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# Responses of Soil Microorganisms and Enzymes to Repeated Applications of Chlorothalonil

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Introduction of anthropogenic chemicals into soil may have lasting effects on soil microbial activities and thus soil health. This study was conducted with chlorothalonil to evaluate its effects on soil bacterial, fungal, and actinomycete populations and soil enzymes (acid phosphatase, alkaline phosphatase, urease, catalase, and invertase) after repeated applications. After the first addition of chlorothalonil, the soil bacterial and actinomycete populations were significantly reduced, whereas the population of soil fungi was unchanged. The most marked inhibition on soil microorganisms was observed after the second pesticide addition. However, after initial variations, soil bacteria, fungi, and actinomycetes adapted gradually to chlorothalonil, and the negative effects became transient and weaker following the third and fourth treatments. The inhibitory effect of repeated chlorothalonil applications on soil enzymes followed a similar trend to that on soil microorganisms. Repeated chlorothalonil applications did not result in significant changes in its persistence. Three bacterial strains capable of utilizing chlorothalonil as a sole source of carbon and energy for growth were isolated 21 days after the fourth treatment with chlorothalonil, which indicated that the capability of soil microorganisms for degrading chlorothalonil was formed during the experiment.

**KEYWORDS: Chlorothalonil; soil; microorganism; enzyme; degradation**

## INTRODUCTION

The maintenance of soil quality, fertility, and structure is essential for the protection of the biodiversity and integrity of terrestrial ecosystems (*1*). Natural and artificial factors may affect directly or indirectly microbial activities, the populations of soil microorganisms, and soil enzyme activities. As agents for the control of plant pathogens, fungicides are frequently introduced into agricultural systems and the soil environment. Like other pesticides, fungicides may not only interfere with the biochemical and physiological reactions of the target plant pathogens but also influence populations or activities of other nontarget microorganisms in soil. Although many studies have reported on the effects of pesticides on soil microorganisms and soil enzymes (*2*−*5*), inconsistent trends or patterns are often found (*4*, *6*).

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a widely used fungicide for the control of foliar pathogens in agricultural systems, especially in the greenhouse production of vegetables. The residual behavior of chlorothalonil and its effects on soil microorganisms after a single application have been previously investigated. The half-life of chlorothalonil in soil varies from 5 to 90 days, depending on the soil type (*7*−*9*). Chlorothalonil residues have been shown to potentially influence soil bacterial and fungal populations (*9*) and the total number of soil microorganisms and activities of soil enzymes (*10*, *11*). Use of chlorothalonil often involves multiple applications in the same growing season or repeated treatments on an annual basis (*12*). Previous studies show that the rate of pesticide degradation is often altered after repeated applications due to stimulating or inhibitory effects brought upon by the pesticide on soil microorganisms and biochemical reactions (*10*, *13*). Although the effects of repeated chlorothalonil applications at a rate of 10 mg/kg on its degradation and total soil microorganisms were investigated (*10*, *11*), so far, little information is available on the behavior of chlorothalonil in soil following repeated field applications and the potential influences of such treatments on soil microorganisms and enzymes.

The objectives of this study were to determine the behavior of chlorothalonil in soil and to evaluate the responses of soil microorganisms and enzymes to repeated applications. This information will be useful for predicting the environmental fate of this widely used fungicide from continuous use and for understanding the potential adverse effects of intensive treatments with chlorothalonil on general soil microbial and biochemical properties.

## MATERIALS AND METHODS

**Chemicals and Soil.** Chlorothalonil (98.20%) was purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China. Commercial formulation of chlorothalonil (75% active ingredient, SDS Biotech K. K, Tokyo, Japan) was used for soil treatment. Other reagents were all of analytical grade.

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Surface soil (0−20 cm) was freshly collected from a vegetable field on the Agricultural Farm of Zhejiang University, Hangzhou, China and contained no detectable amount of chlorothalonil residues. The field for the collection of soil was used for an investigation about behaviors of chlorpyrifos on vegetables in greenhouses from 2000 to 2005. It was only treated with organophosphorus insecticide chlorpyrifos and with no history of chlorothalonil. The collected soil was air-dried, passed through a 2 mm sieve, and then stored at room temperature for 48 h before use. The physicochemical properties of the soil were determined at the Institute of Environmental Resource and Soil Fertilizers, Zhejiang Academy of Agricultural Sciences, Hangzhou, China, by using standard methods (*14*, *15*). 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 (WHC), 39.4%; cationic exchange capacity (CEC), 10.6 cmol/kg; total nitrogen, 0.14%; and pH, 6.8.

**Soil Treatment.** To determine the initial concentration of chlorothalonil to be treated in the soil, chlorothalonil was once applied using field samples at the rate of 4000 g/ha with a dose of 3 kg of active ingredient per hectare, as the recommended dosage on the label for the control of vegetables. Chlorothalonil residues in soil were analyzed 2 h after the treatment. The mean value of chlorothalonil residues in a pakchoi (*Brassica Chinensis* L.) and a tomato (*Lycopersicum esculentum* Mill.) vegetated soils was found to be 1.5 mg/kg. This value was used for chlorothalonil spiking in this study. About 1.5 kg of the air-dried soil was placed in a 2 L flower pot and treated with a predetermined volume of the commercial chlorothalonil formulation following proper dilution with distilled water. The final soil water content was adjusted to 60% of its water holding capacity, and the containers were covered with aluminum foil. Controls without chlorothalonil treatment were similarly prepared. Three replicates were prepared for the control and pesticide treatments. The soils were all incubated at 25 ± 1 °C in the dark. Chlorothalonil was applied again with the same dose 21, 42, and 63 days after the first treatment, respectively. At appropriate intervals following chlorothalonil treatment, soil samples were removed for the measurement of microbial populations, enzyme activities, and residual chlorothalonil concentrations.

**Measurement of Soil Microbial Populations.** Nutrient agar medium (*16*) was used for the isolation and counting of soil bacteria. The medium was composed of beef extract (3 g), peptone (5 g), agar (18 g), and prepared in 1000 mL of 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), $MgSO_4{\cdot}7H_2O$ (0.5 g), $FeSO_4{\cdot}7H_2O$ (0.01 g), sucrose (30.0 g), KCl (0.5 g), and agar (18.0 g) in 100 mL of distilled water at natural pH was used. The modified starch nitrate agar medium, composed of NaCl (0.5 g), $KNO_3$ (1 g), $K_2HPO_4$ (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), $FeSO_4{\cdot}7H_2O$ (0.01 g), soluble starch (20.0 g), and agar (18.0 g) in 1000 mL of distilled water at pH 7.0 was used for the isolation and counting of actinomycetes from soil. The number of soil microorganisms was enumerated by the most-probable-number technique (*17*). The inoculated agar plates (three replications) were incubated at 30 ± 1 °C for 2 days for bacteria and fungi and 13 days for actinomycetes before the colonies were counted.

**Measurement of Soil Enzyme Activities.** Activities of urease, acid, and alkaline phosphatase were assayed with the methods described by Tabatabai et al. (*18*). Activities of invertase and catalase were assayed according to the procedures of Guan (*19*). Briefly, to measure urease activity, fresh soil (5.0 g) was mixed with 5 mL of tri(hydroxymethyl) aminomethane buffer solution, 0.2 mL of toluene, and 1 mL of urea (0.2 M) in a 50 mL flask. After incubation at 37 °C for 2 h, 35 mL of KCl-$Ag_2SO_4$ was added to the mixture, and the volume was increased to 50 mL. The concentration of $NH_4$-N was determined with titration by $H_2SO_4$ (0.005 M) after distillation of the mixture with 0.2 g of MgO.

For the measurement of acid and alkaline phosphatases activities, 1.0 g of fresh soil was mixed with 0.2 mL of toluene, 4 mL of modified universal buffers (MUB) of pH 6.5 and 11.0 respectively, and 1.0 mL of *p*-nitrophenyl phosphate (0.025 M) and incubated for 1 h at 37 °C. After incubation, the mixture was mixed well with 1.0 mL of $CaCl_2$ (0.5 M) and 4.0 mL of NaOH (0.5 M) and then filtered through a filter paper. The concentration of *p*-nitrophenyl (PNP) in the filtrate was determined colorimetrically at 402 nm.

The invertase activity in soils was determined by salicylic acid colorimetry. A 5.0 g aliquot of air-dried soil was mixed with 15 mL of 8% sucrose solution, 5 mL of phosphate buffer (pH 5.5), and 0.1 mL of toluene. After incubation for 24 h at 37 °C, the mixture was centrifuged (3000*g*, 5 min). One milliliter of the supernatant fraction was mixed with 3.0 mL of 3,5-dinitrosalicylic acid, and then the production was measured colorimetrically at 508 nm.

The catalase activity in soils was measured by the $KMnO_4$ titrimetric method (*19*). A 2.0 g aliquot of air-dried soil was mixed with 40 mL of distilled water and 5.0 mL of 0.3% $H_2O_2$ solution and shaken for 20 min on a rotary mixer at 150 rpm. The suspension was treated with 5 mL of 1.5 M $H_2SO_4$ and then filtered. The residual $H_2O_2$ was determined by titration with $KMnO_4$ in the presence of $H_2SO_4$.

**Determination of Chlorothalonil Residues in Soil.** For the extraction of chlorothalonil residues from soil, 10 g of soil was taken from the microcosms and transferred to a 250 mL Erlenmeyer flask, followed by the addition of 100 mL of acetone. The soil-solvent mixture was shaken for 2 h at 200 rpm on a mechanical shaker, and the mixture was filtered through a 7 cm Buchner funnel. The filter cake was washed twice with 40 mL of acetone. The filtrates were collected in a 250-mL separatory funnel containing 50 mL of 2% sodium sulfate and then extracted three times with 40, 30, and 30 mL of petroleum ether (60−90 °C). All extracts were then passed through a layer of anhydrous sodium sulfate and collected in a flat-bottomed flask. The sample extract was concentrated to about 2 mL on a rotary evaporator, and a 1 $\mu$L aliquot was injected for GC analysis.

A Shimadzu GC-9A (Shimadzu corporation, Japan) equipped with an electron capture detector (ECD) was used for the GC analysis. A HP-5 glass capillary column (length 25 m, internal diameter 0.53 mm, and film thickness 5.0 $\mu$m, Agilent Technologies) was used for the separation. The operating temperatures were as follows: injection port, 250 °C; column, 210 °C; and detector, 250 °C. Nitrogen of 40 mL/min was used as the carrier gas.

**Isolation of Chlorothalonil-Degrading Strains.** Soil samples before chlorothalonil treatment and after the fourth treatment of chlorothalonil were diluted with 100 mL of sterilized water in a 250 mL Erlenmeyer flask, respectively. A 1 mL aliquot of soil supernatants was used as an inoculant into a 100 mL Erlenmeyer flask containing 20 mL of sterile mineral salts medium ($MgSO_4{\cdot}7H_2O$, 0.40 g; $FeSO_4{\cdot}7H_2O$, 0.002 g; $K_2HPO_4$, 0.20 g; $(NH_4)_2SO_4$, 0.20 g; $CaSO_4$, 0.08 g; $H_2O$, 1000 mL; and pH, 7.2) supplemented with 50 mg/L of chlorothalonil as the sole source of carbon and energy. After incubation for 1 week at 30 °C on a rotary shaker (150 rpm) and if it turned turbid, the culture was inoculated into 20 mL of sterile mineral salts medium with chlorothalonil (100 mg/L) and incubated for another 1 week under the same conditions. The cultures were repetitively acclimated in sterile mineral salt medium with increasing concentrations of chlorothalonil, ranging from 100 to 200 mg/L. Subsequently, the cultures were cultivated onto Luria−Bertani (LB) plates (yeast extract, 5.0 g; peptone, 10.0 g; NaCl, 5.0 g; distilled water, 1000 mL; and pH, 7.0) and incubated for 3 days at 30 °C. Chlorothalonil-degrading strains were isolated from colonies formed on the plates.

**Recoveries Assay.** For the study on extraction efficiency of the methods established, recovery analyses were conducted in the mineral salts medium (20 mL) and soil (10 g) at spiking levels of 0.1, 1.0, and 10.0 mg/kg of chlorothalonil. Extraction and analysis were performed in triplicate as described previously.

## RESULTS AND DISCUSSION

**Recovery Evaluation.** Average recoveries of chlorothalonil from the mineral salts medium at levels of 0.1, 1.0, and 10.0 mg/L were measured to be 94.3 ± 4.9, 104.6 ± 4.5, and 99.6 ± 3.7%; the corresponding recoveries from soil at levels of 0.10, 1.0, and 10.0 mg/kg were determined to be 88.6 ± 2.7, 94.5 ± 4.9, and 108.5 ± 9.2%, respectively. The limits of detection and quantitation for chlorothalonil were 0.001 and 0.01 mg/kg of dry soil, respectively. These data indicated that the extraction procedure is efficient in extracting chlorothalonil residues from mineral salts medium and soil.

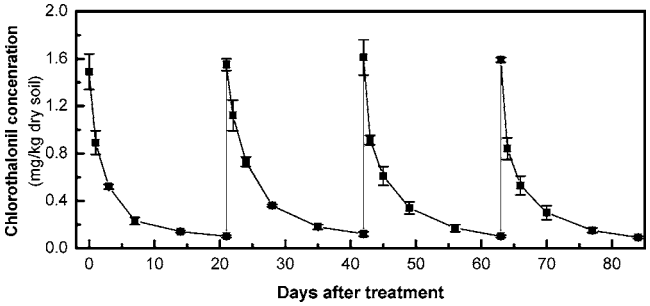


**Figure 1.** Degradation of chlorothalonil in soil after its repeated applications.

**Degradation of Chlorothalonil in Soil after Repeated Applications. Figure 1** shows the disappearance of chlorothalonil from the test soil. The dissipation kinetics was fitted to a first-order decay model by linear regression analysis of the logarithmic transformed concentrations against incubation time. The half-life of chlorothalonil for the first treatment was estimated to be 5.7 ± 0.33 days. Following subsequent applications of chlorothalonil, the degradation rate of chlorothalonil remained essentially unchanged, with the estimated first-order half-lives for the second, third, and fourth treatments as 5.8, 5.6, and 5.5 days, respectively.

It is well-known that enhanced degradation of a pesticide may occur after its repeated applications (*13*, *20*−*29*). In this study, degradation of chlorothalonil in the soil was largely unaffected, which was similar to that observed for metolachlor after repeated applications (*13*). This observation was contradictory to Singh et al. (*10*, *11*), who found that degradation of chlorothalonil was inhibited by its repeated applications, with its half-life extended from 8.6 to 21.5 days in one study (*10*) and from 12.3 to 20.9 days in another study (*11*).

To explore the possible factors contributing to the difference between our results and previous studies, the isolation of indigenous microorganisms capable of degrading chlorothalonil was conducted before and after the experiment. No microbial strain was isolated from the tested soil before chlorothalonil application. However, three bacterial strains utilizing chlorothalonil as the sole carbon and energy sources were isolated from the soil 21 days after the fourth treatment with chlorothalonil, and one bacterial strain (Y1) was a rod-shaped bacterium and further used in a biodegradation experiment. The capability of

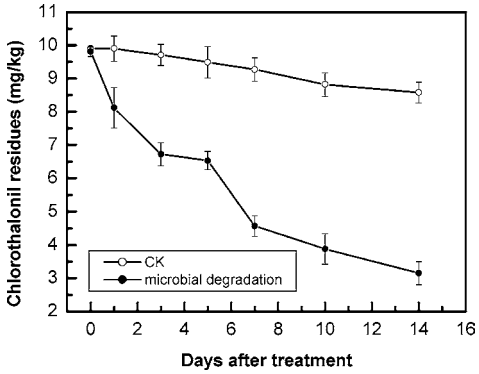


**Figure 2.** Degradation of chlorothalonil at a concentration of 10 mg/kg by a isolated strain Y1 in pure culture medium in the dark under conditions of pH 7.0, 30 °C with a rotary shaking of 150 rpm after inoculation ($OD_{610\ nm}$ = 0.20).

Y1 for degrading chlorothalonil was demonstrated in pure culture (**Figure 2**). While only 13.4% of the added chlorothalonil was lost in the control treatment, about 67.9% of the spiked chemical was degraded in the mineral salts medium after 14 days of incubation at 30 ± 1 °C and pH 7.0 in the dark. These results indicated that some indigenous microorganisms became adapted to chlorothalonil after its repeated applications, even though statistically significant acceleration was not observed in chlorothalonil degradation.

**Responses of Microbial Populations to Repeated Chlorothalonil Additions.** The responses of soil microbes to the first and subsequent additions of chlorothalonil were investigated by monitoring the population sizes of soil bacteria, fungi, and actinomycetes (**Table 1**). Most of soil microorganisms are not culturable by the methods used in this study. However, these methods are classical approaches for the counting of soil microorganisms and used by many people to indicate potential effects of pesticides (*2*).

After the first addition of chlorothalonil, the populations of soil bacteria and actinomycetes were significantly ($P \leq 0.05$) reduced by 37.4 and 59.4% on day 1 when compared to the untreated control (**Table 1**). The population of bacteria in the chlorothalonil treated soil remained significantly smaller than that in the control soil 7 days after the treatment, and the population of soil actinomycetes was substantially inhibited 14 days after the treatment. Thereafter, the populations of both

**Table 1.** Effects of Repeated Chlorothalonil Applications on Soil Microorganism Populations[a]

| chlorothalonil addition | days after treatment | bacteria ($10^7$/g of dry soil) | | fungi ($10^5$/g of dry soil) | | actinomycetes ($10^5$/g of dry soil) | |
|---|---|---|---|---|---|---|---|
| | | 0 mg/kg | 1.5 mg/kg | 0 mg/kg | 1.5 mg/kg | 0 mg/kg | 1.5 mg/kg |
| first addition at day 0 | 1 | 23.8 ± 5.9a | 14.9 ± 5.3b | 7.7 ± 2.0a | 5.2 ± 0.4a | 43.5 ± 6.9a | 17.7 ± 6.4b |
| | 7 | 26.0 ± 3.5a | 15.8 ± 3.8b | 7.6 ± 1.9a | 9.8 ± 1.3a | 40.0 ± 7.2a | 20.0 ± 3.7b |
| | 14 | 26.9 ± 7.7a | 20.4 ± 3.8a | 8.1 ± 2.3a | 9.5 ± 3.4a | 41.1 ± 7.8a | 26.4 ± 7.1b |
| | 21 | 24.4 ± 6.6a | 30.1 ± 0.3a | 7.4 ± 3.3a | 8.6 ± 3.1a | 40.1 ± 6.0a | 40.1 ± 6.5a |
| second addition at day 21 | 22 | 23.2 ± 6.1a | 9.1 ± 3.6b | 7.5 ± 2.0a | 4.5 ± 1.5a | 40.4 ± 9.5a | 18.4 ± 5.3b |
| | 28 | 22.5 ± 7.4a | 13.0 ± 4.7b | 7.5 ± 3.5a | 4.7 ± 1.4a | 40.8 ± 6.7a | 14.9 ± 5.0b |
| | 35 | 21.4 ± 6.2a | 20.6 ± 3.2a | 8.1 ± 2.2a | 8.9 ± 3.0a | 39.4 ± 9.9a | 31.1 ± 6.7a |
| | 42 | 20.9 ± 4.1a | 21.0 ± 3.7a | 8.1 ± 2.0a | 9.0 ± 3.2a | 41.9 ± 8.2a | 42.0 ± 7.5a |
| third addition at day 42 | 43 | 21.3 ± 8.9a | 16.0 ± 3.8a | 7.6 ± 3.5a | 7.9 ± 2.1a | 41.3 ± 7.6a | 30.3 ± 5.9a |
| | 49 | 20.5 ± 3.4a | 18.0 ± 0.2a | 7.6 ± 3.6a | 9.4 ± 3.5a | 41.1 ± 7.4a | 42.0 ± 10.0a |
| | 56 | 20.5 ± 3.5a | 20.6 ± 3.6a | 8.0 ± 2.2a | 10.1 ± 1.4a | 41.0 ± 6.9a | 39.2 ± 3.6a |
| | 63 | 22.7 ± 7.1a | 22.8 ± 7.2a | 8.0 ± 2.1a | 7.5 ± 3.8a | 39.2 ± 9.2a | 35.2 ± 3.5a |
| fourth addition at day 63 | 64 | 22.8 ± 3.8a | 20.9 ± 3.7a | 8.1 ± 2.2a | 6.7 ± 2.4a | 41.5 ± 7.6a | 33.5 ± 7.5a |
| | 70 | 22.4 ± 9.9a | 20.7 ± 7.9a | 7.8 ± 2.5a | 8.2 ± 2.3a | 41.0 ± 7.3a | 47.9 ± 9.0a |
| | 77 | 22.5 ± 7.1a | 22.7 ± 3.6a | 7.8 ± 2.6a | 13.2 ± 4.8b | 42.8 ± 9.8a | 43.2 ± 10.4a |
| | 84 | 20.2 ± 3.8a | 22.3 ± 3.4a | 7.9 ± 2.2a | 7.5 ± 3.6a | 44.3 ± 6.9a | 44.6 ± 9.6a |

[a] All values represent mean ± SD of triplicate samples. Values in horizontal rows followed by the same letter are not statistically different ($P < 0.05$) as compared to the controls.

bacteria and actinomycetes recovered gradually and reached similar levels to the controls after 21 days of incubation. In comparison, soil fungi seemed to be more resistant to chlorothalonil treatment. The average population of soil fungi decreased by 29.9% on day 1, but the decrease was not statistically significant at $\alpha = 0.05$. The overall population of fungi even increased and exceeded the level found in the control soil after day 1. This observation was in agreement with previous studies, in which the treatment of chlorothalonil also had an insignificant effect on soil fungi or even the stimulated soil fungal population (*30*, *31*).

The effect of a pesticide on soil microorganisms depends on the properties of both the pesticide and soil, as well as the concentration of the pesticide (*4*, *6*). Sigler and Turco (*9*) found that both enhancement and inhibition could occur in soil microbial community after a single chlorothalonil application. A persistent inhibition on soil microbes was observed after chlorothalonil application at a high rate of 10 mg/kg (*10*). In this study, the concentration of chlorothalonil used (1.5 mg/kg) was substantially lower than that in Singh et al. (*10*), which may contribute to the quick recovery of the soil microbial communities within 21 days, along with the disappearance of chlorothalonil from the soil.

The inhibitory effect of chlorothalonil on soil bacteria, fungi, and actinomycetes caused by the second treatment became more pronounced when compared to the first treatment (**Table 1**). The populations of soil bacteria, fungi, and actinomycetes were decreased by 60.8, 40.0, and 54.5% 1 day after the treatment when compared to the untreated control, and the decreases in populations of bacteria and actinomycetes were statistically significant at $\alpha = 0.05$. After 14 days of incubation following the second pesticide treatment, the populations of soil bacteria and fungi were recovered to levels similar to those in the control soils. However, the population of actinomycetes remained at a relatively low level throughout the 14 day incubation and was reduced by 63.5% on day 7 and recovered to the control level on day 21 (**Table 1**).

The adverse effects of the third and fourth chlorothalonil applications on soil microorganisms were much smaller when compared to the first two treatments. Following the third application, the populations of bacteria and actinomycetes were 24.9 and 26.6% smaller than those in the untreated controls on day 1 after the pesticide treatment. The populations rapidly recovered to the levels of the control soil on day 7 after the third treatment. As compared to the control treatment, the populations of soil fungi were consistently larger throughout the 21 day incubation period after the third chlorothalonil treatment. Essentially, no significant negative effect of the fourth addition of chlorothalonil was observed for soil microorganisms. The populations of soil bacteria, fungi, and actinomycetes quickly recovered to or exceeded the levels in the control. The reductions observed for bacteria (8.3%), fungi (17.3%), and actinomycetes (19.3%) populations on day 1 after the fourth treatment were transient and became undetectable thereafter.

With repeated applications of a pesticide, soil microorganisms may become adapted to the chemical because of selective pressures (*32*, *33*). As evidence to the potential adaptation, several bacterial strains capable of utilizing chlorothalonil as the sole carbon and energy sources were isolated from the tested soil following repeated pesticide treatments. The adaptation may have contributed to the observation that the third and fourth additions of chlorothalonil did not significantly decrease the overall populations of the different microbial communities. In agreement with our results, enhanced herbicide degradation in soil with repeated applications was observed (*34*). Tworkoski et al. (*35*) reported accelerated degradation of diuron, simazine, and terbacil in soil after their annual applications from 1981 to 1995 under field conditions. In this study, although apparent microbial adaptation occurred in the treated soil, the adaptation did not lead to a quantifiable enhancement in the degradation of chlorothalonil. However, it is likely that enhanced degradation for chlorothalonil may become detectable after further applications or at higher pesticide concentrations.

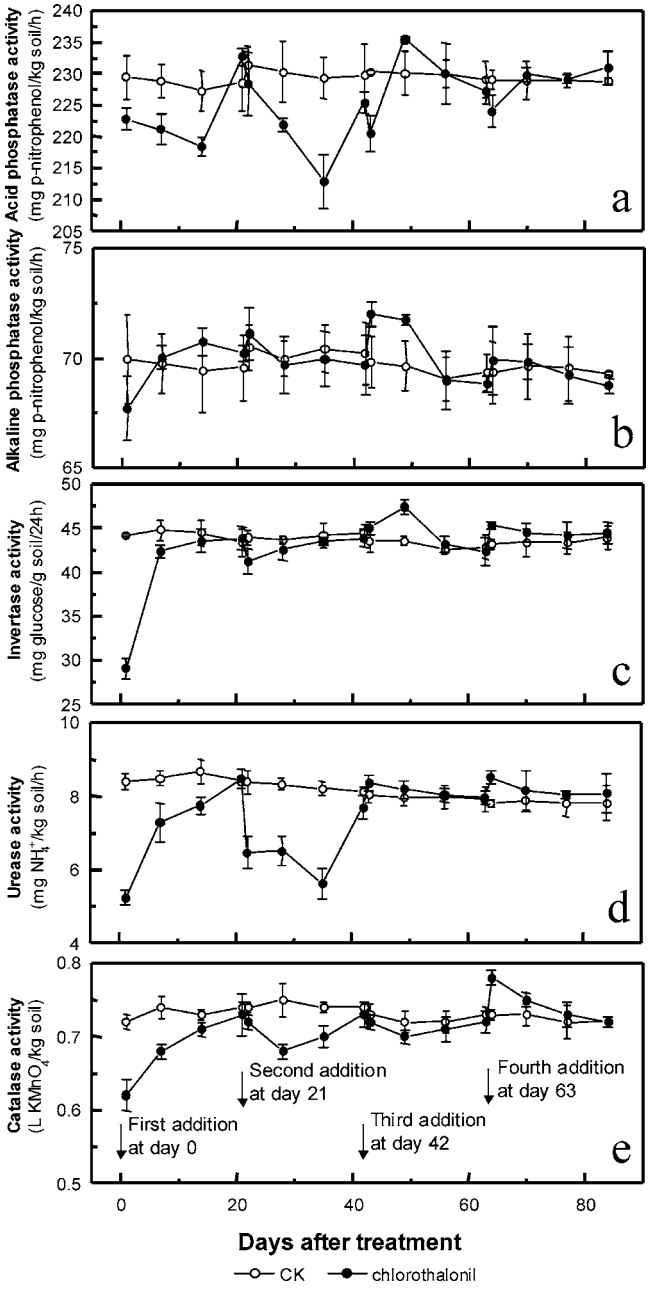


**Figure 3.** Effects of repeated chlorothalonil applications on soil enzymes.

**Responses of Soil Enzymes to Repeated Additions of Chlorothalonil.** The effects of chlorothalonil treatment on the activity of various soil enzymes are shown in **Figure 3**. Following the first addition of chlorothalonil, the activity of urease, catalase, and invertase was significantly ($P = 0.05$) inhibited by 37.7, 13.9, and 34.2%, respectively, on day 1, and the activity did not recover to the levels of the control until day 21. However, no significant inhibitory effect was observed for the activity of acid and alkaline phosphatases. In contrast to our findings, a persistent negative effect of chlorothalonil

on soil phosphatase was observed by Singh et al. (*10*, *11*) following chlorothalonil treatment at a much higher concentration (10 mg/kg). The second pesticide treatment had no significant negative effect on the activity of alkaline phosphatase and invertase but caused significant inhibitions to the activity of acid phoshpatase, urease, and catalase. The most pronounced inhibitions of 7.2, 31.5, and 9.3% for acid phoshpatase, urease, and catalase were observed on day 14, 14, and 7 after the second addition, respectively. Afterward, the activity of these three enzymes recovered to the similar levels of untreated soils on day 21. However, inhibitory effects, if any, after the third and fourth additions of chlorothalonil were negligible, and the enzyme activity was even stimulated as compared to the control for some enzyme groups, including acid phosphatase, alkaline phosphatase, urease, and catalase after the third addition and alkaline phosphatase, urease, catalase, and invertase after the fourth addition.

The inhibition and stimulation patterns of chlorothalonil on the selected soil enzyme groups generally coincided with those on soil microbial populations. Overall, significant inhibition of enzyme activity only occurred after the first or second pesticide treatment, and when detectable, only to certain enzyme groups, most noticeably urease, catalase, and acid phosphatase. Even when significant suppression occurred, the level of enzyme activity was consistently restored to the initial level within 21 days. The recovery of soil enzyme activities may be partially attributed to the fact that as chlorothalonil was degraded in soil, the adverse effects of chlorothalonil on soil microorganisms and biochemical reactivity (e.g., effects on microbial protein synthesis systems) gradually disappeared (*36*). The recovery of enzyme activities may be also a result of microbial adaptation to the anthropogenic chemical.

In conclusion, results from this study indicated that soil microorganisms and enzymes were only affected by the first one or two applications of chlorothalonil at a field relevant application rate. Additional applications of chlorothalonil at the same rate did not inhibit the overall population densities of soil microorganisms or soil enzymatic functions. However, as an important agent for the control of plant pathogens, chlorothalonil is often used at rates much greater than the recommended dosage. Further studies with higher dosages of chlorothalonil and in different soil types under field conditions are needed.

## LITERATURE CITED


(1) Burrows, L. A.; Edwards, C. A. The use of integrated soil microcosms to predict effects of pesticides on soil ecosystems. *Eur. J. Soil Biol.* **2002**, *38*, 245−249.

(2) Omar, S. A.; Abdel-Sater, M. A. Microbial populations and enzyme activities in soil treated with pesticides. *Water, Air, Soil Pollut.* **2000**, *127*, 49−63.

(3) Ibekwe, A. M.; Kapiernik, S. K.; Gan, J.; Yates, S.; Yang, C. H.; Crowley, D. E. Impact of fumigants on soil microbial communities. *Appl. Environ. Microbiol.* **2001**, *67*, 3245−3257.

(4) Sannino, F.; Gianfreda, L. Pesticide influence on soil enzymatic activities. *Chemosphere* **2001**, *45*, 417−325.

(5) Singh, J.; Singh, D. K. Dehydrogenase and phosphomonoesterase activities in groundnut (*Arachis hypogaea* L.) field after diazinon, imidacloprid, and lindane treatments. *Chemosphere* **2005**, *60*, 32−42.

(6) Jjemba, P. K. The effect of chloroquine, quinacrine, and metronidazole on both soybean plants and soil microbiota. *Chemosphere* **2002**, *46*, 1019−1025.

(7) Jury, W. A.; Focht, D. D.; Farmer, W. J. Evaluation of pesticide groundwater pollution potential from standard indices of soil-chemical adsorption and biodegradation. *J. Environ. Qual.* **1987**, *16*, 422−428.

(8) Nelson, E. B. Maximizing disease control with fungicide applications: the basics of turfgrass fungicides, part two: behavior in soil. *Turfgrass Trends.* **1996**, *5*, 5−10.

(9) Sigler, W. V.; Turco, R. F. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. *Appl. Soil Ecol.* **2002**, *21*, 107−118.

(10) Singh, B. K.; Walker, A.; Wright, D. J. Persistence of chlorpyrifos, fenamiphos, chlorothalonil, and pendimethalin in soil and their effects on soil microbial characteristics. *Bull. Environ. Contam. Toxicol.* **2002**, *69*, 181−188.

(11) Singh, B. K.; Walker, A.; Wright, D. J. Degradation of chlorpyrifos, fenamiphos, and chlorothalonil alone and in combination and their effects on soil microbial activity. *Environ. Toxicol. Chem.* **2002**, *21*, 2600−2605.

(12) Kwon, J. W.; Armbrust, K. L. Degradation of chlorothalonil in irradiated water/sediment systems. *J. Agric. Food Chem.* **2006**, *54*, 3651−3657.

(13) Sanyal, D.; Kulshrestha, G. Effects of repeated metolachlor applications on its persistence in field soil and degradation kinetics in mixed microbial cultures. *Biol. Fertil. Soils* **1999**, *30*, 124−131.

(14) Liu, G. S., Ed. *Physical and chemical analysis of soils and profile description* (in Chinese); China Standard Publishing House: Beijing, 1996; pp 266.

(15) Institute of Soil Science, Academia Sinica. *Soil physical and chemical analysis* (in Chinese); Shanghai Science and Technology Press: Shanghai, 1979; pp 593.

(16) Society of American Bacteriologists. *Manual of methods for pure culture of bacteria*; McGraw-Hill: New York, 1951.

(17) Nanjing Institute of Soil, Chinese Academy of Science. *Soil microorganism research methods*; Science Press: Beijing, 1985.

(18) Tabatabai, M. A. Soil enzymes. In *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*; Weewer, R. M., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., Wollum, A., Eds.; Soil Science Society of America: Madison WI, 1994; pp 775−833.

(19) Guan, S. Y.; Zhang, D. S.; Zhang, Z. M., Eds. *Soil enzymes and its methodology*, 1st ed.; Agricultural Press: Beijing, 1986; pp 274−338.

(20) Spain, J. C.; Van Veld, P. A. Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effects of concentration, exposure time, inoculum, and chemical structure. *Appl. Environ. Microbiol.* **1983**, *45*, 428−435.

(21) Kearney, P. C.; Kellog, S. T. Microbial adaptation to pesticides. *Pure Appl. Chem.* **1985**, *571*, 389−403.

(22) Liu, S. Y.; Zhang, R.; Bollag, J. M. Biodegradation of metolachlor in a soil perfusion experiment. *Biol. Fertil. Soils* **1988**, *5*, 276−281.

(23) Samuel, T.; Pillai, M. K. K. Impact of repeated application on the binding and persistence of [$^{14}$C]DDT and [$^{14}$C]HCH in a tropical soil. *Environ. Pollut.* **1991**, *74*, 205−216.

(24) Bromilon, R. H.; Evans, A. A.; Nicholls, P. H.; Todd, A. D.; Briggs, G. G. The effect of soil fertility on repeated applications of pesticides over 20 years. *Pestic. Sci.* **1996**, *48*, 63−72.

(25) Mitchell, J. A.; Cain, R. B. Rapid onset of the accelerated degradation of dicarboximide fungicides in UK soil with a long history of agrochemical exclusion. *Pestic. Sci.* **1996**, *48*, 1−11.

(26) Walker, A.; Welch, S. J.; Roberts, S. J. Induction and transfer of enhanced biodegradation of the herbicide napropamide in soils. *Pestic. Sci.* **1996**, *47*, 131−135.

(27) Arthur, E. L.; Anhalt, J. C.; Anderson, T. A.; Coats, J. R. Enhanced degradation of deethylatrazine in an atrazine soil history of Iowa. *J. Environ. Sci. Health* **1997**, *32*, 599−620.

(28) Mohapatra, S.; Awasthi, M. D. Enhancement of carbofuran degradation by soil enrichment cultures, bacterial cultures, and synergistic interaction among bacterial cultures. *Pestic. Sci.* **1997**, *49*, 164−168.

(29) Bharati, K.; Padhy, S.; Adhya, T. K. Accelerated biodegradation of $\gamma$-hexachlorocyclohexane ($\gamma$-HCH) in a flooded alluvial soil retreated with $\gamma$-HCH or its metabolite 1,2,4-trichlorobenzene (TCB). *Bull. Environ. Contam. Toxicol.* **1998**, *60*, 858−863.

(30) Tu, C. M. Effect of some technical and formulated insecticides on microbial activities in soil. *J. Environ. Sci. Health* **1991**, *26*, 557−573.

(31) Pandey, S.; Singh, D. K. Total bacterial and fungal population after chlorpyrifos and quinalphos treatments in groundnut (*Arachis hypogaea* L.) soil. *Chemosphere* **2004**, *55*, 197−205.

(32) Holden, P. A.; Firestone, M. K. Soil Microorganisms in soil cleanup: how can we improve our understanding? *Environ. Qual.* **1997**, *26*, 32−40.

(33) InSeon, K.; Rodong, P.; YongTack, S. Involvement of adaptive mechanism and an energy-dependent efflux system of *Pseudomonas* sp. LE2 counteracting insecticide lindane. *Pest. Biochem. Physiol.* **2002**, *73*, 140−148.

(34) Roeth, F. W. Enhanced herbicide degradation in soil with repeat application. *Weed Sci.* **1986**, *2*, 45−65.

(35) Tworkoski, T. J.; Welker, W. V.; Vass, G. D. Soil residues following repeat applications of diuron, simazine, and terbacil. *Weed Technol.* **2000**, *14*, 191−196.

(36) Omar, S. A.; Moharram, A. M.; Abd-Alla, M. H. Effects of an organophosphorus insecticide on the growth and cellulolytic activity of fungi. *Int. Biodeterior. Biodegrad.* **1993**, *31*, 305−310.

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