Role of Soil pH in the Development of Enhanced Biodegradation of Fenamiphos

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Repeated treatment with fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate) resulted in enhanced biodegradation of this nematicide in two United Kingdom soils with a high pH (≥ 7.7). In contrast, degradation of fenamiphos was slow in three acidic United Kingdom soils (pH 4.7 to 6.7), and repeated treatments did not result in enhanced biodegradation. Rapid degradation of fenamiphos was observed in two Australian soils (pH 6.7 to 6.8) in which it was no longer biologically active against plant nematodes. Enhanced degrading capability was readily transferred from Australian soil to United Kingdom soils, but only those with a high pH were able to maintain this capability for extended periods of time. This result was confirmed by fingerprinting bacterial communities by 16S rRNA gene profiling of extracted DNA. Only United Kingdom soils with a high pH retained bacterial DNA bands originating from the fenamiphos-degrading Australian soil. A degrading consortium was enriched from the Australian soil that utilized fenamiphos as a sole source of carbon. The 16S rRNA banding pattern (determined by denaturing gradient gel electrophoresis) from the isolated consortium migrated to the same position as the bands from the Australian soil and those from the enhanced United Kingdom soils in which the Australian soil had been added. When the bands from the consortium and the soil were sequenced and compared they showed between 97 and 100% sequence identity, confirming that these groups of bacteria were involved in degrading fenamiphos in the soils. The sequences obtained showed similarity to those from the genera *Pseudomonas*, *Flavobacterium*, and *Caulobacter*. In the Australian soils, two different degradative pathways operated simultaneously: fenamiphos was converted to fenamiphos sulfoxide (FSO), which was hydrolyzed to the corresponding phenol (FSO-OH) or was hydrolyzed directly to fenamiphos phenol. In the United Kingdom soils in which enhanced degradation had been induced, fenamiphos was oxidized to FSO and then hydrolyzed to FSO-OH, but direct conversion to fenamiphos phenol did not occur.

Enhanced biodegradation of pesticides after their repeated application to soils has been widely reported (6, 26). In some circumstances, enhancement of degradation does not occur, and the specific factors that lead to adaptation of microbial communities in some soils and not in others are not known. Pesticide degradation in soil is influenced by both biotic and abiotic factors, which act in tandem and complement one another in the microenvironment. The role of abiotic factors in development and stability of enhanced degradation has received little attention.

Fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate) is an organophosphate insecticide-nematicide, which is widely used for the control of ectoparasitic, endoparasitic, and free-living nematodes in horticultural and other field crops (18). The biological efficacy of fenamiphos has been reported to be significantly reduced by enhanced biodegradation (2, 15, 17, 20). It is oxidized rapidly in soil to fenamiphos sulfoxide (FSO) and fenamiphos sulfone (FSO₂), both of which have similar nematicidal activity to fenamiphos (25). Degradation studies therefore usually include an estimation of total toxic residues (TTR), a combination of the amounts of the parent compound plus the two oxidation products. Chung and Ou (5) reported that in soils showing enhanced biodegradation of

fenamiphos, the parent compound is oxidized to FSO, which is then rapidly hydrolyzed to FSO-phenol (FSO-OH). FSO-OH is subsequently mineralized to CO₂. In this situation, the step involving transformation from FSO to FSO₂ is not important.

There is little information on the microbial population involved in fenamiphos biodegradation in soil, although a microbial consortium has been isolated that degrades fenamiphos in liquid medium (16). This consortium was reported to consist of six different bacteria and required soil particles in the liquid medium itself to allow survival of the consortium and degradation (16). There is no information concerning the conditions that influence the transfer of mixed microbial populations that enhance the degradation of fenamiphos in one soil to another soil.

The present study examines (i) the effects of repeated applications of fenamiphos on the development and stability of enhanced degradation in different soils from Horticulture Research International (HRI), Wellesbourne, United Kingdom; (ii) the role of enhanced degradation in loss of efficacy of fenamiphos in two Australian soils; (iii) the ease of transfer of enhanced biodegradation from one soil to another; and (iv) isolation of fenamiphos-degrading bacteria from an Australian soil. In addition, the degradative pathway of fenamiphos was investigated in the different soils.

MATERIALS AND METHODS

Pesticides and soils and residue analyses. Analytical-grade fenamiphos, FSO, and FSO₂ (Promochem, Ltd., Welwyn Garden City, United Kingdom) were used

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TABLE 1. Soil properties

Soil	pH	Organic matter (%)	Moisture content (%) ^a	Microbial biomass (mgC kg ⁻¹) ^b	Content (%)			Phosphatase activity (μg of PNP/g of soil) ^c	Dehydrogenase activity (μg of TPF/g of soil) ^d
					Sand	Silt	Clay		
United Kingdom soils									
Deep Slade	4.7	2.33	15.9	150	86	8	6	50	135
Deep Slade	5.7	2.67	16.8	172	83	10	7	69	149
Deep Slade	6.7	2.79	16.7	189	84	9	7	101	180
Deep Slade	7.7	3.02	17.7	201	76	10	14	132	194
Deep Slade	8.4	3.12	17.9	200	85	8	7	141	225
Australian soil									
CRF	6.8	5.24	20.1	300	38	28	34	112	254
BEP	6.7	13.89	21.6	349	13	42	45	119	249

^a That is, 50% maximum water-holding capacity.^b mgC, mg of organic carbon.^c PNP, *p*-nitrophenol.^d TPF, triphenyl formazan.

for all incubation and analytical studies. Standard fenamiphos phenol, FSO-OH, and FSO₂-OH were prepared by base hydrolysis of fenamiphos, FSO, and FSO₂, respectively, as described by Ou (15). The soils were collected from field sites at HRI; the samples had no known pretreatment history of fenamiphos. In the field, natural variations in soil pH were seen without major changes in other soil characteristics. Because of this, the site has been selected for a series of studies on the effect of pH on the degradation of different pesticides. Additional soil samples came from two field sites in Australia: (i) Chiquita Racenello Farm (CRF), located 10.5 km south west of Tully, Queensland, and (ii) Buchanan East Palmerston farm (BEP), which is 22.5 km southwest of Innisfail, Queensland. Fenamiphos had been used in these fields for several years to control nematodes and, recently, a loss of efficacy against the target pests has been reported (18). After we received the soils by airmail, we stored them moist in the dark at 4°C until used for experiments (2 weeks). The properties of the soils are listed in Table 1. Soil pH was measured by using a glass electrode in a 1:2.5 soil-distilled-water suspension, and organic matter content was measured by loss on ignition at 450°C. The maximum water-holding capacity (percent weight/weight) was measured after saturation of soil samples (20 g) with water and free drainage for 24 h. Particle size distribution was assessed by laser granulometry on organic matter free samples. Microbial biomass was estimated by the technique of Mele and Carter (13), and enzyme activities were determined as described by Tabatabai (23). Details of the analytical methods for residues of fenamiphos and its degradation products were described previously (19).

Effect of pH on degradation of fenamiphos in HRI soils. The soils used were from Deep Slade field (HRI), and they had similar general physical and chemical characteristics with the exception of pH (Table 1). Samples were collected from the 0- to 20-cm soil layer. They were partially air dried overnight and sieved to pass a 3-mm-pore-size mesh, and their moisture contents and water-holding capacity were then determined. Three replicates (500 g) of each soil were treated with a solution (5 ml) of fenamiphos in methanol to give a concentration of 45 mg of active ingredient kg of soil⁻¹. This is approximately equivalent to the maximum recommended dose for fenamiphos (45 kg ha⁻¹), assuming incorporation into the top 7 cm of soil. Each soil sample for treatment was spread on a polyethylene sheet, and the fenamiphos solution was applied to a small area of the soil surface. The soil samples were then left for 3 to 4 h on a laminar flow bench for the methanol to evaporate, after which they were mixed by hand and passed through a 3-mm mesh. Distilled water was added to adjust the moisture content to 40% of the maximum water-holding capacity (Table 1). The samples were incubated at 20°C, and moisture contents were maintained throughout the experiment by regular additions of distilled water. Subsamples were extracted periodically over a period of 72 days, and extracts were analyzed for fenamiphos, FSO, and FSO₂. After 33 days, when >50% TTR was lost from three of the soils, 200-g amounts were taken from each replicate and treated again with fenamiphos to achieve a concentration of 45 mg kg⁻¹. This second treatment was sampled and analyzed at intervals for 39 days. Soil samples (100 g) were taken from this second subsample after 45 days and again treated with fenamiphos to adjust the concentration to 45 mg kg⁻¹. For the third treatment, residue analysis was carried out for 27 days. This procedure allowed evaluation of the effect of repeated application of fenamiphos on its degradation rate. Separate samples (100 g) of all five soils were treated with FSO and FSO₂ to achieve a concentration of 35 mg kg of soil⁻¹. The mixing, handling, and incubation conditions

were the same as for the experiment described above. Samples were analyzed over a 72-day period. In addition, subsamples of the soils (100 g) that had been incubated with three applications of fenamiphos over a 72-day period were also treated separately with FSO and FSO₂ and incubated as before with analysis at intervals for the subsequent 30 days.

Role of microorganisms in fenamiphos degradation in HRI soils. Subsamples of soils with different pH were fumigated with chloroform to establish the role of microorganisms in degradation of fenamiphos and its oxidation products. Soil samples (100 g) were treated with 2 ml of liquid chloroform in sealed Duran bottles. After 7 days at 30 °C, the chloroform was removed by repeated evacuations in a vacuum desiccator. Samples (100 g) from the different pH soils were also treated with chloramphenicol (antibacterial) or cycloheximide (antifungal) to identify the main microbial component responsible for fenamiphos degradation. Aqueous solutions of chloramphenicol (2.5 ml; 4,800 mg liter⁻¹) or cycloheximide (2.5 ml; 4,800 mg liter⁻¹) were added to the soil to achieve an antibiotic concentration of 120 mg kg⁻¹. Fumigated and antibiotic-treated soil samples were then treated with a fenamiphos solution to give a concentration of 45 mg kg⁻¹. All samples were incubated at 20°C and 40% of water-holding capacity.

Effect of a change in soil pH on fenamiphos degradation. Three subsamples (250 g) of the two acidic HRI soils (pH 4.7 and 5.7; Table 1) were mixed with CaCO₃ at the rate of 10 g kg of soil⁻¹ to increase the pH (10). Fenamiphos was added on three successive occasions at the rate of 45 mg kg⁻¹, followed by incubation as described above, with residue analysis at regular intervals.

Enhanced degradation of fenamiphos in two Australian soils. Triplicate (500-g) soil samples from the two Australian field sites (CRF and BEP; Table 1) were treated with a fenamiphos solution in methanol to yield a concentration of 45 mg kg of soil⁻¹. All soil samples were handled as described for the United Kingdom soils. Soil samples were retreated a second and a third time with fenamiphos after 7 and 11 days, respectively. FSO and FSO₂ were also incorporated into separate subsamples of both soils in order to study the rate of degradation of the metabolites. Soils were fumigated with chloroform or were treated with antibiotics to establish the role of microorganisms in fenamiphos degradation.

Soil pH and the transfer and stability of degrading ability. The enhanced degrading ability of the BEP Australian soil was activated by three successive applications of fenamiphos as described above. Three subsamples (190 g) of the five soils from the Deep Slade field (soils 1 to 5, Table 1) were mixed with 10 g of this activated soil. All mixtures were treated with fenamiphos and incubated for 21 days with regular analyses for fenamiphos and its degradation products. To study the persistence of the microbial system responsible for enhanced degradation after the mixing of the BEP soil into the five HRI soils, a further experiment was carried out 90 days after preparation of the initial mixing experiment described above. All of the soil samples that had received a single dose of fenamiphos were retreated with the nematocide at this time to give a concentration of 45 mg kg of soil⁻¹. The soils were sampled at regular intervals over the subsequent 30 days to determine the rate of pesticide degradation. Fenamiphos metabolites formed during degradation were identified by comparing high-pressure liquid chromatography (HPLC) profiles with those for standard fenamiphos, FSO, FSO₂, fenamiphos phenol, FSO-OH, and FSO₂-OH. At the end of the 30-day incubation period, the bacterial community structure was examined by PCR-denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene from total extractable DNA.

FIG. 1. Degradation of three successive applications of fenamiphos in soils of pH 4.7 (A), pH 5.7 (B), pH 6.7 (C), pH 7.7 (D), and pH 8.4 (E). The columns show degradation of fenamiphos, accumulation of FSO, accumulation of FSO₂, and dissipation of TTR. Symbols: ◆, first treatment; ■, second treatment; ▲, third treatment.

Isolation of a fenamiphos-degrading microorganisms from an Australian soil (BEP). A mixed microbial population responsible for fenamiphos degradation in the BEP soil was isolated by standard enrichment culture techniques using liquid mineral salt medium (MSM) (7) with fenamiphos as the sole source of carbon and nitrogen (MSM-F). Fenamiphos was added directly to the medium (without any organic solvent) and was dissolved by shaking. Liquid medium was inoculated with 0.5% of enhanced BEP soil and incubated at 25°C. Immediately after a 50% loss of fenamiphos from inoculated MSM-F, a 0.5-ml aliquot was transferred into 20 ml of fresh MSM-F. After three such transfers, a 10-fold dilution series was prepared, and an aliquot (0.1 ml) was spread on MSM-F agar (containing 1% bacteriological agar) and nutrient agar. Plates were incubated at 25°C for up to 6 days. Several attempts were made to identify whether the pure bacterial isolates obtained could degrade fenamiphos by transferring single colonies from plates to liquid MSM-F (20 ml). Samples were incubated at 25°C. Degradation of fenamiphos and the growth of the bacterial isolate were monitored for up to 8 weeks postinoculation.

The fenamiphos-degrading culture from the Australian soil was maintained by sequentially transferring 0.5 ml of culture to fresh MSM-F repeatedly (more than

20 times). DNA was extracted for 16S rRNA gene profiling of the bacterial community, and further attempts were made to isolate fenamiphos-degrading pure cultures from the stable enrichment culture. A 10-fold dilution series was made, 0.1 ml was spread onto MSM-F agar and nutrient agar, and plates were incubated at 25°C for up to 6 days. Single colonies from agar plates were transferred into fresh MSM-F to test their degrading ability.

DGGE. DGGE was carried out to investigate changes in the microbial communities in soils and to separate and to identify the bacterial components of the isolated fenamiphos-degrading consortium. The changes in microbial community structure were investigated by using DGGE of the 16S rRNA gene with DNA extracted directly from the soil samples. The samples investigated in this way were (i) untreated soils from the Deep Slade field (Table 1); (ii) soils from the Deep Slade field treated once with fenamiphos; (iii) soils from the Deep Slade field mixed with the rapidly degrading BEP soil, treated with fenamiphos, and incubated for 90 days, followed by a second treatment with fenamiphos; (iv) Australian BEP and CRF soils treated three times with fenamiphos; and (v) a soil sample from the Deep Slade field (soil 4, Table 1) in which enhanced biodegradation of fenamiphos had been induced. Soil samples (1 g) were taken

TABLE 2. Estimated half-lives of fenamiphos and its degradation products in HRI soil

Treatment	Half-life (days) ^a at pH:						
	4.7	5.7	6.7	7.5	7.7	8.4	8.6
Natural soils							
Fenamiphos							
First treatment	10.7 ^{Aa}	13.7 ^{Ab}	13.1 ^{Ac}		12.6 ^{Ad}	13.9 ^{Abe}	
Second treatment	13.1 ^{Ba}	12.8 ^{Aa}	11.4 ^{Bb}		4.0 ^{Bc}	3.8 ^{Bc}	
Third treatment	10.8 ^{Aa}	10.8 ^{Ba}	10.9 ^{Ca}		4.1 ^{Bb}	3.8 ^{Bc}	
TTR							
First treatment	75.3 ^{Aa}	53.7 ^{Ab}	40.3 ^{Ac}		21.6 ^{Ad}	16.2 ^{Ad}	
Second treatment	130.8 ^{Ba}	110.0 ^{Ba}	58.2 ^{Bb}		5.2 ^{Bc}	4.8 ^{Bc}	
Third treatment	144.4 ^{Ba}	94.9 ^{Bb}	34.1 ^{Cc}		5.0 ^{Bd}	4.9 ^{Bd}	
FSO	39.6 ^a	30.1 ^b	22.0 ^c		16.0 ^d	11.3 ^c	
FSO ₂	20.3 ^a	16.5 ^b	14.4 ^c		7.9 ^d	7.5 ^d	
Changed-pH soils							
Fenamiphos							
First treatment				9.2 ^{Aa}			9.8 ^{Aa}
Second treatment				3.3 ^{Ba}			3.1 ^{Ba}
Third treatment				3.9 ^{Ba}			3.7 ^{Ba}
TTR							
First treatment				24.6 ^{Aa}			21.9 ^{Ab}
Second treatment				5.2 ^{Ba}			4.9 ^{Ba}
Third treatment				4.3 ^{Ca}			3.8 ^{Cb}

^a Values were calculated as described by Singh et al. (19). Within any one of the five data sets, values followed by the same superscript capital letter in the column or by the same superscript lowercase letter in the row are not significantly different ($P = 0.05$). In the case of the "changed-pH soil" values, the pH 4.7 and 5.7 columns correspond to the pH 7.5 and 8.6 columns, respectively (each pair of pH values constituting a single data set).

from each replicate of these soils, and DNA was extracted by using the soil DNA clean kit (Mo Bio, Carlsbad, Calif.) according to the manufacturer's instructions. Bacterial cells from the isolated fenamiphos-degrading consortium were pelleted by centrifugation, and DNA was extracted by the same method. PCR amplification of the 16S r-DNA prior to DGGE was performed as described by Muyzer et al. (14). Thermocycling consisted of 35 cycles of 92°C for 45 s, 55°C for 30 s, and 68°C for 45 s, with 10 pmol of each of the primers. The primers amplified eubacterial 16S rRNA regions corresponding to *Escherichia coli* nucleotide positions 341 to 534. PCR samples (40 µl) were loaded onto 8% (wt/vol) polyacrylamide gels in TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA [pH 7.4]). The polyacrylamide gels were made with a denaturing gradient ranging from 40 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). The gel was run for 16 h at 60 V and 60°C (Bio-Rad Laboratories, Richmond, Calif.). Separate gels were run for soil samples and isolated consortia. After electrophoresis, the gels were stained in distilled water containing ethidium bromide (0.5 mg liter⁻¹) and destained in water for 15 min. Images were captured by UV illumination and a charge-coupled device camera. The central portion from strong DGGE bands from the mixed soil samples and from the isolated consortium were excised with a sterile razor blade and then soaked in 50 µl of purified water (Milli-Ro, Bedford, Mass.) overnight. A subsample (5 µl) was used as a template for reamplification. The PCR products were purified by QIAquick PCR purification kit (Qiagen, Ltd., West Sussex, United Kingdom). The purity of individual bands was checked by DGGE. DNA was sequenced by using individual amplification primers, a *Taq* DyeDeoxy terminator cycle sequencing kit, and an ABI automated sequencer (Applied Biosystems). The sequences obtained were edited by using DNASTar and were compared to sequences in the EMBL and Ribosomal Database Project (RDP) II databases (the FASTA and MATCH programs, respectively).

DNA sequences. The partial 16S rRNA sequences, generated from DGGE bands within the soil profiles and the isolated consortia, have been deposited in the EMBL database under accession numbers AJ581120 to AJ581123.

RESULTS

Effect of pH on degradation of fenamiphos in HRI soils. The results from the experiment with repeated application of fe-

namiphos to the soils are presented in Fig. 1. Half-lives (Table 2) were derived from the data for loss of fenamiphos and dissipation of TTR following linear regression of the log concentration remaining against time. Of the 52 fitted lines, 38 were statistically significant at $P < 0.001$, 8 were statistically significant at $P < 0.01$; and the remaining 6 were statistically significant at $P < 0.05$.

The oxidation of fenamiphos to FSO was rapid in the soil with pH 4.7 (Fig. 1A), with a half-life of 10.7 days (Table 2). Oxidation of FSO to FSO₂ was relatively slow, and the calculated half-life of TTR was 75.3 days. Repeated application of fenamiphos did not effect the rate of fenamiphos oxidation and FSO and FSO₂ accumulated in the soil samples. The degradation of TTR was slower in the second and third treatments, with half-lives of 131 and 144 days, respectively (Table 2). The oxidation of fenamiphos to FSO at soil pH 5.7 was also rapid (Fig. 1B). The degradation of FSO and FSO₂ and the overall dissipation of TTR were faster at pH 5.7 than at pH 4.7. Fenamiphos oxidation in neutral soil (pH 6.7) was similar to that observed in the two acidic soils, but the degradation of FSO and FSO₂ and therefore the dissipation of TTR was faster (Fig. 1C), with a half-life for TTR of 40.3 days (Table 2). Oxidation of fenamiphos in the soil with pH 7.7 was similar to that in the other soils, but degradation of the oxidation products was rapid, with a half-life for TTR of 21.6 days (Fig. 1D and Table 2). Repeated treatment with the nematicide led to rapid degradation of fenamiphos and enhanced degradation of TTR, with half-lives for TTR of 5.24 and 5.02 days for the second and third treatments, respectively. FSO did not accumulate with repeated application, and FSO₂ was not detected

FIG. 2. Degradation of three successive applications of fenamiphos (TTR) in fumigated (◆) and nonfumigated (■) CRF soil (A) and BEP soil (B).

in the soil samples after the second treatment (Fig. 1D). Repeated application of fenamiphos also led to enhancement of biodegradation of both the parent compound and its metabolites in the soil with pH 8.4 (Fig. 1E). FSO did not accumulate after the first treatment, and no FSO₂ was detected in the soil samples after the second treatment (Fig. 1E).

The degradation rate of both FSO and FSO₂, when incubated separately in the different soils, increased with the increase in soil pH (Table 2). The calculated half-lives for FSO decreased from 39.6 to 11.3 days as pH increased from 4.7 to 8.4, and those for FSO₂ decreased from 20.3 to 7.5 days over this pH range. When incubated in the soils that had received three previous applications of fenamiphos, the half-lives of FSO and FSO₂ at acidic and neutral pH were similar to those above, but in the soils with pH 7.7 and 8.4, these two metabolites were degraded very rapidly with half-lives of less than 1 day (data not shown).

Role of microorganisms in fenamiphos degradation in HRI soils. There was negligible degradation of fenamiphos, and little production of FSO or FSO₂ in any of the soil samples fumigated with chloroform (data not shown). Treatment of soils with the antibacterial compound chloramphenicol inhibited degradation, whereas treatment with the antifungal compound cycloheximide had no effect.

Effect of a change in soil pH on fenamiphos degradation. Addition of lime to the pH 4.7 soil raised its pH to 7.5, and a similar addition to the pH 5.7 soil increased its pH to 8.6. The degradation rates of fenamiphos and the formation and behavior of metabolites in these two soil samples were similar to those recorded in the respective alkaline United Kingdom soils (Table 2). Degradation occurred relatively slowly after the first application of fenamiphos, and the half-lives for TTR were

24.6 and 21.9 days at pH 7.5 and 8.6, respectively. Subsequent treatments resulted in enhanced degradation in both soils with half-lives for TTR of 5.2 and 4.9 days for the second treatments and of 4.3 and 3.8 days for the third treatments at pH 7.5 and 8.6, respectively (Table 2).

Enhanced degradation of fenamiphos in two Australian soils. Fenamiphos degradation in the two Australian soils was rapid (Fig. 2). In the CRF soil, small amounts of FSO were formed initially (2.5 mg kg⁻¹) after the first treatment, but after 7 days no FSO was extracted from this soil. No FSO₂ was detected in any of the soil samples throughout the incubation experiments. More than 50% of the applied pesticide was degraded within 4 days in the first treatment. The second and third treatments gave an accelerated degradation rate, and >50% of the applied fenamiphos was degraded within 3 and 2 days, respectively (Fig. 2A). The rate of degradation was also rapid in the BEP soil in which >50% of fenamiphos was degraded by 4, 3, and 2 days after the first, second, and third treatments, respectively (Fig. 2B). Neither FSO nor FSO₂ were detected in the BEP soil samples during the incubation study. Fumigation of the CRF or BEP soils with chloroform resulted in total inhibition of fenamiphos degradation (Fig. 2). Prior treatments of the enhanced soil samples with chloramphenicol also led to complete inhibition of fenamiphos degradation, whereas cycloheximide had no effect (data not shown).

Soil pH and the transfer and stability of degrading ability. Degradation of fenamiphos in the Deep Slade soils when mixed with 5% of enhanced BEP soil was rapid (Fig. 3A). More than 50% of the applied fenamiphos was degraded within 5, 2, 2, 1, and 1 days at soil pH 4.7, 5.7, 6.7, 7.7, and 8.4, respectively. Degradation of fenamiphos when reapplied 90 days after the first mixing gave different degradation rates in the different pH soils, with rapid rates of loss only in the alkaline soils (Fig. 3B). Repeated application failed to reinduce enhanced degradation in the two acidic soils. However, in the neutral pH (6.7) soil, enhanced degradation was reinduced after the third treatment (data not shown).

Isolation of a fenamiphos-degrading microorganisms from an Australian soil (BEP). After several dilution and enrichment cycles in MSM, a stable microbial consortium was obtained that could utilize fenamiphos as a sole source of carbon and nitrogen. The consortium degraded fenamiphos by direct hydrolysis to fenamiphos phenol which, in turn, was degraded completely without the accumulation of any other intermediate. The growth of the consortia was fast and the degradation of fenamiphos was rapid when 35 mg of fenamiphos liter⁻¹ was degraded within 12 h. This consortium was also able to degrade FSO and FSO₂ (data not shown). Attempts to isolate a single pure culture that degraded fenamiphos from the enriched medium or from the stable consortium were unsuccessful.

DGGE analysis of total microbial DNA. The 16S rRNA profiles of the bacterial populations present in the different soils are shown in Fig. 4 and the profile for the isolated consortium is presented in Fig. 5. Between 20 and 40 bands were detected for each soil sample. Differences in soil pH resulted in minor changes in DGGE banding patterns, and the addition of fenamiphos to these soils did not change these bands dramatically. The PCR-DGGE analysis of DNA from Australian soils and the UK soils mixed with 5% of Australian BEP soil (in-

FIG. 3. (A) Degradation of fenamiphos (TTR) in HRI soils with different pHs immediately after being mixed with 5% enhanced BEP soil; (B) degradation of fenamiphos (TTR) in HRI soils with different pHs 90 days after the first mixing. Symbols: ◆, pH 4.7; ■, pH 5.7; ▲, pH 6.7; □, pH 7.7; △, pH 8.4.

FIG. 5. DGGE profile for isolated BEP consortium (in triplicate lanes 1, 2, and 3). Four marked bands (1 to 4) were sequenced for identification.

cubated for 90 days) resulted in major changes in the DGGE band profiles at pH 7.7 and 8.4 and, to a lesser extent, at pH 6.7. This indicates that the prominent bacterial population of the Australian soil had colonized the Deep Slade soil at these pH values. At a lower pH this did not occur. Some overlapping bands were detected in the two Australian soils and the enhanced Deep Slade soil, indicating that common bacteria may be present. The nonoverlapping bands within the samples suggest that other bacteria have been enriched in these soils. DGGE fingerprinting of the isolated consortium gave four distinct bands (Fig. 5) that migrated to the same general po-

FIG. 4. DGGE analysis of bacterial communities in control, treated, and mixed HRI and Australian soils. Lanes 1 and 20 show the DGGE bacterial marker. HRI soils at pH 4.7 (lanes 2 to 4), pH 5.7 (lanes 5 to 7), pH 6.7 (lanes 8 to 10), pH 7.7 (lanes 11 to 13), and pH 8.4 (lanes 14 to 16) are also shown. The samples were left untreated (lanes 2, 5, 8, 11, and 14), treated with fenamiphos (lanes 3, 6, 9, 12, and 15), or mixed with BEP soil and treated with fenamiphos (lanes 4, 7, 10, 13, and 16). Samples of BEP soil (lane 17), CRF soil (lane 18), HRI soil (lane 19) are also shown. The markers consisted of *Pseudomonas fluorescens* (arrow 1), *Sphingomonas yanoikuyae* (arrow 2), *Bacillus subtilis* (arrow 3), *Burkholderia phenazinium* (arrow 4), *Paenibacillus amyloticus* (arrow 5), *Agrobacterium rhizogenes* (arrow 6), and *Arthrobacter polychromogenes* (arrow 7). Dominant bands from BEP soil and their persistence in high pH HRI soils 90 days after the first mixing are boxed.

TABLE 3. Characterization of DGGE bands within fenamiphos-degrading systems^a

Clone	RDP group	Isolated consortia			Mixed soil	
		Subgroup	FASTA-EMBL database ID	% ID	Band(s)	% ID
BEP1	<i>Pseudomonas</i> and relatives	<i>Pseudomonas</i>	<i>P. aeruginosa</i> PAO1 (AE004844) and <i>P. putida</i> (AF447394)	98	1 and 2	99
BEP2	<i>Pseudomonas</i> and relatives	<i>Pseudomonas</i>	<i>P. putida</i> 21DINH (AF307868)	98	2	97
BEP3	<i>Bacteroides</i> and <i>Cytophaga</i>	<i>Cytophaga</i>	<i>Flavobacterium</i> sp. (PPL252729)	97	3	97
BEP4	<i>Caulobacter</i>	<i>Caulobacter</i>	<i>C. crescentus</i> (AE005930)	100	4	100

^a The four major bands obtained in the isolated consortia were sequenced, and similarities to known groups on the Ribosomal Database Project (RDP) database and the EMBL database are shown. In addition, the sequence similarities to equivalent bands (boxed in the DGGE gel) that dominated the United Kingdom and Australian activated soils are presented (mixed soil). ID, identity.

sition as the highlighted bands in the Australian soil and the Australian enhanced United Kingdom soils. When the bands from the isolated consortium were sequenced (Table 3), the results indicated that pseudomonads and *Cytophaga* and *Caulobacter* species were involved in the degradation process. When the equivalent bands from the mixed soil sample were sequenced, they demonstrated between 97 and 100% sequence identity to the consortium bands. This finding confirmed that the mixed soil DGGE bands highlighted represented the groups of bacteria that are involved in degrading fenamiphos in soil. Although bacteria from the same genera are likely to be present in the HRI soil and faint bands can be seen in the positions marked on the gel in these areas, their reproducible prominence in the DGGE profile in United Kingdom soils with a high pH that also degrade fenamiphos clearly suggests that these bacteria originated from the BEP soil.

Identification of metabolites formed during degradation of fenamiphos. The HPLC profiles for the standard compounds and extracts from the different soil samples showed that the major metabolite peak in the two Australian soil samples treated with fenamiphos was fenamiphos phenol. Small concentrations of FSO-OH were also detected. No peaks for FSO, FSO₂ or FSO₂-OH were detected in extracts from either of the Australian soils after the second treatment. In the HRI soil in which enhanced degradation had been induced, the major metabolite peak was FSO-OH. No peak for FSO₂, fenamiphos phenol, or FSO₂-OH was observed in extracts from this soil. In nonenhanced soils, degradation of fenamiphos was slow and major metabolites were identified as FSO, FSO₂, and FSO₂-OH. When the five soils of different pH were mixed with 10% of the Australian BEP soil, the pattern of metabolite formation after the initial application of fenamiphos was identical to that in the BEP soil alone. However, when the second application of fenamiphos was made to the mixed soils 90 days after the first, only the soils with a higher pH (6.7 and above) showed a degradation pathway similar to that observed in the original BEP soil.

DISCUSSION

Repeated application of fenamiphos to soil has been reported to result in its enhanced degradation (17). Fenamiphos degrades quickly in both enhanced and nonenhanced soils, but FSO₂ is rarely formed in enhanced soils (15). This suggests that rapid disappearance of the oxidation products is the main contributor to enhanced degradation of fenamiphos TTR. Davis et al. (8) reported that enhanced degradation of fenamiphos TTR was due primarily to an increase in the disappear-

ance rate of FSO in soil samples collected from field sites treated on two occasions with fenamiphos. In the present study, the different soil samples from the Deep Slade field had similar general physical and chemical characteristics other than soil pH. The degradation rate of fenamiphos initially was relatively independent of pH (Table 2), and three consecutive treatments did not result in the development of enhanced degradation of the parent compound in soils with a pH of ≤6.7. However, in the two alkaline soils, the second and third treatments with fenamiphos resulted in much more rapid degradation than with the first treatment. This observation suggests that if all other general soil properties are similar, soil pH will play an important role in the development of enhanced degradation.

There have been previous reports of a nonspecific relationship between high pH and rapid biodegradation of carbamate insecticides (21), dicarboximide fungicides (26), substituted urea herbicides (27, 28), and triazine herbicides (12). The important effect of pH was further supported in our studies by the results from the experiments in which the pH of two soils (pH 4.7 and 5.7) was increased by addition of CaCO₃, and these soils behaved like the original United Kingdom soils with a higher pH. Several studies have shown that an increase in soil pH results in an increase in soil microbial biomass and enzymatic activities (3, 29), and the present results with soils from Deep Slade (Table 1) are consistent with this. Acosta-Martinez and Tabatabai (1) also reported that the addition of CaCO₃ to acidic soils both increased soil pH and resulted in an increase in the activities of 14 soil enzymes. Bending et al. (4) showed that pH-mediated spatial variability in isoproturon degradation across a field was linked to the distribution of pesticide-degrading *Sphingomonas* spp. The observations of these authors, together with the results from the present experiments, suggest that alkaline pH in soil supports higher microbial biomass and enzymatic expression, which in turn helps the microbial community to adapt and develop gene-enzyme systems for the enhanced degradation of pesticides. Further evidence for the importance of soil pH was provided by the experiment in which the rapid-degrading Australian soil was mixed into the previously untreated soils with different pHs. These results demonstrated that the soils capacity for rapid degradation was stable only at alkaline pH, and the DGGE profiling of bacterial DNA from the mixed soils showed that the bacterial population from the Australian soil was transferred and stable for >90 days in the high-pH United Kingdom soils. Since methanol was used to dissolve fenamiphos prior to addition to the soil, it is also possible that methanol degraders were enriched

FIG. 6. Proposed pathways for fenamiphos degradation in soil samples used in this study. Pathway A operates in the nonenhanced HRI soils, pathway A-1 operates in enhanced HRI soils, and pathway B operates in the enhanced Australian soil and in the isolated consortium. The broken line within the lower arrows indicates unknown multiple degradation steps.

during soil incubation. This effect was minimized, since the methanol that was introduced would have evaporated quickly from the soil after application. During culture in MSM-F, no methanol was used. The isolation and characterization of a fenamiphos-degrading consortium from the Australian soil, which utilized fenamiphos as a sole source of carbon and nitrogen, confirmed that the bands identified in the soil were fenamiphos degraders. Four DGGE bands from the higher-pH mixed soils matched bands from the isolated consortium. It is not unexpected that all bands did not have 100% sequence identity, since two successive rounds of PCR were required to obtain pure bands for sequencing from both samples. In this way a small number of amplification errors may have occurred in the PCR products. Alternatively, the results may indicate

that we have isolated a subpopulation of the degradative bacteria through the enrichment procedure. As such the isolated consortia may represent a less-diverse bacterial population than that originally present in the soil sample. For example, the BEP soil may contain a range of pseudomonads capable of fenamiphos degradation, dominated at the time of sampling by strains with the two sequences we obtained. Enrichment resulted in the dominance of similar but not identical strains of *Pseudomonas*. This possibility is further supported by the general observation that pesticide-degrading genes are normally associated with plasmids that can move between bacterial strains, thus enhancing the diversity of the degraders. The degradative pathways of fenamiphos in the mixed soils also support this observation, since only higher pH soils were able

to follow the BEP pathways 90 days after mixing. Acidic soils mixed with the Australian soil, showed the degradative pathway common to the original HRI soils. Failure to isolate a fenamiphos-degrading pure culture could be attributed to several factors. There have been several previous reports of xenobiotic degradation by bacterial consortia (11, 22, 24). In the present study, even when the individual components of the consortia were grown independently, they either did not grow on or did not degrade the xenobiotic. It is well known that degradation of several chemicals is carried out by communal interaction between different components of consortia (9, 11, 16, 22).

In the soil from HRI in which enhanced degradation of fenamiphos had been induced, the parent compound was rapidly oxidized to FSO, which in turn was quickly degraded. The rate-limiting step was conversion of fenamiphos to FSO, since the half-lives for fenamiphos and TTR were almost identical. The degradation study with FSO and FSO₂ in enhanced soil supports this observation, since the half-lives for both metabolites were <1 day. Furthermore, the major fenamiphos metabolite peak observed on HPLC had a retention time identical with that of FSO-OH. No FSO₂ was detected, a finding in accord with results from a previous study (5). No fenamiphos phenol was detected in any of the HRI soils. Degradation of fenamiphos in the two Australian soils was rapid. Continuous applications of fenamiphos for several years in these two neutral pH soils had clearly resulted in the development of robust microbial systems, which degrade fenamiphos quickly. The most significant observation was the lack of formation of FSO in these two soils during fenamiphos degradation. In previous studies, enhanced degradation of fenamiphos TTR was attributed to enhanced degradation of FSO (5, 8). In the present study, little FSO and FSO-OH was formed during the first incubation in CRF soil, and thereafter no FSO was detected throughout the incubation study. In BEP soil neither FSO nor FSO-OH was detected at any time during incubation. The major metabolite peak for fenamiphos, identified by HPLC, was fenamiphos phenol, suggesting that fenamiphos may be directly converted to fenamiphos phenol, which in turn is metabolized to CO₂ and water. The proposed pathways of fenamiphos degradation in different soils are presented in Fig. 6. There is therefore a fundamental difference between the pathways of degradation in the Australian soils and in the enhanced HRI soil. In the HRI soil, fenamiphos oxidation is the rate-limiting reaction, and it is enhanced degradation of FSO that leads to enhanced degradation of fenamiphos TTR. In the Australian soils, loss of TTR was due to enhanced degradation of fenamiphos with an apparent alteration to the pathway of metabolism.

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