



Degradation of chlorpyrifos alone and in combination with chlorothalonil and their effects on soil microbial populations

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Abstract

In practice, pesticides are usually applied simultaneously or one after another for crop protection, and this type of pesticide application often leads to a combined contamination of pesticide residues in the soil environment. A laboratory study was conducted to investigate the influence of chlorothalonil on chlorpyrifos degradation and its effects on soil bacterial, fungal, and actinomycete populations. Under the experimental conditions here, the half-lives of chlorpyrifos alone, and in combination with chlorothalonil, at the recommended and double dosages, were measured to be 3.24, 2.77, and 2.63 d, respectively. Chlorpyrifos degradation was not significantly altered by its combination with chlorothalonil. However, the inhibitory effect of chlorpyrifos on soil microorganisms was increased by its combination with chlorothalonil, and the increase was related to the levels of chlorothalonil added. Compared to those in the controls, the populations of bacteria, fungi, and actinomycetes were significantly reduced by 44.1%, 61.1%, and 72.8%, respectively, on the first day after treatment (DAT) by chlorpyrifos alone. With the addition of chlorothalonil, the inhibition was increased to 55.2%, 79.3%, and 85.8% at the recommended dosage, and 86.0%, 94.1%, and 90.8% at the double dosage, at one DAT, respectively. The results suggested that combined effects should be taken into account to assess the actual impacts of pesticide applications.

Key words: pesticide; soil microorganism; chlorpyrifos; chlorothalonil; combination

Introduction

Synthetic pesticides are purposely introduced into agricultural systems to protect crops against weeds, insects, fungi, and other pests (Yang *et al.*, 2007). However, the majority of the applied pesticides, even if sprayed on foliage of crop plants and weeds, will eventually reach the soil, which may affect the growth and activity of soil microbial communities (Cope, 1971; Omar and Abdel-Sater, 2001; Singh and Singh, 2005). Microorganisms are an important biological component of the soil ecosystem and play vital roles in soil fertility through their roles in nutrient cycling and organic matter decomposition (Wainwright, 1978). Generally, any actions that alter the numbers and activities of soil microbes by pesticides could affect the soil biochemical process and ultimately influence soil fertility and plant productivity (Wainwright, 1978; Moonman, 1989). The extensive and excessive use of pesticides has aroused concern on the fate of pesticides in soil and possible side-effects on the soil microbial communities. Although a number of studies have been conducted, most of these studies have only focused on the individual pesticide (Wainwright, 1978; Hill and Stratton, 1991; Getenga *et al.*, 2000; Xie *et al.*, 2004; Gundi *et al.*, 2005). Under

actual agricultural practices, however, different classes of pesticides, such as, insecticides, fungicides, and herbicides are often applied simultaneously or one after another for the purpose of crop protection and these chemicals may interact with each other within the soil systems (Fließbach and Mäder, 2004). The degradation behavior of a single pesticide may be changed after it interacts with other pesticides coexisting in the soil, and such changes in degradation behavior would have different side-effects on the biological function of the soil. There is, therefore, an increasing concern on the behaviors of combined pesticide residues in soil and their potential effects on soil quality.

Chlorpyrifos is a broad-spectrum organophosphate insecticide and acaricide, and is widely used for pest control on grain, cotton, fruit, and vegetable crops, as well as, lawns and ornamental plants (Fang *et al.*, 2006). It is moderately persistent in soils with half-life from less than 1 d to more than 240 d, depending on soil types, soil moisture, soil pH, and initial concentrations (Racke *et al.*, 1990; Racke, 1993; Awasthi and Prakash, 1997; Singh *et al.*, 2003). On the other hand, chlorothalonil is a broad-spectrum fungicide used to control fungal diseases, on vegetables, trees, small fruits, turf, ornamentals, and other agricultural crops. The half-life of chlorothalonil in soil varies from less than 1 d to more than 90 d, depending on

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the soil type (Potter *et al.*, 2001; Sigler and Turco, 2002). Chlorpyrifos and chlorothalonil are usually applied one after another for the control of insects and disease pests in the production of vegetables. Therefore, these two chemicals may exist together in the soil environment at a given point. Previous studies have shown that, as a nonselective and broad-spectrum fungicide, chlorothalonil has the potential to cause critical changes in soil microbial populations (Katayama *et al.*, 1991; Takagi *et al.*, 1991; Chen *et al.*, 2001; Motonaga *et al.*, 2002). Chlorothalonil, therefore, may have altered the degradation behavior of chlorpyrifos through its effect on the chlorpyrifos-degrading microbes.

The objectives of this study were to examine the influence of chlorothalonil, applied at its recommended dosage and double dosage, on the degradation of chlorpyrifos and to assess the effects of chlorpyrifos alone and in combination with chlorothalonil on soil bacterial, fungal, and actinomycetes populations.

1 Materials and methods

1.1 Chemicals

Commercial formulation of chlorpyrifos (Chlorpyrifos[®], 40% EC) and chlorothalonil (Dacotech[®], 75% WP) used in this study were obtained from Xinnong Chemical Co., Zhejiang, China, and SDS Biotech K. K., Japan, respectively. A standard sample of chlorpyrifos (99.5%) was purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture, China. All chemicals and solvents were of analytical reagent grade and the solvents were redistilled before use.

1.2 Soil

The soil was from a greenhouse on the Huajiachi Campus, Zhejiang University, Hangzhou City, China. Surface soil (0–15 cm) was collected randomly and passed through a 2-mm sieve without air drying. The physico-chemical properties of the soil were determined at the Institute of Environmental Resource and Soil Fertility, Zhejiang Academy of Agricultural Science, China. The soil was classified as loam soil and its characteristics were summarized as follows: sand 21.5%; silt 71.1%; clay 7.4%; organic matter 3.05%; water holding capacity 36.61%; total N 0.14%; cation exchange capacity 10.6 cmol/kg, and pH 6.77.

1.3 Soil treatment

After preincubation in the dark for one week at 25°C, chlorpyrifos or/and chlorothalonil were applied to soils with a predetermined volume of their commercial formulation, following proper dilution with distilled water, to give a certain level of pesticides and to obtain a soil moisture of 60% water holding capacity. According to the pre-determination of chlorpyrifos and chlorothalonil residues after applications of these two pesticides at their recommended dosages, the normal concentrations of chlorpyrifos and chlorothalonil in soils were both set to be 2 mg a.i./kg. Four treatments including control, chlorpyri-

fos alone at recommended dosage (CPYR), combination of chlorpyrifos and chlorothalonil at their recommended dosages (CPYR + CTHR), and chlorpyrifos at recommended dosage in combination with double recommended dosage of chlorothalonil (CPYR + CTHD), were used in this experiment. The control treatments received the same amount of sterilized distilled water without pesticides. Each treatment including the control was performed in triplicate. The dosed soil (1 kg dw) mixed by hand initially passed through a 2-mm sieve twice, and then transferred to 1.5-L polypropylene containers. Each container was covered with aluminum foil, with several pinholes. The treated soil was incubated in the dark, at 25°C. Soil moisture was determined and maintained by regular addition of sterilized water every two days. At fixed intervals of 2 h, 1, 3, 5, 7, 14, 21, and 28 d after treatment (DAT), aliquots of the soil sample (50 g) were collected for the determination of chlorpyrifos residues and soil microbial populations.

1.4 Determination of chlorpyrifos residues in soil

Soil sample (25.0 g) was weighed into a 250-ml Erlenmeyer flask, and 100 ml of acetone-petroleum ether mixture (1:1, V/V) was added. After shaking at 150 r/min for 2 h on a rotary shaker, the mixture was decanted and filtered through a 7-cm Buchner funnel, and the filter cake was rinsed successively, thrice, with 20 ml of acetone-petroleum ether mixture (1:1, V/V). The filtrates were collected in a 250-ml separatory funnel containing 50 ml of 3% sodium sulfate and then extracted thrice with 50, 40, and 40 ml of petroleum ether. The organic phase was collected in a 250-ml, flat-bottom flask through anhydrous sodium sulfate, and concentrated on a rotary evaporator till it was almost dry. Petroleum ether was used to redissolve the residues, the final volume was made up to 10.0 ml, and then subjected to GC-ECD analysis.

The residue of chlorpyrifos was determined with Shimadzu GC-9A gas chromatography (Shimadzu Corp., Japan) equipped with Ni⁶³ electron capture detector (ECD) and a fused silica capillary column (SE-30, Lanzhou ATECH Technologies Co. Ltd., China) (15 m in length, 0.32 mm internal diameter, and 0.33 μm film thickness). Operating conditions were as follows: injector port, 280°C; detector, 280°C; column, 240°C; carrier gas (N₂) flow rate, 100 ml/min; injection volume, 1 μl.

1.5 Recovery assay

Three replicate analyses were carried out at four different spiking levels to test the validity of the method described earlier, for extraction of chlorpyrifos from the soil. Soil samples that had not been treated with chlorpyrifos previously were spiked with 0.1, 1.0, 5.0, and 10.0 mg/kg chlorpyrifos, respectively. The extraction and analyses of chlorpyrifos was conducted as described in Section 1.4.

1.6 Microbial population enumerating

Nutrient agar medium (Society of American Bacteriologists, 1951) was used for isolation and counting of soil bacteria. The medium was composed of beef extract (3.0

g), peptone (5.0 g), agar (18.0 g) in 1000 ml distilled water at pH 7.0. For the isolation and counting of soil fungi, Czapek's agar medium consisting of NaNO_3 (2.0 g), K_2HPO_4 (1.0 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g), sucrose (30.0 g), KCl (0.5 g), and agar (18.0 g) in 1000 ml distilled water at pH 7.0 was used. The modified starch nitrate agar medium, composed of NaCl (0.5 g), KNO_3 (1.0 g), K_2HPO_4 (0.5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g), soluble starch (20.0 g), and agar (18.0 g) in 1000 ml distilled water at pH 7.0 was used for isolation and counting of actinomycetes from soil. The number of soil microorganisms was enumerated by the most-probable-number (MPN) technique (Nanjing Institute of Soil, Chinese Academy of Sciences, 1985). The inoculated agar plates (three replications) were incubated at $30 \pm 1^\circ\text{C}$ for 2 d for bacteria and fungi, and 13 d for actinomycetes, before the colonies were counted.

2 Results and discussion

2.1 Recovery assay

Figure 1 shows the chromatograms of blank and chlorpyrifos fortified soil samples. The average recoveries of chlorpyrifos fortified in soil are shown in Table 1. Recoveries of chlorpyrifos were 92.08%–100.35% with relative standard deviations of 0.82%–4.32%. All these data are generally considered to be satisfactory for chlorpyrifos residue determination.

2.2 Degradation of chlorpyrifos in soil

The degradation curves of chlorpyrifos in soil are shown in Fig. 2. Usually, the disappearance of a pesticide in soil is interpreted with the first-order kinetics. The corresponding kinetic data based on the first-order equation are listed in Table 2. Considering the fact that the determination coefficient (R^2) derived from the first-order equation is less than 0.7. The first-order kinetics is not satisfactory for the description of chlorpyrifos in soil (Beulke and Brown, 2001). In this study, the degradation of chlorpyrifos in soil shows biphasic characteristics of decreasing slowly after an initial rapid decline (Fig. 2), its degradation is subjected to the biexponential equation (Table 2, all the data have been analyzed with the SPSS 11.5 software package). This is in agreement with the previous observations of Laabs *et al.* (2000) and Fang *et al.* (2006), who reported that the dissipation of chlorpyrifos could be better described by the biexponential model.

Half-life ($T_{1/2}$) of chlorpyrifos alone in soil was calculated to be 3.26 d, which is consistent with half-lives

Table 1 Average recoveries and RSD of chlorpyrifos fortified in soil

Fortified level (mg/kg)	Sample weight (g)	Average recovery (% , mean \pm SD)*	RSD (%)
0.1	25.0	92.08 \pm 3.98	4.32
1.0	25.0	95.66 \pm 1.37	1.38
5.0	25.0	100.35 \pm 0.82	0.82
10.0	25.0	95.86 \pm 1.81	1.89

* Mean \pm standard deviation of three replications; SD: standard deviation; RSD: relative standard deviation.

reported previously (Getzin, 1981; Laabs *et al.*, 2000; Fang *et al.*, 2006). The half-lives of chlorpyrifos combined with chlorothalonil at the recommended and double dosages were 2.77 and 2.61 d, respectively (Table 2). Although chlorpyrifos degradation may be slightly enhanced by the addition of chlorothalonil, no significant effect was found. The results indicated that chlorpyrifos degradation rate was not significantly affected by chlorothalonil.

Many studies have focused on the persistence of one pesticide in soil and its impact on soil microbes after its application. However, pesticides are usually applied simultaneously or one after another and thus may interact together within the soil systems. The combination of pesticide residues may alter the behavior of a given pesticide and its effect on the soil ecological system. Singh *et al.* (2002) has reported that the degradation of chlorpyrifos

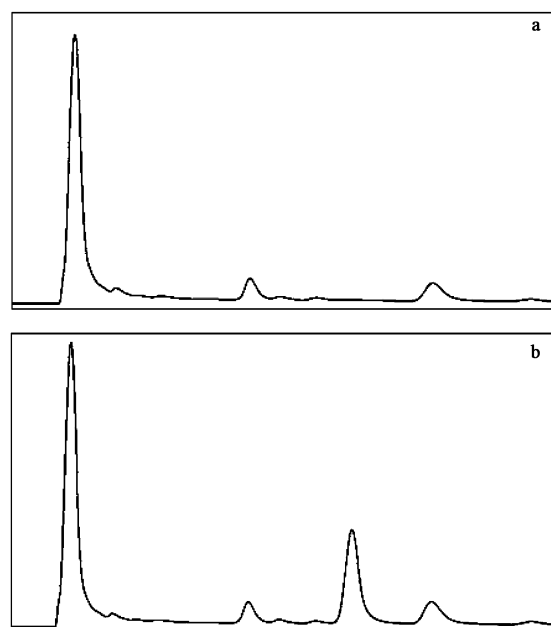


Fig. 1 Chromatograms of blank (a) and chlorpyrifos fortified (b) soil samples.

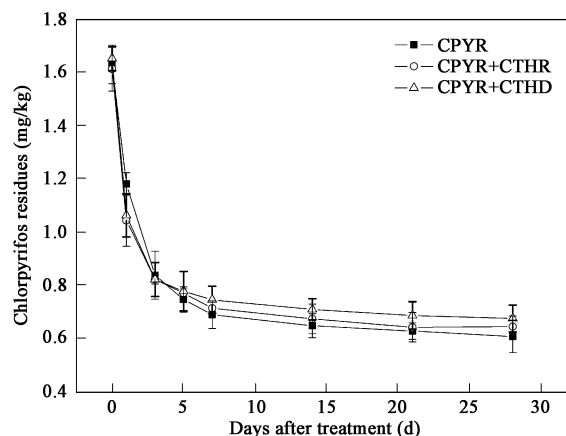


Fig. 2 Degradation of chlorpyrifos in soil treated with chlorpyrifos alone (CPYR) and in combination with recommended dosage chlorothalonil (CPYR+CTHR) and double recommended dosage of chlorothalonil (CPYR+CTHD). Values given are the means of data obtained from three replications.

Table 2 Degradation equations, determination coefficients (R^2), and derived half-lives ($T_{1/2}$) of chlorpyrifos in soil

Treatment	Equation	R^2	$T_{1/2}$ (d)
CPYR	$C_t=1.2317e^{-0.0492t}$	0.6120	
CPYR + CTHR	$C_t=1.1517e^{-0.0485t}$	0.5016	
CPYR + CTHD	$C_t=1.1543e^{-0.0414t}$	0.5052	
CPYR	$C_t=0.7196e^{-0.0063t} + 0.9080e^{-0.6511t}$	0.9995	3.26 a*
CPYR + CTHR	$C_t=0.7824e^{-0.0083t} + 0.8279e^{-1.0888t}$	0.9960	2.77 a
CPYR + CTHD	$C_t=0.7909e^{-0.0063t} + 0.8598e^{-1.1125t}$	0.9989	2.61 a

CPYR: chlorpyrifos at recommended dosage; CTHR: chlorothalonil at recommended dosage; CTHD: chlorothalonil at double dosage. *: half-lives followed by the same letter are not significantly different ($P < 0.05$).

is significantly inhibited by the addition of chlorothalonil, meanwhile soil microbial activities are inhibited by the application of chlorothalonil. It is possible that the inhibitory effect of chlorothalonil on soil microorganisms causes decreased degradation of chlorpyrifos. However, in the present study, the degradation of chlorpyrifos has not been significantly altered by its combination with chlorothalonil. This may partially be because of the lower concentration of chlorothalonil used in this study compared to that used in the study of Singh *et al.* (2002). Additionally, the effect of a pesticide on soil microorganisms depends on the properties of both the pesticide and soil (Sannino and Gianfreda, 2001; Jjemba, 2002; Singh *et al.*, 2003).

2.3 Effects of chlorpyrifos on soil microbial populations

Effects of chlorpyrifos on soil bacterial, fungal, and actinomycetes populations are shown in Table 3. Bacterial population was significantly reduced by 44.1% at 1 DAT by chlorpyrifos, compared to the control. The inhibitory

effect did not disappear until 7 DAT. At 14 DAT, the bacterial population was returned to a level similar to that of the control. The total count of the soil fungi was slightly increased just after the addition of chlorpyrifos. However, the effect became inhibitory after longer periods. The population of soil fungi was significantly ($P < 0.05$) reduced by 61.1% on day 1 as compared to that in the control (Table 3), and did not recover until 14 d. The response of soil actinomycetes population to chlorpyrifos treatment was almost similar to that of the soil fungi. It was also significantly inhibited during the period from 1 to 7 DAT by chlorpyrifos, and subsequently it was recovered to a similar level of control.

The results revealed that chlorpyrifos possessed an inhibitory effect on soil bacteria, fungi, and actinomycetes during the initial periods after its treatment. In agreement with these results, similar inhibitory effects of chlorpyrifos on bacteria were also observed in the previous studies (Tu, 1970; Pandey and Singh, 2004; Shan *et al.*, 2006).

The effect of a pesticide on soil microorganisms depends

Table 3 Effects of chlorpyrifos alone and in combination with chlorothalonil on total counts of soil bacteria, fungi, and actinomycetes

Soil micro-organisms	Pesticide treatment	Days after treatment (d)			
		0*	1	3	5
Bacteria ($\times 10^7/g$ dw)	CK	5.45 \pm 0.02 a	4.20 \pm 1.02 a	4.38 \pm 0.91 a	6.47 \pm 2.34 a
	CPYR	4.43 \pm 1.26 a	2.35 \pm 0.65 b	2.18 \pm 0.34 b	3.26 \pm 0.72 b
	CPYR + CTHR	4.63 \pm 0.94 a	1.88 \pm 0.90 bc	1.95 \pm 0.39 bc	2.20 \pm 0.23 b
	CPYR + CTHD	4.20 \pm 0.60 a	0.59 \pm 0.21 c	1.24 \pm 0.50 c	2.23 \pm 0.37 b
Fungi ($\times 10^6/g$ dw)	CK	14.35 \pm 3.7 ab	15.41 \pm 7.75 a	14.74 \pm 2.74 a	13.76 \pm 4.62 a
	CPYR	16.71 \pm 2.44 a	5.99 \pm 2.63 b	4.56 \pm 0.92 b	6.09 \pm 2.76 b
	CPYR + CTHR	9.45 \pm 3.48 bc	3.19 \pm 1.26 b	2.99 \pm 0.60 b	3.27 \pm 0.72 b
	CPYR + CTHD	7.40 \pm 3.46 c	0.91 \pm 0.13 b	2.99 \pm 0.61 b	1.17 \pm 0.27 b
Actinomycetes ($\times 10^6/g$ dw)	CK	15.96 \pm 3.86 ab	15.41 \pm 7.75 a	12.74 \pm 4.62 a	13.53 \pm 4.8 a
	CPYR	19.92 \pm 9.48 a	4.19 \pm 1.21 b	6.34 \pm 1.74 b	4.07 \pm 0.35 b
	CPYR + CTHR	15.48 \pm 5.59 ab	2.19 \pm 0.35 b	3.39 \pm 0.34 b	2.00 \pm 0.77 b
	CPYR + CTHD	6.40 \pm 2.26 b	1.42 \pm 0.52 b	2.03 \pm 0.31 b	1.34 \pm 0.44 b
		7	14	21	28
Bacteria ($\times 10^7/g$ dw)	CK	4.82 \pm 0.59 a	3.82 \pm 0.91 a	4.82 \pm 1.05 a	4.19 \pm 0.58 a
	CPYR	3.22 \pm 0.69 b	4.44 \pm 0.32 a	5.45 \pm 2.42 a	3.20 \pm 0.71 a
	CPYR + CTHR	2.22 \pm 0.35 bc	3.83 \pm 0.34 a	3.62 \pm 1.58 a	6.27 \pm 4.77 a
	CPYR + CTHD	1.57 \pm 0.42 c	5.44 \pm 1.60 a	3.19 \pm 1.25 a	2.99 \pm 1.04 a
Fungi ($\times 10^6/g$ dw)	CK	16.48 \pm 2.83 a	14.25 \pm 3.38 a	13.66 \pm 4.54 a	15.00 \pm 5.29 a
	CPYR	7.26 \pm 3.73 b	14.94 \pm 5.65 a	11.09 \pm 5.40 a	10.98 \pm 2.40 a
	CPYR + CTHR	3.63 \pm 1.21 bc	12.91 \pm 1.65 a	11.49 \pm 2.35 a	18.56 \pm 5.65 a
	CPYR + CTHD	1.49 \pm 0.56 c	11.27 \pm 2.73 a	14.89 \pm 5.13 a	12.74 \pm 4.69 a
Actinomycetes ($\times 10^6/g$ dw)	CK	11.46 \pm 2.43 a	11.24 \pm 2.42 a	13.44 \pm 6.96 a	11.38 \pm 2.48 a
	CPYR	5.03 \pm 0.38 b	12.71 \pm 4.83 a	9.08 \pm 4.17 a	9.78 \pm 1.37 a
	CPYR + CTHR	2.82 \pm 0.69 bc	10.29 \pm 3.17 a	12.92 \pm 1.34 a	14.47 \pm 3.17 a
	CPYR + CTHD	1.81 \pm 0.01 c	13.49 \pm 7.00 a	10.92 \pm 2.03 a	9.34 \pm 3.44 a

* After 2 h treatment; CK: control. All values are means \pm SD of triplicate samples, means followed by the same letter within a column are not significantly different according to LSD's Multiple Range Test ($P < 0.05$).

not only on the chemical itself, but also on the pesticide concentration, soil type, and microbial composition in tested soil. Therefore, inconsistent trends or patterns of a pesticide are often observed. In contrast to the results here, significant stimulation of soil bacteria and fungi by chlorpyrifos has also been reported (Tu, 1991; Pozo *et al.*, 1995; Pandey and Singh, 2004; Shan *et al.*, 2006).

2.4 Effects of chlorpyrifos combined with chlorothalonil on soil microbial populations

The inhibitory effect of chlorpyrifos on soil microorganisms was enhanced by its combination with chlorothalonil. Compared to the control, the populations of bacteria, fungi, and actinomycetes in the treatment of CPYR + CTHR were significantly decreased by 55.2%, 79.3%, and 85.8% at 1 DAT, respectively (Table 3). Moreover, the enhancement in the inhibitory effect was related to the level of chlorothalonil. With the increase of chlorothalonil concentration, the corresponding reduction in the microbial populations was amplified to 86.0%, 94.1%, and 90.8% at 1 DAT in the treatment of CPYR + CTHD, respectively.

The combination of chlorpyrifos and chlorothalonil may lead to a joint action against soil microorganisms and thus increase their toxicities to soil microbes. The authors' previous results indicated that soil microbial population and soil enzyme activities would be inhibited by chlorothalonil applications (Feng *et al.*, 2006; Yu *et al.*, 2006). Synergistic toxicity to microbes may be formed by the presence of chlorpyrifos and chlorothalonil together in soil, and thus induce a more toxic effect than those of individuals.

Pesticides are frequently used in agricultural fields and thus may have lasting effects on soil microbial communities and their functions, which are directly related to soil health and fertility. A profound observation in this study is that the inhibitory effect of chlorpyrifos on soil microorganisms is enhanced after its combination with chlorothalonil and the enhancement is related to the levels of chlorothalonil applied. Although more pesticide combinations in more types of soil should be conducted, it can be concluded that the combination of different pesticides may give altered effects from those obtained from just a single pesticide. This observation suggests that the combined effects should be taken into account, to assess the actual impacts of pesticide applications.

3 Conclusions

In this study, degradation of chlorpyrifos alone and in combination with chlorothalonil and their effects on soil microbial populations were investigated. Although chlorpyrifos degradation in the tested soil was not significantly altered by the addition of chlorothalonil, the inhibitory effect of chlorpyrifos on soil microorganisms was largely increased by its combination with chlorothalonil. The results indicated that the combination of pesticide residues might have a more toxic effect on soil microorganisms, which should be taken into account to estimate the real influence of pesticides.

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