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Degradation of chlorpyrifos in laboratory soil and its impact on soil microbial functional diversity

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Abstract

Degradation of chlorpyrifos at different concentrations in soil and its impact on soil microbial functional diversity were investigated under laboratory condition. The degradation half-live of chlorpyrifos at levels of 4, 8, and 12 mg/kg in soil were calculated to be 14.3, 16.7, and 18.0 d, respectively. The Biolog study showed that the average well color development (AWCD) in soils was significantly ($P < 0.05$) inhibited by chlorpyrifos within the first two weeks and thereafter recovered to a similar level as the control. A similar variation in the diversity indices (Simpson index $1/D$ and McIntosh index U) was observed, but no significant difference among the values of the Shannon-Wiener index H' was found in chlorpyrifos-treated soils. With an increasing chlorpyrifos concentration, the half-life of chlorpyrifos was significantly ($P \leqslant 0.05$) extended and its inhibitory effect on soil microorganisms was aggravated. It is concluded that chlorpyrifos residues in soil had a temporary or short-term inhibitory effect on soil microbial functional diversity.

Key words: Biolog; chlorpyrifos; community-level physiological profile; microbial functional diversity
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Introduction

Pesticides have been widely used to control insect pests, plant pathogens, and weeds over the past 50 years. Some of these pesticides will eventually reach the soil following field application, even sprayed on the foliage of crop plants and weeds, and most of them have an adverse effect on soil microbial functional diversity and ultimately influence soil fertility and plant growth, which pose a serious threat to the sustainability of agricultural soils (Kennedy and Gewin, 1997; Johnsen *et al*., 2001; Singh *et al*., 2002a). It has been reported that soil microbial diversity is regarded as one of the most important parameters of soil microorganisms and has been suggested as an indicator of soil health (Kennedy and Smith, 1995; Brussaard *et al*., 2007). Therefore, the impact of pesticides on soil microbial functional diversity has raised considerable public concern.

Chlorpyrifos (*o*,*o*-diethyl-*o*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a broad-spectrum organophosphate insecticide and acaricide, which is widely used to control insect pests on grain, cotton, fruit, nut, and vegetable crops, as well as lawns and ornamental plants in China. Because of its intensive use, a wide range of terrestrial ecosystems may be contaminated with chlorpyrifos, which has increased public concern and there is a need to evaluate its environmental behavior and effects. The dissipation (Redondo *et al*., 1997), adsorption (Van Emmerik *et al*., 2007), leaching (Li *et al*., 2005), photolysis (Graebing and Chib, 2004), and biodegradation (Singh *et al*., 2006) of chlorpyrifos in soil ecosystems have been extensively investigated. The half-life of chlorpyrifos in soil varies greatly from less than 1 d to more than 100 d depending on the soil type, soil microorganisms, and climatic condition (Singh *et al*., 2002a). The effects of chlorpyrifos on soil microbial characteristics (including microbial biomass carbon and nitrogen, microbial populations, microbial respiration, enzymatic activities, and nitrogen cycling) have also been frequently studied (Singh *et al*., 2002b; Menon *et al*., 2004; Adesodun *et al*., 2005; Menon *et al*., 2005; Shan *et al*., 2006). It had been reported that soil microbial biomass was reduced by 25% and 50% after chlorpyrifos treatment at concentrations of 10 and 50 mg/kg, respectively, in an Italian biobed (Vischetti *et al*., 2007). Menon *et al*. (2004) reported that nitrogen mineralization in the loamy sand and sandy loam was significantly inhibited after chlorpyrifos application. Shan *et al*. (2006) also indicated that soil bacterial, fungal, and actinomycete populations were inhibited by chlorpyrifos at a concentration of 10 mg/kg. However, little information is available on the impact of chlorpyrifos on soil microbial diversity.

In the present study, Biolog EcoPlate™ was used to

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monitor community-level physiological profiles (CLPPs) of soil microorganisms as carbon source utilization patterns. Average well color development (AWCD) and diversity indices ($1/D$, H', and U) were employed to assess soil microbial functional diversity. The objectives of this study were: (1) to understand the chlorpyrifos degradation dynamics in laboratory soil; (2) to examine the impact of chlorpyrifos on soil microbial functional diversity; and (3) to evaluate the effect of chlorpyrifos on soil health.

1 Materials and methods

1.1 Chemicals

Technical grade chlorpyrifos (99.5% purity) was purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China. Acetone and petroleum ether (60–90°C) of analytical grade were purchased from Dafang Chemical Co., Hangzhou, China. The organic solvents were all redistilled in a full glass system before use.

1.2 Soils

The soil samples used in this study were collected from a field site located at Huajiachi Campus, Zhejiang University, Hangzhou, China, and contained no detectable amount of chlorpyrifos residues. Soil samples taken from the top layer (0–15 cm) were air-dried at room temperature, mixed thoroughly and sieved (2 mm) to remove stones and debris. Soil samples were stored at room temperature for 7 d before use. The physico-chemical properties of the soil were determined according to the standard protocols (Institute of Soil Science, Academia Sinica, 1979). The soil was classified as silt loam, and its properties were as follows: sand, 21.5%; silt, 71.1%; clay, 7.4%; organic matter content, 3.05%; water holding capacity, 39.4%; cationic exchange capacity, 10.6 cmol/kg; total nitrogen, 0.14% and pH 6.8.

1.3 Soil treatment and sampling

Soil samples (1.5 kg dw) were treated with an acetone solution of chlorpyrifos coupled with the appropriate amount of sterile distilled water to give a final concentration of 4, 8, and 12 mg/kg of dry soil, corresponding to the recommended dose (3750 mL/hm^2), twice the recommended dose (7500 mL/hm^2), and three times the recommended dose (11250 mL/hm^2), respectively. Chlorpyrifos concentrations (R) were calculated according to Eq. (1):

$$R = D \times C/(H \times \rho \times 100) \quad (1)$$

where, D represents the application rate of chlorpyrifos, C is the active ingredient content of chlorpyrifos formulation (48%), H is soil penetration depth of pesticide (5 cm), and ρ is soil density (0.95 g/cm^3 dw) (Burrows and Edwards, 2004). Soil samples containing chlorpyrifos were mixed thoroughly using sterile plastic spoons, then passed through a 2-mm mesh to ensure uniform distribution of the added pesticide. The samples were left for 1 h on a laminar flow bench to evaporate the solvent, and then transferred to 3-L polypropylene flowerpots and covered with aluminum foil. Soil samples which received the same amount of sterilized water were regarded as controls. Soil water content was adjusted to 60% of the water-holding capacity which was maintained by the periodic addition of sterile distilled water. All treatments were in triplicate and the samples were incubated at (25 ± 1)°C in the dark. At the intervals of 0, 1, 3, 5, 7, 14, 21, and 35 d, soil samples (20 g) from each treatment were obtained using a soil auger (2-cm diameter) to determine chlorpyrifos residues. In addition, 10 g of the soil were sampled to measure substrate utilization patterns using Biolog microplates on 7, 14, 21, and 35 d, respectively.

1.4 Biolog assay

A 10^{-1} dilution solution was prepared by suspending a 10-g dw soil sample in 100 mL of sterile physiological saline (0.85% NaCl, *W/V*) on an orbital shaker at 150 r/min and 25°C for 1 h to ensure homogeneous dispersion of the soil particles. The solution was then allowed to settle for 30 min to clear the supernatant. Serial dilutions were subsequently performed to the 10^{-3} dilution. A 150-μL aliquot of the 10^{-3} dilution was immediately inoculated into each of the 96 wells of the Biolog microplates, and the microplates were then incubated at (25 ± 1)°C in the dark. Color development in the plates was measured every 24 h at 590 nm for 7 d using a BIO-TEK Elx808 automated microplate reader (BIO-TEK Instruments Inc., USA).

1.5 Extraction of chlorpyrifos from soil

Soil samples (20 g dw) were placed into a 250-mL Erlenmeyer flask, and 20 mL of distilled water and 50 mL of acetone were successively added. The samples were then shaken on a rotary shaker at 150 r/min for 2 h at 25°C. The soil mixture in each sample was filtered through a Buchner funnel, and the filter cake was washed 3 times with 25 mL of acetone. All filtrates were collected in a 250-mL flat-bottom flask and evaporated on a vacuum rotary evaporator to remove the acetone. The solution left in each flask was subsequently transferred into a 250-mL separating funnel and extracted 3 times with 50 mL of petroleum ether. The organic phase was dried through anhydrous sodium sulphate and collected in a 250-mL flat-bottom flask. The extract was then concentrated until almost dry under a gentle nitrogen flow and redissolved in 5 mL of redistilled petroleum ether for gas chromatograph (GC) analysis.

1.6 Determination of chlorpyrifos

Gas chromatograph analyses were performed using an Agilent 6890N gas chromatograph (Agilent Technologies, USA), equipped with an electron capture detector (ECD). A fused silica capillary column (DB-1701, 30 m × 0.32 mm × 0.25 μm) was employed. The oven temperature was initially 80°C, held for 0.5 min, and raised to 230°C at 10°C/min and held for 10 min. The injector and detector were set at 230 and 300°C, respectively. Nitrogen was used as a carrier gas at a constant flow rate of 50 mL/min.

1.7 Recovery study

A recovery experiment was conducted to confirm the validity of the methods described above. Known concentrations of chlorpyrifos were spiked into 20 g of dry soil to certain concentration: 0.01, 0.1, 1.0, and 10.0 mg/kg. Extraction and analyses were performed in triplicate as described above.

1.8 Data analysis

For CLPPs analysis, AWCD was calculated by Eq. (2):

$$\text{AWCD} = \sum \text{OD}_i/31 \tag{2}$$

where, OD_i is the optical density value from each well by water blank substraction (Garland and Mills, 1991). The absorbance values at 72 h were used to calculate diversity indices and principal component analysis (PCA). Soil microbial diversity was calculated by Eqs. (3)–(5):

$$\text{Simpson index} \quad D = \sum \frac{(n_i(n_i-1))}{(N(N-1))} \tag{3}$$

$$\text{Shannon-Weaver index} \quad H' = -\sum p_i(\ln p_i) \tag{4}$$

$$\text{McIntosh index} \quad U = \sqrt{(\sum n_i^2)} \tag{5}$$

where, n_i refers to absorbance value, N is total absorbance values of all wells. p_i is the proportional absorbance value of the well over total absorbance value of all wells, and the Simpson index was expressed as the reciprocal ($1/D$) (Magurran, 1988). PCA was used to characterize community level profiles. AWCD and diversity indices were compared using one-way ANOVA with SPSS 11.5 (SPSS Inc., USA), respectively.

2 Results and discussion

2.1 Evaluation of recovery

Figure 1 shows gas chromatograms of the standard, blank soil, and chlorpyrifos-fortified soil. No interference was observed in the control sample during analysis of chlorpyrifos. The average recoveries of chlorpyrifos from soil are presented in Table 1. The recoveries of chlorpyrifos in soil ranged from 92.0% to 105.4% with a relative standard deviation (RSD) less than 7.0%. The limits of detection and quantification were measured to be 0.001 and 0.01 mg/kg dry soil, respectively. These data indicated that the extraction method was considered to be satisfactory for analysis of chlorpyrifos residues.

Table 1 Recoveries of chlorpyrifos from the spiked soils

Fortification level (mg/kg)	Sample weight (g)	Average recovery* (%)	RSD (%)
0.01	20	105.4	7.0
0.10	20	104.8	5.2
1.00	20	95.0	3.0
10.00	20	92.0	0.3

* Each value is a mean of three replicates.

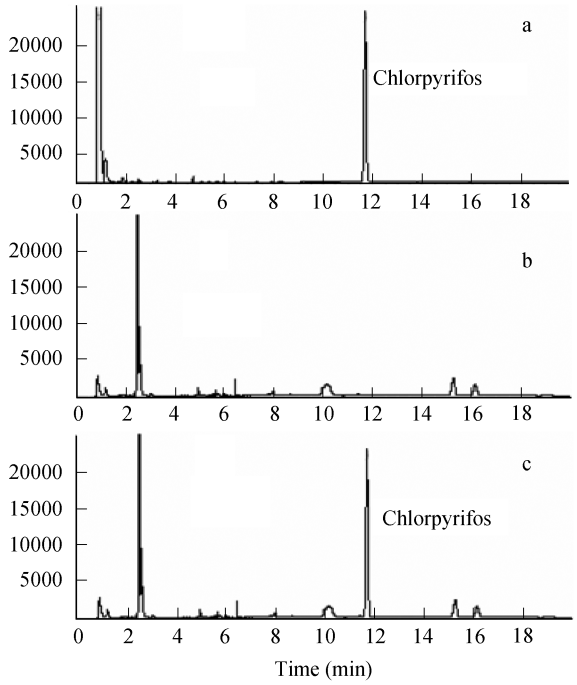

Fig. 1 Gas chromatograms of standard (a), blank soil (b), and chlorpyrifos-fortified soil (c).

2.2 Degradation of chlorpyrifos in soil

The disappearance patterns of chlorpyrifos at different concentrations in laboratory soil are shown in Fig. 2. Degradation of chlorpyrifos in soil was subjected to the first-order model. The kinetic data of chlorpyrifos

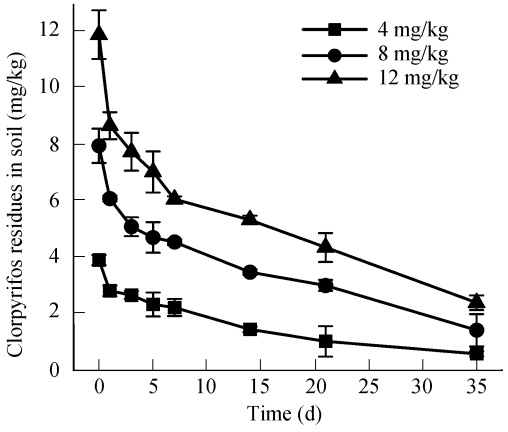

Fig. 2 Degradation of chlorpyrifos at concentrations of 4, 8, and 12 mg/kg in laboratory soil.

Table 2 Kinetics data of chlorpyrifos degradation in soil

Chlorpyrifos concentration (mg/kg)	Dynamic function	DT_{50}* (d)	r^2
4	$C = 3.20e^{-0.0484t}$	14.3 a	0.9720
8	$C = 6.46e^{-0.0415t}$	16.7 b	0.9538
12	$C = 9.26e^{-0.0385t}$	18.0 c	0.9381

* The degradation of chlorpyrifos in soil was described by the first-order function ($C = C_0 \times e^{-kt}$). The degradation half-live of chlorpyrifos (DT_{50}) in soil was obtained by the function $DT_{50} = \ln 2/k$. Each value is a mean of three replicates, DT_{50} followed by a different letter within a column are significantly different ($P \leqslant 0.05$).

degradation in Table 2 show that chlorpyrifos at the initial level of 4, 8, and 12 mg/kg in soil was degraded by 83.8%, 81.6%, and 79.5% with half-live of 14.3, 16.7, and 18.0 d after treatment for 35 d, respectively. The half-lives were significantly ($P \leqslant 0.05$) extended with increasing chlorpyrifos concentration. This was most likely because of inhibition of soil microbial communities by chlorpyrifos at high concentration. It had been shown by Shan *et al*. (2006) that chlorpyrifos at a high concentration (10 mg/kg) had a greater impact on the inhibition of soil microbial populations than lower concentrations (2 and 4 mg/kg).

2.3 Substrate utilization patterns and functional diversity of soil microorganisms

AWCD reflects the oxidative capacity of soil microorganisms developing in the Biolog microplates and is usually used as an indicator of overall microbial activity (Rodriguez and Toranzos, 2003). The AWCD values from each treatment are shown in Fig. 3. At the early stage of treatment (day 7), AWCD values from the chlorpyrifos-treated soils were smaller compared with the control. On day 14, no significant effect on the AWCD was observed in chlorpyrifos-treated samples at 4 and 8 mg/kg. However, the AWCD from the chlorpyrifos-treated sample at 12 mg/kg was significantly ($P \leqslant 0.05$) smaller than that of the control. On day 21, the AWCD values in all treatments recovered to the control level, suggesting that overall microbial activity recovered to the control level. After 35 d of chlorpyrifos treatment, the chlorpyrifos-treated soils showed higher AWCD values than the control, which indicated that soil microbial activities in chlorpyrifos-treated soils were obviously enhanced.

The diversity indices ($1/D$, H', and U) are used to assess soil microbial functional diversity (Gomez *et al*., 2006). The $1/D$ is used to emphasize the dominant population of soil microorganisms (Simpson, 1949), and H' indicates the richness of soil microorganisms (Shannon and Weaver, 1949), while U indicates the evenness or homogeneity of soil microorganisms (McIntosh, 1967). The changes in soil microbial diversity indices from chlorpyrifos-treated soils are summarized in Table 3. Compared with the control, the diversity indices in soil treated with chlorpyrifos at 4, 8, and 12 mg/kg were significantly ($P \leqslant 0.01$) decreased by 18.2%, 30.5%, 27.7% for $1/D$, and 47.9%, 48.7%, 55.6% for U on day 7, respectively. On day 14, the $1/D$ values in chlorpyrifos-treated soils were still obviously ($P \leqslant 0.05$) smaller than those in the control soil, but there was no significant difference among the three chlorpyrifos-treated soils. After chlorpyrifos treatment for 21 d, $1/D$ and U recovered to or exceeded those of the control level. Throughout the experiments, no significant fluctuation in the H' was observed in any of the soils, indicating that

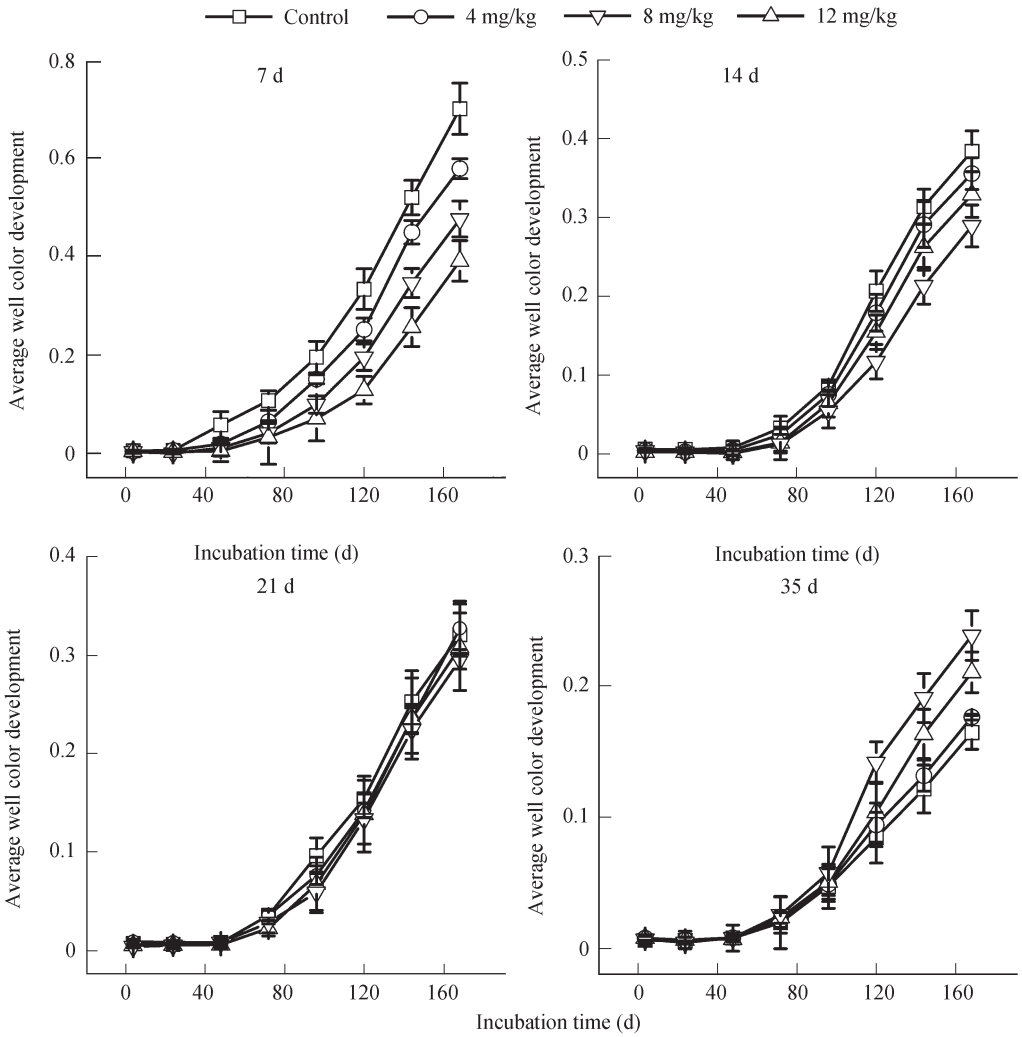

Fig. 3 Average well color development (AWCD) of soil samples at different sampling times.

the richness of soil microorganisms was not apparently effected. In agreement with this result, Singh *et al*. (2002a) reported that chlorpyrifos at a concentration of 10 mg/kg had little effect on the richness of soil microorganisms under control condition.

As shown in Table 3, 1/*D* and *U* decreased during the initial two weeks after chlorpyrifos treatment, and subsequently recovered gradually to the control level. At an early stage of the experiment (14 d), the dominant populations of soil microorganisms were obviously suppressed, and the microbial evenness declined. This may be attributed to the presence of chlorpyrifos residues in the soil. Similar findings have been demonstrated by Zhang *et al*. (2006) who reported that the dominant populations and evenness of soil microorganisms decreased in methyl parathion-contaminated soil. However, contrary results had been reported by Singh *et al*. (2002a), where the overall metabolic diversity and evenness of soil microorganisms were little affected in soil after chlorpyrifos application at a concentration of 10 mg/kg. In this study, at a later stage in the experiment (21 d), the dominant populations and evenness of soil microorganisms recovered. It is possible that some soil microorganisms may develop tolerance and adapt to the pesticide applied due to selective pressure of the chemical. These soil microorganisms may utilize chlorpyrifos as a source of carbon and energy and proliferate substantially in vacant niches. A similar phenomenon had been revealed in fungal populations and denitrifying bacteria which can tolerate chlorpyrifos residues at a range of 10 to 300 mg/kg in an agricultural loam (Martinez-Toledo *et al*., 1992).

2.4 Principal component analysis of substrate utilization patterns

To distinguish the carbon utilization patterns of soil microorganisms, PCA analyses for each treatment were conducted (Fig. 4). Throughout this study, the first principal component (PC1) had more important power than the second principal component (PC2). PC1 mainly reflected the consumption of carbohydrate with an average score of 0.85. As shown in Fig. 4, a dispersed or separated pattern was clearly observed on day 7, which indicated the significant differences in microorganisms between the chlorpyrifos-treated soils and the control soil. The values of PCA were gradually grouped on day 14, which suggested that the soil microbial communities in chlorpyrifos-treated soils recovered gradually to those of the control soil. On day 21, a grouped or convergent pattern was observed, which suggested that the soil microbial communities in chlorpyrifos-treated soils had recovered to the control level. The treatments did not show any significant differences in their ordination. On day 35, the scores of chlorpyrifos-treated soils in PC1 were higher than the control soil, suggesting that chlorpyrifos treatment caused proliferation of soil microorganisms.

The results obtained in this study indicated that a small but significant inhibition of soil microbial communities was observed during the first two weeks after chlorpyrifos treatment. Rehabilitation or recolonization of soil microbial communities occurred thereafter. A similar trend had been reported by Pandey and Singh (2004), where chlorpyrifos at a dose of 4 L/hm^2 had a short-term inhibitory effect on the total bacterial population which recovered within 45 d after soil treatment. In this study, the half-live of chlorpyrifos had a positive correlation with chlorpyrifos concentration, and its inhibitory effect on soil microbial communities followed a positive dose-response pattern. Therefore, it could be presumed that the degradation rate of chlorpyrifos was greatly correlated with soil microbial communities. Vischetti *et al*. (2002) had reported that the relationship between soil microbial biomass and imazamox and benfluralin degradation followed a parabolic curve in three different soils. However, the functional diversity of soil microorganisms was also affected by many biotic and nonbiotic factors. Firstly, chlorpyrifos concentration was mainly responsible for the changes in soil microbial communities. This was confirmed in previous studies which demonstrated that the inhibitory effects of chlorpyrifos on soil microorganisms were enhanced with increasing chlorpyrifos concentration

Table 3 Diversity indices of soil microbial community after chlorpyrifos treatment

Treatment		Simpson index (1/*D*)	Shannon-Wiener index (*H′*)	McIntosh index (*U*)
Time (d)	Concentration (mg/kg)			
7	Control	10.54 ± 0.41 a	2.67 ± 0.19 a	2.34 ± 0.18 a
	4	8.62 ± 0.38 b	2.60 ± 0.29 a	1.22 ± 0.34 b
	8	7.33 ± 0.45 c	2.51 ± 0.12 a	1.20 ± 0.33 b
	12	7.62 ± 1.12 c	2.52 ± 0.10 a	1.04 ± 0.06 b
14	Control	9.36 ± 1.49 a	2.72 ± 0.12 a	0.93 ± 0.11 a
	4	6.34 ± 0.77 b	2.39 ± 0.31 a	0.88 ± 0.08 a
	8	6.05 ± 0.48 b	2.57 ± 0.30 a	0.70 ± 0.07 b
	12	6.03 ± 0.64 b	2.26 ± 0.31 a	0.69 ± 0.04 b
21	Control	8.95 ± 0.56 a	2.51 ± 0.34 a	0.68 ± 0.05 a
	4	9.14 ± 0.97 a	2.74 ± 0.03 a	0.71 ± 0.07 a
	8	9.54 ± 0.42 a	2.68 ± 0.10 a	0.54 ± 0.11 a
	12	10.43 ± 0.27 a	2.76 ± 0.21 a	0.60 ± 0.28 a
35	Control	4.95 ± 0.65 a	2.30 ± 0.17 a	0.63 ± 0.02 a
	4	14.03 ± 0.51 b	2.92 ± 0.23 a	0.49 ± 0.18 a
	8	12.08 ± 0.45 b	2.83 ± 0.26 a	0.50 ± 0.17 a
	12	12.65 ± 0.65 b	2.75 ± 0.51 a	0.51 ± 0.03 a

All values are mean ± SD of three replicates. Data in the same column followed by different letters are significantly different ($P \leqslant 0.05$).

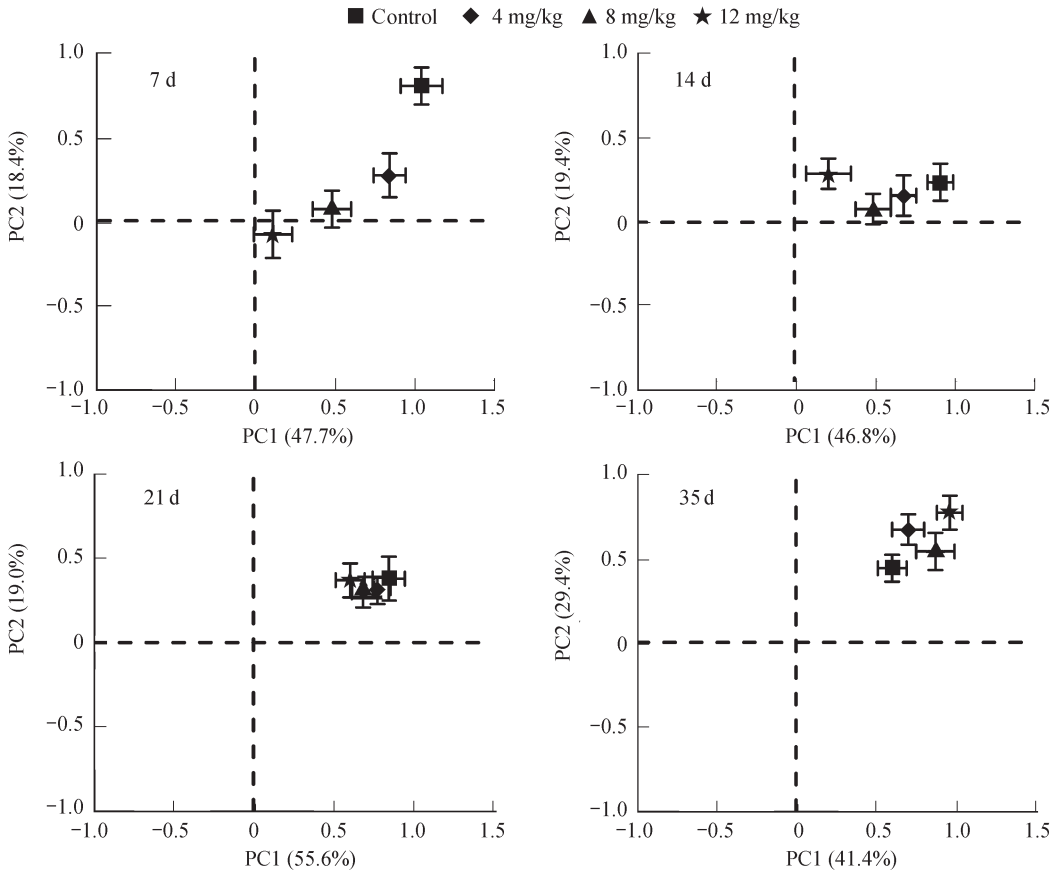

Fig. 4 Principal component analysis of substrate utilization patterns from each treatment at different sampling times. PC1 and PC2 represent the first and second principal components in the PCA profiles, respectively. The value in parentheses represents explained percentage of the variance.

(2, 4, and 10 mg/kg) (Shan *et al*., 2006). Simultaneously, soil characteristics (soil type, organic matter content, pH and salinity) and environmental factors (relative humidity, evapotransition, and temperature) also affected the abundance of soil microorganisms (Akhtar *et al*., 2004; Pandey and Singh, 2004; Cycon and Piotrowska-Seget, 2007). In addition, soil sampling methods played a key role in the diversity studies of soil microbial communities, and temporal and spatial heterogeneity of soil samples should be considered in detail (Johnsen *et al*., 2001). It is, therefore, difficult to obtain a clear and quantitative relationship between chlorpyrifos concentration and its effect on soil microorganisms.

3 Conclusions

Degradation of chlorpyrifos in laboratory soil was described by the first-order model. The half-live of chlorpyrifos in soil was significantly extended with increasing chlorpyrifos concentration. The results indicated that a short-term or temporary inhibitory effect on soil microbial diversity was observed during the initial two weeks after chlorpyrifos treatment. Soil microbial diversity recovered to the control level thereafter. Therefore, chlorpyrifos application had no lasting impact on soil health. However, further studies on the resistance and resilience of soil microorganisms to repeated applications of chlorpyrifos are required to evaluate the recovery and sustainable development of intensively used horticultural soils.

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