DEGRADATION OF CHLORPYRIFOS, FENAMIPHOS, AND CHLOROTHALONIL ALONE AND IN COMBINATION AND THEIR EFFECTS ON SOIL MICROBIAL ACTIVITY

Brajesh Kumar Singh,†‡ Allan Walker,*† and Denis J. Wright‡
†Soil and Environmental Sciences Department, Horticulture Research International, Wellesbourne, Warwick CV35 9EF, United Kingdom
‡Department of Biological Science, Imperial College at Silwood Park, Ascot, Berks SL5 7PY, United Kingdom

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Abstract—The effects of repeated application and of combinations of pesticides on their degradation rates in soil and on some soil microbial properties were studied. Repeated application of chlorpyrifos did not modify its degradation rate, whereas repeated applications of fenamiphos and chlorothalonil suppressed their own rates of degradation. When applied in combination, the presence of chlorothalonil reduced the degradation rate of both chlorpyrifos and fenamiphos, and the half-life of chlorothalonil was extended in the presence of chlorpyrifos. The dynamics of residues of the major metabolites of the different compounds were also affected by the pesticide combinations and, particularly, by the presence of chlorothalonil. The measured soil microbial parameters (enzyme activities and total microbial biomass) were stable in the pesticide-free control soils throughout the 90-d incubation period, but they were all adversely affected by the presence of chlorothalonil in the soil. The effects from fenamiphos or chlorpyrifos on the soil microbial characteristics were either very small or insignificant.

Keywords—Chlorpyrifos  Fenamiphos  Chlorothalonil  Degradation  Microbial characteristics

INTRODUCTION

Most studies regarding the environmental fate of pesticides are done with single applications of one compound. However, in practice, different pesticides are used to protect the crop from weeds, pathogens, and insect pests. Repeated application of individual pesticides is also a common occurrence, particularly in tropical regions with many horticultural crops and with major cash crops like cotton and sugarcane. Repeated application of a pesticide at the same site can often influence the activities of the soil microorganisms responsible for its biodegradation, and the phenomenon of enhanced or accelerated biodegradation is a common occurrence [1]. Enhanced biodegradation can lead to extremely rapid rates of loss and, in its extreme form, can result in failure to control the target pest, disease, or weed. In some circumstances, repeated application can have a deleterious effect on the pesticide-degrading microbial community so that inhibition of degradation becomes apparent. This has been demonstrated to occur with the fungicide chlorothalonil, and marked reductions in degradation rate in soil after repeated application have been reported [2]. This raises the possibility that pesticides in mixture with chlorothalonil may show different degradative properties than when the same compounds are applied individually. As well as effects on pesticide biodegradation rates, repeated applications of individual or combinations of chemicals may also affect the wider activities of the soil microbial community, as has been demonstrated recently for the fungicides benomyl, captan, and chlorothalonil [3].

The compounds studied in the present experiments were chlorpyrifos, chlorothalonil, and fenamiphos, which were selected to provide a representative insecticide, fungicide, and nematicide, respectively. All three compounds are widely used, and in tropical agriculture, they are frequently used in combination. The objectives of the experiments were, first, to investigate the effects of mixtures of chlorpyrifos, chlorothalonil, and fenamiphos on the degradation rates of the parent compounds and the dynamics of their metabolites in cultivated soils and, second, to evaluate the effects of the chemicals, both alone and in combination, on some soil microbial processes. Particular emphasis was placed on simulating repeated pesticide application to soil.

MATERIALS AND METHODS

Pesticides

Commercial formulations of chlorpyrifos (Dursban 4, 48% active ingredient; Dow AgroSciences, Wantage, UK) and chlorothalonil (Cropguard, 44.1% active ingredient; Atlas, Doncaster, UK) and analytical-grade fenamiphos (99% purity; Promocem, Welwyn Garden City, UK) were used throughout these studies. Analytical-grade samples of the parent compounds and their major metabolites (>99% purity; provided by Dow AgroSciences, Bayer Agrochemicals [Leverkusen, Germany], or purchased from Promocem) were used for preparation of analytical standards.

Soil

The soil used was collected from a site at Imperial College (Silwood Park, Ascot, UK). It was a sandy loam (67% sand, 24% silt, and 9% clay) with 5.12% organic matter, pH 6.7, and a gravimetric water-holding capacity of 39.7%. Soil pH was measured in a soil:water (1:1, w/v) suspension, organic matter content by loss on ignition, and water-holding capacity following saturation and free drainage for 24 h.

Pesticide incubations

Three replicate samples of topsoil were collected from the 0- to 10-cm layer at locations approximately 10 m apart. They were partially air-dried overnight and sieved to pass a 3-mm
mesh, and their moisture contents and water-holding capacity were determined. A separate subsample (1 kg) of each of the replicate soils was treated with chlorpyrifos, chlorothalonil (10 ml; 1,000 mg/L suspended in water), or fenamiphos (2.5 ml; 4,000 mg/L solution in methanol) to give a concentration of 10 mg active ingredient/kg dry soil. Further subsamples of each replicate soil were treated with the pesticides at a rate of 10 mg/kg in the combinations chlorpyrifos + fenamiphos, chlorpyrifos + chlorothalonil, fenamiphos + chlorothalonil, or chlorpyrifos + fenamiphos + chlorothalonil.

The concentration of pesticide (10 mg/kg) was selected to represent the lowest dose of each compound when used for agricultural purposes and is equivalent to 1.0 kg/ha of active ingredient incorporated to a depth of 1 cm in the soil. All samples treated with the fenamiphos solution in methanol were left for 3 to 4 h for the solvent to evaporate. Distilled water was added to all soils to adjust the final moisture contents to 40% of water-holding capacity. Three replicate samples without any pesticide treatment were kept as controls. Soils were mixed by hand initially and then passed through a 3-mm mesh sieve, after which they were transferred to loosely capped polypropylene containers (1-L capacity) and incubated at 20°C. The treated soils were sampled periodically for 98 d and analyzed for pesticides and their metabolite concentrations. After 33 d or when 50% of a specific pesticide had disappeared, if this occurred later, the soil was retreated with another dose of the appropriate chemical at 10 mg/kg. When appropriate, a third treatment was applied 33 d after the second treatment irrespective of residue concentration in the soil samples.

**Residue analyses**

Single samples of soil (20 g) from each incubation container were shaken with acetonitrile:water (90:10, v/v; 25 ml) for 1 h on a wrist-action shaker. They were centrifuged for 5 min at 6,000 rpm, after which subsamples of clear supernatant were analyzed directly by high-performance liquid chromatography using Kontron series 300 equipment (Kontron Instruments, Milan, Italy). The column used was Lichrosorb-RP18 (250 × 5 mm; Merck, Darmstadt, Germany) with an isocratic mobile-phase flow rate of 1 ml min⁻¹. The mobile phase for analysis of chlorpyrifos was acetonitrile:water (85:15, v/v) with a detection wavelength of 235 nm. The mobile phase for 3,5,6-trichloro-2-pyridinol (TCP; the major degradation product of chlorpyrifos), chlorothalonil, hydroxy-chlorothalonil, and fenamiphos was acetonitrile:water:orthophosphoric acid (75:25: 0.5, v/v/v) with detection wavelengths of 235, 235, 235, and 250 nm, respectively. Fenamiphos sulfoxide and fenamiphos sulfone were separated using a mobile phase of acetonitrile:water (60:40, v/v) and a wavelength of 225 nm. The recovery of chlorpyrifos, fenamiphos, and chlorothalonil following addition to soil at 10 mg/kg averaged 97, 98, and 94%, respectively, and analytical data were not corrected for recovery.

**Microbial studies**

All the soil-pesticide combinations described above, together with the untreated controls, were investigated to determine possible effects of the pesticides on the soil microbial population and its activity. For this purpose, soil dehydrogenase and phosphatase activity as well as total microbial biomass were determined 30, 60, and 90 d after the first pesticide applications.

**Soil dehydrogenase.** The method suggested by Tabatabai [4] was used with little modification to determine the soil dehydrogenase activity. Single amounts of soil (2 g) from each replicate of all treatments, including the controls, were mixed with 20 mg of CaCO₃, 2 ml of distilled water, and 1 ml of 3% (w/v) triphenyl tetrazolium chloride (TTC) solution. Additional samples of each replicate of the control were used to provide blanks, and everything was added except TTC solution. All samples were mixed thoroughly by vortex machine followed by incubation at 37°C for 24 h. The formazan produced was then extracted from the samples into methanol (three amounts of 5 ml) using a vortex machine. The combined methanol extracts were centrifuged and made up to 25 ml, and formazan concentration was determined from absorption measurements at 485 nm. Control absorbance was taken as zero. Standard solutions of formazan were prepared by making a 50-ml stock solution at a concentration of 1 mg/ml (Sol-1). In turn, Sol-1 was diluted by a factor of 10 (Sol-2), and Sol-2 was further diluted to provide concentrations of formazan of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml, which were used to derive a calibration curve.

**Soil phosphatase.** The method used to determine soil phosphatase activity was also described by Tabatabai [4]. Replicate samples of each soil treatment (0.5 g) were incubated with 1 ml of 25 mM p-nitrophenyl phosphate and 4 ml of distilled water for 30 min. The reaction was then stopped with 4 ml of 0.5 M NaOH and 1 ml of 0.5 M CaCl₂. Controls were maintained by adding everything except p-nitrophenyl phosphate. Samples were centrifuged, and absorbance was measured at 420 nm. A standard solution was made by dissolving p-nitrophenol (p-NP; 1 g) in 1 L of distilled water. Several dilutions were made to obtain different concentrations of p-NP (0, 10, 20, 30, 40, 50, 100, 200, and 500 μg p-NP/ml). Absorbance of the standards was measured at 420 nm, and a calibration curve was constructed.

**Total microbial biomass.** Total microbial biomass of the soil samples was determined by measurement of ninhydrin-reactive N following fumigation with liquid chloroform and extraction with potassium chloride as described by Mele and Carter [5]. The chloroform was first purified by removal of the preservative ethanol. Subsamples of soil (10 g) from the different treatments were weighed into conical flasks, and 1 ml of chloroform was added. All flasks were sealed with parafilm and incubated for 10 d at 30°C. Samples without chloroform were also incubated as controls. After the 10 d of incubation, the fumigated samples were evacuated in a vacuum desiccator five times for 2 min. A solution of potassium chloride (2 M; 25 ml) was added to each flask, and the samples were shaken for 1 h and then centrifuged. An aliquot (1 ml) was transferred to a boiling tube, and 0.5 ml of ninhydrin reagent was added. The samples were transferred to a beaker of boiling water for 20 min and then cooled for 10 min. Water: ethanol (50:50, v/v; 5 ml) was added and the final volume made to 10 ml. Absorbance was measured at 570 nm and compared with a standard curve of absorbance against the amino-nitrogen concentration of leucine (2–10 mg amino-N/L). Biomass carbon was calculated as described earlier [5].

**Statistical analysis of data**

The data for effects on soil microbial parameters were analyzed by one-way analysis of variance (ANOVA) using Genstat (Ver 5.3; Lawes Agricultural Trust, IACR Rothamsted, UK), and least significant differences from the untreated controls were derived at p = 0.05. Data for pesticide degradation were assumed to conform to the first-order rate equation, and
For any one pesticide, half-lives followed by the same letter in either the row or the column were not significantly different at the time period of the experiment. Concentrations of TCP were greater in the treatments involving chlorothalonil than in those involving chlorpyrifos alone or chlorpyrifos + fenamiphos.

Residues of fenamiphos in the different incubations are shown in Figure 2a, and the first-order half-lives are listed in Table 1. They show an initial half-life in the first incubations of approximately 8 to 13 d, with the longer half-lives associated with the treatments involving chlorothalonil. The rate of degradation slowed in all treatments with repeated application and was particularly pronounced with the third treatment. Fenamiphos was first transformed into fenamiphos sulf-

Table 1. Correlation coefficients ($r^2$) for the lines of best fit to the first-order rate equation and the derived first-order rate constants and half-lives for the different pesticides

<table>
<thead>
<tr>
<th></th>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Treatment 3</th>
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<tbody>
<tr>
<td></td>
<td>Rate constant (d$^{-1}$)</td>
<td>Half-life (d)</td>
<td>Rate constant (d$^{-1}$)</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
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<td></td>
<td></td>
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<td>Chlorpyrifos</td>
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<td>63.6A</td>
<td>0.984 0.0115</td>
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<td>0.986 0.0102</td>
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<td>117.5B</td>
<td>0.971 0.0042</td>
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<tr>
<td>Chlorpyrifos + fenamiphos + chlorothalonil</td>
<td>0.889 0.0036</td>
<td>192.5C</td>
<td>0.864 0.0031</td>
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<tr>
<td>Fenamiphos (parent compound)</td>
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</tr>
<tr>
<td>Fenamiphos</td>
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<td>0.962 0.0828</td>
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<td>0.945 0.0513</td>
<td>13.5A</td>
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</tr>
</tbody>
</table>

*For any one pesticide, half-lives followed by the same letter in either the row or the column were not significantly different at $p = 0.05$. 

Degradation of chlorpyrifos, fenamiphos, and chlorothalonil

Fig. 2. Fenamiphos degradation in soil treated with the nematicide alone or in combination with other pesticides. a. Transformation of fenamiphos. b. Formation of metabolites. c. Total toxic residues (TTR). Solid lines indicate fenamiphos sulfoxide and dashed lines fenamiphos sulfone. Vertical bars represent ± one standard error (n = 3); where none is apparent, the error is smaller than the size of the symbol.

oxide and then into fenamiphos sulfone (Fig. 2b). Formation and persistence of the sulfoxide and sulfone were different in soil treated with chlorothalonil or with chlorpyrifos + chlorothalonil than when fenamiphos was applied alone or in combination with chlorpyrifos. The concentrations of the sulfone degradation product in the treatments with chlorothalonil and with the chlorpyrifos + chlorothalonil mixture were increased by more than 75% compared to those in the treatments with fenamiphos alone or with the fenamiphos + chlorpyrifos combination.

In terms of total toxic residues (TTR; fenamiphos + fenamiphos sulfoxide + fenamiphos sulfone), the suppressive effect of chlorothalonil was similar whether used by itself or in combination with chlorpyrifos (Fig. 2c). The transformation rate of fenamiphos was not affected by the presence of chlorpyrifos. The effective half-lives of fenamiphos and the TTR were approximately 8 and 36 d, respectively, when the soil was treated with fenamiphos alone. However, less than 10% of total TTR was lost during 98 d of incubation when fenamiphos was applied in combination with chlorothalonil or with chlorpyrifos + chlorothalonil.

The half-life of chlorothalonil following the first application was 12 to 13 d, with little difference between treatments (Table 1). The degradation rates of the second and third applications were slower than that of the first in most of the treatments (Fig. 3a and Table 1). Following the first application, more than 75% of the applied chlorothalonil had disappeared from the soil after 14 d of incubation. However, after the third application, the half-life of the chlorothalonil was greater than 20 d in all treatments. There appeared to be a suppression of chlorothalonil degradation when applied in combination with chlorpyrifos, but this was less marked in the triple combination of chlorothalonil + chlorpyrifos + fenamiphos. The dissipation rate of chlorothalonil was the same when applied alone or in combination with fenamiphos. The concentration of hydroxy-chlorothalonil increased with time and with repeated applications (Fig. 3b). The concentrations of the metabolite were greatest in the chlorothalonil + chlorpyrifos treatment; other treatments had similar concentrations.

Microbial studies

A marked reduction in dehydrogenase activity (p < 0.05) was observed in all treatments involving chlorothalonil (Fig.
DISCUSSION

Although occasional studies report the effects of pesticides on soil microbial processes, these usually involve single applications of realistic doses of the pesticides and by inclusion of pesticide combinations. The half-life of chlorpyrifos in the Silwood Park soil when applied alone was 64 d, which is consistent with half-lives in other soils reported previously [6]. The concentration of the metabolite TCP was never greater than 1 mg/kg. The rate of chlorpyrifos dissipation when applied in combination with fenamiphos was unaffected. However, it was suppressed in the presence of chlorothalonil. This suppression in the rate of chlorpyrifos degradation was substantial. The calculated half-lives when applied in combination with chlorothalonil or chlorothalonil + fenamiphos were 118 and 193 d, respectively, for the first treatment and 165 and 224 d, respectively, for the second treatment (Table 1). Chlorpyrifos alone had a half-life of approximately 60 d for both the first and second treatments. Chlorothalonil has been reported previously to suppress its own degradation when applied repeatedly [2], and the present results indicate that this suppressive effect can extend to other compounds in mixture with chlorothalonil. When all three pesticides were applied together, the rate of chlorpyrifos degradation was suppressed further. This suppression can be explained as a synergistic effect. An additive effect cannot explain the results, because fenamiphos has no effect on chlorpyrifos degradation when applied in the absence of chlorothalonil (Fig. 1a and Table 1).

Fenamiphos degradation was the same whether applied alone or in combination with chloropyrifos. Repeated application of fenamiphos to this soil did not result in induction of accelerated degradation, which contrasts with previous findings by Smelt et al. [7]. As shown in Table 1, the half-life of fenamiphos was 8.4 and 23.9 d for the first and third treatments, respectively, when applied alone. The transformation rates were similar when applied in combination with chloropyrifos (half-lives of 9.8 and 24.5 d in the first and third treatments, respectively). However, the rate of transformation was reduced when treated with chlorothalonil or the chlorothalonil + chloropyrifos combination. Dissipation rates of TTR were also considerably reduced when the nematicide was incubated in soil in combination with chlorothalonil or with chlorothalonil + chloropyrifos. These effects were solely the result of the presence of chlorothalonil, because chloropyrifos had no effect on fenamiphos degradation.

The degradation rate of chlorothalonil when applied alone was suppressed by repeated application, confirming the earlier observation [2]. These earlier authors suggested that accumulation of hydroxy-chlorothalonil may play a role in the suppression of chlorothalonil degradation after repeated application. Hydroxy-chlorothalonil also accumulated steadily during the present incubations (Fig. 3b), although no direct evidence suggests that the presence of the metabolite is responsible for the suppression in the degradation rate of the parent compound. Motonaga et al. [2] cautioned that their pesticide application rate was high (40 mg/kg) and that further study was required with lower doses and different soil types. Our results suggest that the suppression of chlorothalonil degradation also occurs at a lower application rate (10 mg/kg). None of the pesticide combinations had any effect on chlorothalonil degradation in the first treatment. However, a slight reduction in the dissipation rate was observed following the second application in combination with chloropyrifos. The dynamics of residues of the intermediate metabolites of all the pesticides studied were altered by the treatment regime, and in general, the concentrations of metabolites were significantly

Fig. 4. The effect of pesticide treatments on (a) soil dehydrogenase activity (least significant difference [LSD] = 17.0, p = 0.05), (b) phosphatase activity (LSD = 8.1, p = 0.05), and (c) total microbial biomass (LSD = 29.4, p = 0.05) after 30, 60, and 90 d. Vertical bars represent ± one standard error (n = 3). CHP = chlorpyrifos; CTH = chlorothalonil; FEN = fenamiphos; PNP = p-nitrophenol; TPF = 1,3,5-triphenylformazan.

4a). Dehydrogenase activity was suppressed by up to 50% in soil treated with chlorothalonil alone or with chlorothalonil + chloropyrifos. Fenamiphos alone or in combination with chloropyrifos had no adverse effect on dehydrogenase activity; however, chloropyrifos alone had a small suppressive effect.

The effects of the pesticides on soil phosphatase activity are summarized in Figure 4b. Soil phosphatase activity was suppressed with the application of chlorothalonil alone or in combination with chloropyrifos. Application of fenamiphos gave a significant increase (p < 0.05) in soil phosphatase activity whether alone or in combination with chloropyrifos. Small reductions in phosphatase activity were observed in the chloropyrifos treatment, especially after 90 d. In general, no effect was observed with fenamiphos + chlorothalonil or with chloropyrifos + fenamiphos + chlorothalonil.

All treatments involving chlorothalonil (alone or in combination with the other pesticides) had a significant effect (p < 0.05) on soil microbial biomass after 60 and 90 d (Fig. 4c). Fenamiphos alone or in combination with chloropyrifos had no effect on microbial biomass. A small reduction in biomass was observed in soil treated with chlorothalonil after the second application.

No significant change in microbial biomass or in enzyme activities was observed in the control soils throughout the 90-d period of the experiments.
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higher whenever the degradation rates of the parent compound was reduced.

Persistence of pesticide residues in the soil may have a significant impact on soil microbial communities and their functions such as the activity of enzymes, which are directly related to soil health and fertility and also to the removal of contaminants [8,9]. Different management practices appear to have a profound influence on the size and activity of the microbial biomass in arable soil. In the present study, the microbial characteristics examined were stable in the control soils throughout the 90-d incubation period; hence, any significant reductions in enzyme activity or microbial biomass can be ascribed to direct effects from the pesticides. All microbial characteristics were adversely affected by chlorothalonil treatment, whether applied alone or in combination with the other pesticides. These findings are in agreement with the results of previous studies [2,10] in which soil respiration and total bacterial counts as well as invertase, amylase, dehydrogenase, and phosphatase activity were reduced following application of chlorothalonil. However, our results contrast with recent reports [3] that chlorothalonil can stimulate dehydrogenase activity. Chlorpyrifos and fenamiphos had little significant effect on the measured microbial properties of the soil, although fenamiphos application resulted in a slight increase in phosphatase and dehydrogenase activity. This result with fenamiphos is, again, in agreement with the data from previous studies [11].

A further significant observation in the present experiments was that the effects of pesticide combinations on degradation rates were occasionally synergistic, but the effects on the soil microbial properties examined were generally additive. The results also highlight the differential effect of chemicals on the soil microflora. For example, all measured microbial parameters were adversely affected by chlorothalonil treatment, but treatment with fenamiphos had either no effect (dehydrogenase and microbial biomass) or a slight, positive effect (phosphatase). Whenever these two chemicals were applied together, the overall effect on soil microbial characteristics was intermediate between the two.

The present results have added an additional dimension to the study of the environmental fate and ecotoxicology of pesticides. Simulated combinations of different pesticides in conjunction with repeated application gave effects different from those observed with just a single application of a single compound. The results suggest that such interactions should be taken into account when assessing potential environmental implications of pesticide use. In our experiments, however, the combinations of pesticides examined were limited, and the studies were done in a single soil type. Further studies with other pesticide combinations and in other soil types are needed.

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