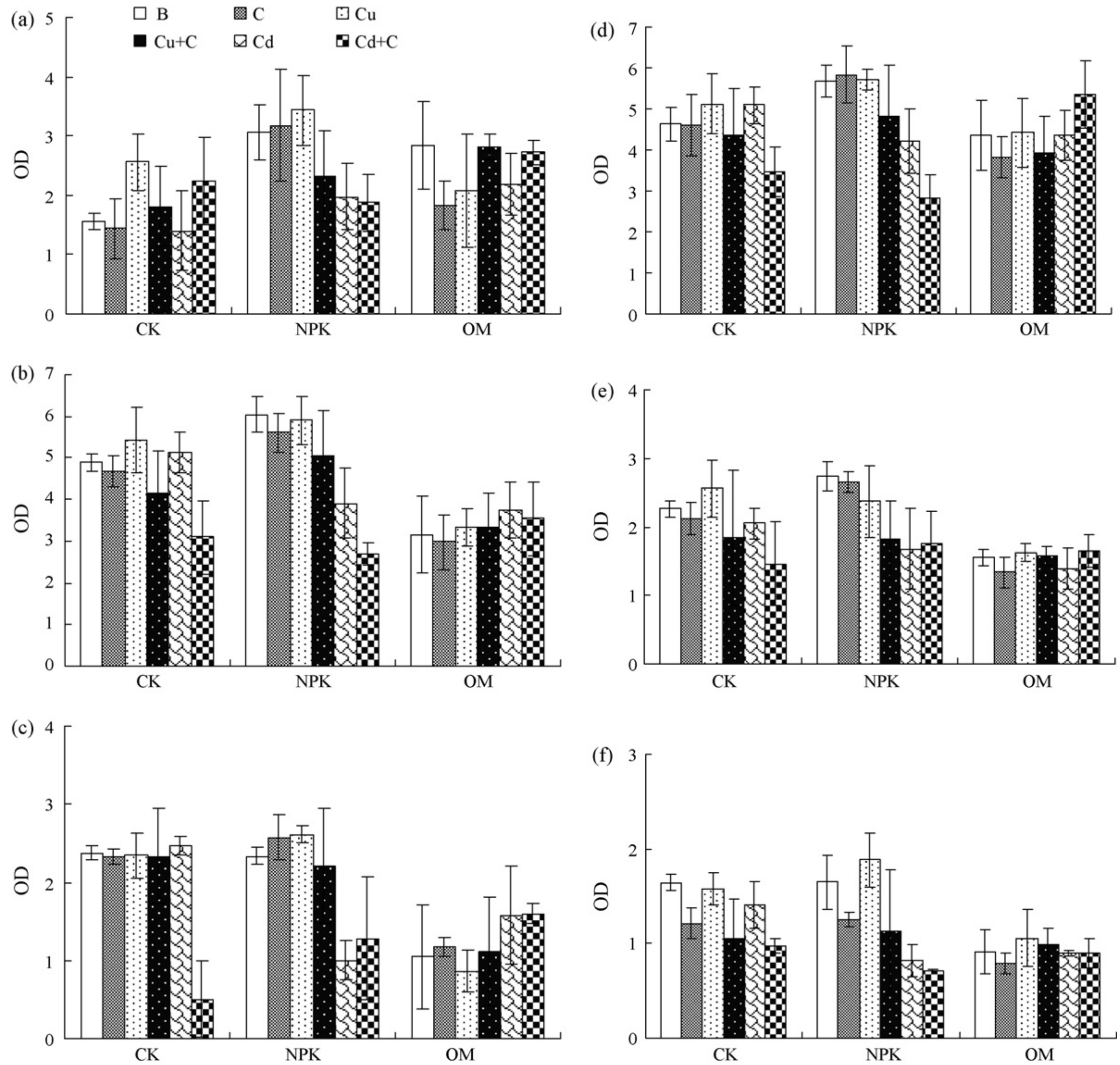

Fig. 3. Microbial substrate utilization potential (OD) in three fertilized soils following addition of Cu, Cd and cypermethrin singly or in combination. Substrates: (a) carbohydrates, (b) carboxylic acids, (c) amines, (d) amino acids, (e) polymers, (f) miscellaneous. Error bars represent the standard deviation. Fertilization mode: CK, no fertilization; NPK, fertilization with NPK fertilizers; OM, fertilization with organic manure. Treatments: B, the control (without contamination); C, cypermethrin contamination; Cu, Cu^{2+} contamination; Cd, Cd^{2+} contamination; Cu + C, combination of Cu^{2+} and cypermethrin; Cd + C, combination of Cd^{2+} and cypermethrin.

organic matter, are important drivers of soil microbial community composition (Steenwerth et al., 2008). Fertilization-induced changes to the soil properties affected soil nutrient availability greatly, such as N, P and organic matter (Table 1). Previous studies have also reported that chemical nature and diversity of soil organic matter were influenced by fertilization (Ellerbrock et al., 1999; Vineela et al., 2008), which in turn induced changes of microbial substrate utilization (Cooksona et al., 2008; Steenwerth et al., 2008). In OM soil, the organic matter content was high because of the compost application, as well as its fraction of carbohydrate was likely to increase during wheat straw degradation. This soil environment had benefits to promote DHA (Gil-Sotres et al., 2005) and the growth of carbohydrate-utilized microbe, however the growth of other microorganisms would be suppressed due to nutrition limitation, therefore the microbial diversity reduced. In NPK and CK soils, the organic matter was mainly from root turnover and exudates and its contents were relatively low. These may create soil conditions to maintain or improve the ability of microbial substrate utilization (Malhi et al., 2006; Cooksona et al., 2008). The variation in the microbial functional diversity was from complex interactions between soil properties that mediated substrate availability and microbial nutrient demand (Cooksona et al., 2008).

There were some limitations of the Biolog method in application, mainly including its focusing on bacterial species that are able to respond rapidly to the substrates and carbon sources that are different from the contaminants found in soils (Preston-Mafham et al., 2002). But due to the high sensitivity and efficiency, the effects of contaminants on CLSU can be regarded as the early warning signals for soil evaluation (Kong et al., 2006). Therefore, the persistence of organic pollutants, in our point of view, might enhance with the lower carbon source utilizations in organic manured soil. Despite of no fertilization over a long period of time, the microbial capability of carbon source utilization is relative high in CK soil.

4.2. Effects of Cu, Cd and cypermethrin on soil microbial activity

Due to the widespread contamination by heavy metals and pesticides in cropland soils, it is essential to eliminate or decrease the potential risk of these compounds in the environment. In the present study, the responses of microorganisms in three fertilized soils to Cu, Cd and cypermethrin and their combinations were different. Cu of 100 mg kg^{-1} decreased DHA significantly ($P < 0.05$), and the decrease extents were in an order of NPK > CK > OM. In CLSU experiment, there was slight influence of contaminants on soil microbial diversity in OM soil with the exception of Shannon evenness. But in NPK and CK soils, combination of cypermethrin and Cd significantly suppressed AWCD, substrate utilization and Shannon index ($P < 0.05$) and the greater decrease was in NPK soil. Furthermore, in NPK soil, combination of Cu and cypermethrin and Cd reduced AWCD and Cd reduced substrate utilization significantly ($P < 0.05$). Therefore, the sensitivity of microorganisms in soils to pollutants followed the order as: NPK > CK > OM. The results indicated that fertilization mode had the great effect on contaminant bioavailability in soil. Little change of soil pH was observed among different treatments in each soil (data not shown). The previous studies have reported soil organic matter can decrease pollutant bioavailability via the formation of complexes between them, and organic manure application is an efficient way to reduce environmental risks of pollutants (Pérez-de-Mora and Madrid, 2007; van Herwijnen et al., 2007; Sannino and Gianfreda, 2001). Hence the low sensitivity in OM soil was a result of compost application. However, higher organic matter content in NPK soil than that in CK soil suggested the effect of contaminants on microbes was controlled by additional regulators. Microbial community responded differently to soil contaminants (Gflier et al., 1998; Wang et al., 2004), so microbial community shifts induced by varied fertilization mode (Marschner et al., 2003; Widmer et al., 2006; Chu et al., 2007) should be taken when assessing contaminant bioavailability. Additionally, in cypermethrin degradation study, the lowest dissipation rate was recorded in NPK soil (Xie and Zhou, 2008), which also contributed to the high sensitivity in NPK soil to some extent.

Although the influence of cypermethrin on the soil microbial diversity was slight, we found that a great synergistic effect could be provoked when applied together with heavy metals, especially with Cd (Table 2). Thus more attentions should be paid to the eco-environmental risk of cypermethrin in heavy metal polluted soils. Heavy metal levels in the present study were the critical doses, which could significantly reduce cypermethrin degradation in soil. Cu of 100 mg kg^{-1} could significantly suppress DHA, while Cd of 5 mg kg^{-1} had relative greater influence on soil microbial diversity. The results suggested that the effect of each pollutant on soil microbes was specific.

5. Conclusions

We concluded that long-term application with different fertilizer types can significantly influence soil microbial activity and the bioavailability of contaminants. Balanced fertilization with chemical NPK fertilizers has benefit to improve soil microbial functional diversity, whereas the bioavailability of contaminants in soil also increases. Conversely, the continuous application of composted straw can decrease the bioavailability of contaminants, but, due to community shift, soil microbial functional diversity seriously reduces. With the scientific interest in organic farming increasing, our finding suggested that more attentions should be paid to the microbial community shifts in this practice.

We also found that the effect of cypermethrin on soil microorganisms was more pronounced in heavy metal polluted soils. Due to the co-occurrence of pesticides and heavy metals in agricultural soils, eco-environmental risk of cypermethrin would arouse more concerns in the future.

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