Factors influencing the ability of *Pseudomonas putida* epI to degrade ethoprophos in soil

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Abstract

An ethoprophos-degrading *Pseudomonas putida* strain (epI) was used in laboratory-based experiments to inoculate soils containing residues of the nematicide. Inoculum densities as low as $10^4$ cells g$^{-1}$ were sufficient to degrade a fresh addition of ethoprophos within 16 days. *P. putida* epI was able to rapidly degrade aged as well as freshly applied ethoprophos residues. However, slower degradation of aged residues was observed in the later stages of incubation suggesting that a fraction of the pesticide residues had become inaccessible to the introduced bacteria. The effects of environmental and soil factors on the ability of *P. putida* epI to degrade ethoprophos were also studied. The bacterium was able to rapidly degrade ethoprophos at 20 and 35°C and at soil water potentials of $-33$ and $-10$ kPa. However, its degrading ability was significantly reduced but not completely inhibited at 5°C or at a soil water potential of $-1500$ kPa. The *P. putida* strain was active in soils with pH 6.8 and 8.3, but there was a complete loss of degrading ability in a soil with pH 5.4. Efficient degradation of ethoprophos by *P. putida* epI was observed in soils with organic matter contents varying from 0.3 to 8.5%. However, degradation was somewhat slower in the soil with the higher organic matter content, presumably due to increased pesticide adsorption and decreased bioavailability. The results indicate that the ethoprophos-degrading *P. putida* epI was efficient as a bioremediation agent in a range of environmental and soil conditions. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Pseudomonas putida* epI; Ethoprophos degradation; Bioremediation; Environmental factors

1. Introduction

An important environmental concern with pesticides is their potential to contaminate surface or groundwater resources. Some important sources of surface water contamination, in particular, are associated with sprayer operations, such as spills during spray preparation, and the disposal of pesticide waste such as that generated by left-over spray suspensions, sprayer washing, or used pesticide containers (Mason et al., 1999). A possible way to solve the problems of pesticide waste, washings or contamination of soils by spillage is bioremediation with pesticide-degrading microorganisms. Previous studies concerning bioremediation of pesticide contamination have concentrated on residues in soil, and several researchers have demonstrated that the degradation in soil of pesticides including parathion (Barles et al., 1979), 2,4,5-T (Chatterjee et al., 1982) and atrazine (Radosevich et al., 1997) can be accelerated by inoculation with appropriate microorganisms. However, others have reported the failure of inoculants to enhance the dissipation of pesticide in natural soil environments (Macrae and Alexander, 1965; Goldstein et al., 1985). Successful inoculation has been shown to depend on inoculum density (Duquenne et al., 1996), on pollutant bioavailability (Greer and Shelton, 1992), and on soil conditions such as moisture, temperature, pH and organic matter content (van Veen et al., 1997). Biological factors such as competition with the indigenous micro-

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flora for substrates (Gunalan and Fournier, 1993), antagonism and predation (Goldstein et al., 1985) also affect inoculum survival and activity. The nature of the pesticide-degrading microorganisms can also affect bioremediation success. Fast-growing microorganisms, which carry constitutive enzymes and are able to degrade a wide range of pesticides with similar chemical structures, can be the most useful in bioremediation strategies (Struthers et al., 1998).

Previous studies with the organophosphorus nematicide ethoprophos (\(O\)-ethyl-S,S-dipropyl-phosphorodithioate) in a soil from Northern Greece led to the isolation of two \textit{Pseudomonas putida} strains (epI and epII) which were able to degrade the compound rapidly in liquid culture (Karpouzas, Ph.D. Thesis, 1999). In our study, one of these isolates (\textit{P. putida} epI) was used as a soil inoculant in order to investigate the influence of soil and environmental factors on its ethoprophos-degrading ability.

2. Materials and methods

2.1. Soils and pesticides

The soils used came from three field locations in the UK and from two locations in Greece. Their properties are listed in Table 1. Organic matter content was measured by loss on ignition at 450°C, and pH was measured using a glass electrode in a soil:distilled water (1:1) suspension. The chloroform fumigation–extraction method described by Mele and Carter (1996) was used for measuring soil microbial biomass. Analytical grade ethoprophos (Promochem, UK) was used in all incubation and analytical studies. Recoveries of ethoprophos added in soil ranged from 92 to 97%.

2.2. Ethoprophos-degrading isolate

The ethoprophos-degrading isolate (EDI) used in all experiments was \textit{P. putida} epI which had been isolated from a soil with an extensive history of ethoprophos applications over 30 years (Karpouzas, Ph.D. Thesis, 1999). The bacterium was able to degrade ethoprophos in a range of concentrations within 48 h either in mineral salts medium supplemented with nitrogen (MSMN) with the pesticide as sole source of carbon, or in soil extract medium (SEM). Better growth was obtained in SEM, and this medium containing ethoprophos (10 mg l\(^{-1}\)) was used for preparation of the inoculum in all of our experiments. Soil from the field site in Northern Greece from which the EDI was obtained was used for the preparation of SEM. Soil and distilled water were mixed in a 1:1 (w/v) ratio and sterilized for 30 min at 121°C. The supernatant was centrifuged and then autoclaved again (30 min at 121°C). The pH of SEM was 6.2. After cooling, 20 ml of a sterile aqueous solution of ethoprophos (500 mg l\(^{-1}\)) was mixed with 980 ml of soil extract to give a final concentration of 10 mg l\(^{-1}\). Soil extract agar containing ethoprophos (10 mg l\(^{-1}\)) was prepared in a similar way to SEM except that Difco Bacto Agar (15 g l\(^{-1}\)) was added. Inoculum was prepared from a culture of \textit{P. putida} epI grown on SEM agar plates containing ethoprophos (10 mg l\(^{-1}\)) for 48 h at 25°C. Growth was washed from the plates with 1 ml of SEM, and 0.2 ml aliquots of the bacterial suspension was used to inoculate fresh soil extract liquid medium (10 ml) containing ethoprophos (10 mg l\(^{-1}\)). Cultures were incubated at 20°C on a platform shaker at 150 rpm, and ethoprophos degradation was monitored by regularly removing 0.5 ml of medium which was mixed with 0.5 ml of hexane in an HPLC vial. The contents of the vial were vortexed, and ethoprophos residues were measured in 3 \(\mu\)l aliquots of the hexane layer by GLC as described by Karpouzas et al. (1999). When more than 50% degradation had occurred, an aliquot of the culture (0.2 ml) was transferred into fresh liquid medium containing ethoprophos (10 ml). This procedure was repeated two times more in order to maximize the degrading potential of EDI. Liquid cultures of EDI produced as described above were used as inoculum in all the soil degradation studies described below at the time when 50% of the initially recovered ethoprophos had degraded. Viable cell counts of the

<table>
<thead>
<tr>
<th>Soil type</th>
<th>Site</th>
<th>pH</th>
<th>Microbial biomass (mg C kg(^{-1}) soil)</th>
<th>Organic matter content (%)</th>
<th>Moisture content (40% water holding capacity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy loam</td>
<td>Northern Greece</td>
<td>5.4</td>
<td>145</td>
<td>2.1</td>
<td>15.95</td>
</tr>
<tr>
<td>Sandy loam</td>
<td>Sheep Pens, HRI</td>
<td>6.8</td>
<td>146</td>
<td>2.3</td>
<td>14.80</td>
</tr>
<tr>
<td>Sandy loam</td>
<td>Thessaloniki</td>
<td>8.3</td>
<td>162</td>
<td>3.5</td>
<td>23.50</td>
</tr>
<tr>
<td>Sandy loam</td>
<td>Soakwaters subsoil, HRI</td>
<td>8.1</td>
<td>6.4</td>
<td>0.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Clay</td>
<td>Brimstone farm, Oxford</td>
<td>6.5</td>
<td>NA(^a)</td>
<td>8.5</td>
<td>39.0</td>
</tr>
</tbody>
</table>

\(^a\) NA: not assessed.
inoculum used in all experiments were determined by preparing a 10-fold dilution series in SEM and spreading on triplicate Nutrient Agar (NA) plates, followed by incubation overnight at 25°C.

2.3. Influence of inoculum density

Fifteen samples (100 g) of soil from the Sheep Pens site (Table 1) were treated with 5 ml of a 200 mg l⁻¹ solution of ethoprophos in methanol to give a pesticide concentration of 10 mg kg⁻¹. Samples were left for 3–4 h for the solvent to evaporate and then mixed by hand to ensure uniform distribution of the chemical, and transferred to sterile polyethylene bottles. A culture of EDI in SEM (20 ml) was used as inoculum for the soil samples. Viable cell counts, prepared at the time of inoculation, gave an actual inoculum density of 7.6 × 10⁶ cfu ml⁻¹. A 10-fold dilution series in SEM was prepared, which produced cultures of EDI with densities of 7.6 × 10⁵, 7.6 × 10⁴ and 7.6 × 10³ cfu ml⁻¹. Volumes of 2.5 ml from the initial culture were added to three soil samples to give an inoculum density in soil of 1.9 × 10⁶ cells g⁻¹. Similarly, aliquots of 2.5 ml from the cultures of EDI produced by serial dilution were added to three replicate samples of soil, corresponding to densities in soil of 1.9 × 10⁵, 1.9 × 10⁴ and 1.9 × 10³ cells g⁻¹. The final three samples received the same volume of SEM without bacteria to serve as uninoculated controls. Distilled water was added to all samples to adjust moisture content to 40% of the water holding capacity (WHC). After initial preparation, individual soil samples were thoroughly mixed with sterile plastic spoons and incubated at 4°C overnight. Next day, the polyethylene containers were transferred to an incubator at 20°C. Immediately before transfer, and at regular intervals thereafter, subsamples (10 g) were removed from each container and analyzed for ethoprophos residues as before (Karpouzas et al., 1999).

2.4. Degradation of “aged” ethoprophos residues in soil

Three samples of soil from Sheep Pens field (200 g) were treated with a solution of ethoprophos in methanol (5 ml; 1000 mg l⁻¹) corresponding to a dose of 25 mg kg⁻¹. Further, three samples (200 g) were treated with the same amount of methanol without ethoprophos to serve as controls. Soils were handled as before; and following adjustment of their moisture content to 40% of the WHC, they were incubated at 15°C for 70 days. After this time, each soil sample was divided into two amounts of 100 g. All initially untreated samples received a fresh addition of ethoprophos (1 ml; 500 mg l⁻¹ in methanol) equivalent to the amount of ethoprophos that remained in the samples treated initially but incubated for 70 days. Recovery of the fresh ethoprophos addition was 96%. All the 12 samples were transferred to polyethylene bottles, and triplicate samples from each treatment were amended with 2.5 ml of an EDI culture in SEM. Viable cell counts, prepared at the time of inoculation, gave an actual density of 10⁸ cells ml⁻¹ in the SEM, which corresponded to a density of 2.5 × 10⁶ cells g⁻¹ soil. Moisture content of the samples was adjusted to 40% of the soil WHC, by addition of distilled water and incubated at 20°C for 10 days. Subsamples (10 g) were removed at frequent intervals and ethoprophos residues were measured as before.

To examine whether differences in substrate availability affected the efficiency of EDI to degrade fresh or aged ethoprophos residues, the concentration of ethoprophos present in the soil solution of the samples was also measured. Subsamples of 25 g were removed from two of the three samples from each treatment immediately on transfer to the 20°C incubator and placed in sterile glass jars (250 ml, Merck). Their moisture content was raised to 60% of their water holding capacity by addition of appropriate amounts of sterile distilled water. Samples were then transferred to the incubator at 20°C and left to equilibrate for 3 days, when each sample was divided into two subsamples of 10 g. The first subsample was used for extraction of the total ethoprophos residues as described before. The second subsample was placed in the body of a 10 ml disposable plastic syringe with the outlet plugged with non-absorbent cotton wool. Soil solution was extracted by centrifugation at 10,000 rpm for 10 min and collected in an HPLC vial attached to the syringe outlet (Walker, 2000). After centrifugation, the volume of water extracted was measured and the same volume of hexane was added. The contents of the HPLC vials were then vortexed for 10 s, and the amounts of pesticide partitioning the hexane layer were measured as before.

2.5. Effect of temperature

Eighteen samples of Sheep Pens soil (100 g) were treated with 10 mg ethoprophos kg⁻¹ as described above, and transferred to sterile polyethylene bottles. Nine of the samples were inoculated with EDI as before, and viable cell counts of the inoculum gave an apparent density of 1.8 × 10⁸ cells g⁻¹ soil. The remaining nine samples received 2.5 ml of SEM without bacterial suspension to serve as uninoculated controls. Soils were handled as described before and incubated at 4°C overnight. Next day, three inoculated and three uninoculated samples were transferred to incubators at 5, 20 and 35°C. Moisture content of the soils was maintained constant by daily additions of distilled water, and degradation of ethoprophos was measured as before.
2.6. Effect of soil moisture

Eighteen samples of soil from the Sheep Pens site (100 g) were treated with ethoprophos (10 mg kg\(^{-1}\)) as described previously. Samples were left for several hours for the soil samples to lose approximately 6 ml water. Nine of the samples were amended with 2.5 ml of an EDI culture in SEM to give a density of 3.2 \times 10^6 cells g\(^{-1}\) soil. The remaining nine samples were supplemented with 2.5 ml of SEM without bacteria to serve as uninoculated controls. The moisture content of three inoculated and three uninoculated samples was adjusted by addition of sterile distilled water to 20% of the maximum WHC (ca. \(-1500\) kPa water potential). Similarly, the moisture content of the remaining two groups of three inoculated and three uninoculated samples was adjusted to either 40 or 60% of the WHC (ca. \(-33\) and \(-10\) kPa water potential). Each sample was mixed thoroughly, stored at 4°C overnight, and incubated at 20°C for 18 days. Subsamples (10 g) were aseptically removed at frequent intervals and analyzed for ethoprophos residues.

2.7. Effect of soil pH

Three soils from different sites (Northern Greece, Thessaloniki Greece, Sheep Pens field HRI, UK) were used to study the influence of soil pH on the degrading ability of EDI. All the three soils were light-textured sandy loams, with the properties shown in Table 1. They provided a pH range from 5.4 to 8.3. Six samples (100 g) of each soil were treated with 10 mg ethoprophos kg\(^{-1}\) soil as before. Three samples of each soil were inoculated with 2 ml of EDI in SEM, which gave an inoculum density of 2.8 \times 10^6 cells g\(^{-1}\) soil. The remaining three samples of each soil were amended with 2 ml of SEM without bacteria. Moisture contents were adjusted to 40% of the appropriate WHC, and after mixing, the samples were stored at 4°C overnight and then incubated at 20°C for 13 days. Subsamples (10 g) were removed at frequent intervals for measuring ethoprophos residues.

2.8. Effect of soil organic matter content

Further, three soils were used to measure the effect of soil organic matter content on the degrading ability of EDI. Soil from the deeper zone (80–90 cm) of the soil profile of Soakwaters field at HRI Wellesbourne, which had a very low organic matter content (0.3%), was used together with soils from Sheep Pens field, HRI Wellesbourne (OM: 2.3%), and Brimstone farm, Oxfordshire, UK (OM: 8.5%). Six samples (100 g) from each soil were treated with 10 mg ethoprophos kg\(^{-1}\) soil. Three samples of each soil were amended with 2 ml of EDI in SEM as before (Section 2.7). The remaining three samples from each soil were amended with 2 ml of SEM to serve as uninoculated controls. Moisture contents were adjusted as before and the samples were incubated at 20°C for 13 days. Subsamples (10 g) were removed at frequent intervals and analyzed for ethoprophos residues.

2.9. Statistical analysis

All experiments involved three replicates and all data points are presented as means ± one standard deviation. The statistical significance of differences between treatment means was determined by one-way analysis of variance, when appropriate.

3. Results

3.1. Effect of inoculum density

The dissipation patterns of ethoprophos in soil inoculated with different densities of EDI are shown in Fig. 1. Degradation of ethoprophos was more rapid in all the inoculated samples compared with the rate of loss in the uninoculated controls. Complete degradation of ethoprophos was observed within 4 days in the soil samples inoculated with approximately 10^6 cells g\(^{-1}\). Degradation of ethoprophos was significantly slower in the samples inoculated with 10^5 or 10^4 cells g\(^{-1}\) in which ethoprophos residues were below the detection limit (5 \(\mu\)g kg\(^{-1}\)) after 9 and 16 days, respectively. The degradation rate of ethoprophos in samples inoculated with 10^3 cells g\(^{-1}\) was initially similar to that in uninoculated control samples with about 80%
recovery of the initial dose 7 days after the start of incubation. However, degradation of ethoprophos proceeded significantly faster \( (P < 0.05) \) thereafter in the inoculated samples. At the end of the study, there was 22% recovery of the initial ethoprophos dose in the samples inoculated with \( 10^3 \) cells g\(^{-1}\) compared with 45% recovery in the uninoculated samples.

### 3.2. Degradation of “aged” ethoprophos residues

The degradation patterns of fresh and aged ethoprophos residues are shown in Fig. 2. The concentration of aged ethoprophos residues at the time of inoculation was between 4.5 and 5.1 mg kg\(^{-1}\), which compares well with the concentration of ethoprophos (5 mg kg\(^{-1}\)) that was applied freshly to the initially untreated samples, since recovery of this fresh addition was ca. 96% (4.8 mg kg\(^{-1}\)). Initial degradation of both fresh and aged ethoprophos residues was faster in the inoculated samples compared with that in the corresponding controls. Less than 30% of the initial concentration of ethoprophos was recovered from the inoculated soils after 1 day, compared with >90% recovery from the uninoculated samples. Subsequently, in both inoculated and uninoculated treatments, significantly less \( (P < 0.05) \) ethoprophos was recovered from the freshly treated samples, than from the aged samples. In the inoculated samples, just 1% of the freshly applied ethoprophos was recovered after 5 days compared with about 12% recovery in the corresponding samples with aged ethoprophos residues. In the uninoculated samples, recoveries of ethoprophos after 5 days were 78 and 90% of the fresh and aged residues, respectively.

The amount of ethoprophos present in the water phase in the uninoculated control samples which had received a fresh dose of the nematicide was equivalent to approximately 15–16% of the total extractable residues after 3 days (Table 2). In comparison, <12% of the aged ethoprophos residues were present in the water phase at this time. When inoculated with EDI, no ethoprophos was found in the water phase (limit of detection was 5 \( \mu \)g l\(^{-1}\)) in soils containing either fresh or aged ethoprophos residues, even though significant solvent-extractable residues were still detectable in the latter soils.

### 3.3. Effect of temperature

The degradation patterns of ethoprophos in soil inoculated with EDI and incubated at 5, 20 or 35°C are presented in Fig. 3. The rate of degradation of ethoprophos was more rapid in all inoculated samples compared with that in the uninoculated controls, irrespective of temperature. Less than 5% of the initial dose was recovered from the samples incubated at 20 or 35°C when measured 2 days after inoculation. In contrast, 97 and 78% recovery was recorded in the corresponding uninoculated samples at the same time. Degradation was significantly slower in the inoculated samples incubated at 5°C, but even at this low temperature, degradation was complete after 13 days. In the uninoculated samples at 5°C, over 90% of the initially applied ethoprophos was still present after 17 days.

![Fig. 3. Ethoprophos degradation in soil inoculated (open symbols) with *P. putida* epl and in uninoculated samples (closed symbols) which were incubated at 5 (□, ■ □ ■), 20 (△, ◆ △ ◆) and 35°C (○, ● ○ ●). Each value is the mean of three replicates. Error bars represent the standard deviation of the mean. Data for the inoculated samples at 20 and 35°C were coincident.](image-url)
3.4. Effect of soil moisture

Degradation patterns of ethoprophos in inoculated and uninoculated soils varying in moisture content are shown in Fig. 4. Degradation was more rapid in all inoculated samples compared with that in the corresponding controls at all moisture contents. Similar patterns of degradation were observed in the inoculated samples when moisture content was maintained at 40 or 60% of the WHC, and less than 5% of the initial amount was still present 2 days after application. In contrast, there was still greater than 90% recovery of the applied ethoprophos from the corresponding uninoculated samples at the same time. Significantly slower degradation was observed in the inoculated samples which were incubated at 20% of the WHC, where about 8% of the initial dose was still present after 18 days. In contrast, more than 90% of the initial dose was recovered from the corresponding control samples at this time.

3.5. Effect of pH

The patterns of degradation of ethoprophos in soils with a range of pH are presented in Fig. 5. There was a rapid loss from the inoculated samples with pH 6.8 (Sheep Pens, Table 1) and 8.3 (Thessaloniki, Table 1) when compared with the corresponding uninoculated controls. Less than 2.5% of the initial dose was recovered 5 days after initial application in the inoculated Sheep Pens and Thessaloniki soil samples in comparison with more than 75% ethoprophos recovery from the corresponding uninoculated controls. Soil residues of ethoprophos were significantly different between the

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ethoprophos in water phase (mg l⁻¹)</th>
<th>Total extractable ethoprophos (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated fresh 1</td>
<td>3.890 (15.8)</td>
<td>4.215</td>
</tr>
<tr>
<td>Uninoculated fresh 2</td>
<td>3.582 (15.0)</td>
<td>4.075</td>
</tr>
<tr>
<td>Uninoculated aged 1</td>
<td>3.113 (11.8)</td>
<td>4.492</td>
</tr>
<tr>
<td>Uninoculated aged 2</td>
<td>2.624 (10.4)</td>
<td>4.295</td>
</tr>
<tr>
<td>Inoculated fresh 1</td>
<td>0.022 (4.2)</td>
<td>0.090</td>
</tr>
<tr>
<td>Inoculated fresh 2</td>
<td>0 (ND)</td>
<td>0.067</td>
</tr>
<tr>
<td>Inoculated aged 1</td>
<td>0 (ND)</td>
<td>0.854</td>
</tr>
<tr>
<td>Inoculated aged 2</td>
<td>0 (ND)</td>
<td>0.667</td>
</tr>
</tbody>
</table>

* Figures in brackets give percentage of the total extractable residues.
$^b$ ND: not detectable.
inoculated samples of these two soils \( (P < 0.05) \) only when measured 1 day after inoculation. No significant difference \( (P = 0.05) \) in soil residues of ethophrophos was observed between inoculated and uninoculated samples of the soil from Northern Greece which had the lower pH (5.4). In the uninoculated samples, the general order of soil residues was pH 5.4 > pH 8.3 > pH 6.8.

3.6. Effect of soil organic matter content

The dissipation patterns of ethophrophos in inoculated and uninoculated control soils with different organic matter contents are shown in Fig. 6. Degradation was more rapid in all inoculated samples relative to their corresponding uninoculated controls. The most rapid rates of degradation were observed in the inoculated Soakwaters (soil 4, Table 1) and Sheep Pens (soil 2, Table 1) samples whose organic matter contents were 0.3 and 2.3\%, respectively. Degradation of ethophrophos was complete within 5 days in the inoculated Soakwaters samples, at that time there was still over 70\% recovery of the applied dose in the corresponding uninoculated control. Degradation of ethophrophos in the inoculated soil from Brimstone farm (soil 5, Table 1), which was characterized by a relatively high organic matter content, was markedly slower than that observed in the other two inoculated soils. In the uninoculated samples, the order of residues was: Brimstone > Sheep Pens > Soakwaters, indicating a trend towards slower degradation with increasing organic matter content.

4. Discussion

Our study confirmed that the EDI \( P. \ putida \) epI was capable of successfully removing ethophrophos residues when reintroduced into the soil. There was a positive relationship between inoculum size and rate of ethophrophos degradation in Sheep Pens soil. Degradation proceeded without a lag phase when approximately \( 10^6 \) cells of \( P. \ putida \) epI were introduced into the soil (Fig. 1). However, a short lag phase of 1–2 days was evident when \( 10^5 \) or \( 10^4 \) cells g\(^{-1}\) were added to the soil. The lag phase observed could indicate a requirement for microbial proliferation, although ethophrophos diffusion, or cell transport limitation, could also be reasons for the initial slow degradation. These data agree with the previous findings of Comeau et al. (1993), who noted a significant reduction in the degradation rate of 2,4-D in soils when inoculum densities lower than \( 10^6 \) cells of \( Pseudomonas \) (\( Bulkholderia \) currently) \( cepacia \) g\(^{-1}\) soil were introduced. The authors attributed this reduced degradation rate to a combination of biological predation, bacterial diffusion limitations, and physical barriers limiting pesticide bioavailability.

An important factor, which determines the effectiveness of any bioremediation system for soil, is the bioavailability of aged pollutant residues. The ethophrophos-degrading strain was able to rapidly degrade both aged and fresh ethophrophos residues (Fig. 2). After an initial rapid phase of degradation, however, dissipation rates were significantly reduced in soils containing the aged residues. Similar results were reported by Cullington and Walker (1999), when a diuron-degrading bacterium was added to soil containing aged diuron residues. However, in those studies, no parallel tests were made with freshly added-diuron. Studies with a range of pesticides have demonstrated that increased residence time of a pesticide in soil can lead to an apparent increase in adsorption and, hence, a reduction in bioavailability (Walker 1987; Blair et al., 1990; Gaillardon et al., 1991). This has been explained by slow diffusion of solute to sites in the soil matrix, and it has been further suggested that these sites may be inaccessible to microorganisms (Scribner et al., 1992). Several workers have shown that the rate of biodegradation of pesticides is related to bioavailability in the aqueous phase, with restricted rates of release from the adsorbed phase leading to reduced rates of degradation over time (Guerin and Boyd, 1992; Sims et al., 1992). The initial fast degradation phase observed in our experiments with both aged and fresh residues (Fig. 2) may be explained by the rapid degradation of the readily bioavailable amounts of ethophrophos. The results suggest that a fraction of the total ethophrophos present in the soil containing aged residues was located in well-protected sites and not.

![Fig. 6. Ethophrophos degradation in soils with 0.3\% (□, ■■■■), 2.3\% (△, ■Δ■Δ) and 8.5\% (○, ■○■○) organic matter content, inoculated (open symbols) or uninoculated (closed symbols) with \( P. \ putida \) epI, respectively. Each value is the mean of three replicates. Error bars represent the standard deviation of the mean.](image-url)
readily available for desorption into the soil solution. This suggestion is supported by the measurements of water phase concentrations of ethophosphos that were made 3 days after inoculation (Table 2). Less ethophosphos was present in the soil solution in the uninoculated controls containing aged residues than in the uninoculated samples containing a fresh addition of ethophosphos at a similar concentration. In addition, there were no measurable residues of ethophosphos 3 days after inoculation in the soils containing aged residues even though significant solvent extractable residues of the nematicide were still present in these soils. Our results are in partial agreement with those of Kontchou and Gshwind (1995), who found that prior incubation of atrazine in soil for 3 weeks significantly lowered its bioavailability and limited its degradation by a *Pseudomonas* strain. However, the difference in degradation rate observed in the atrazine study was much greater than that observed with ethophosphos. The atrazine study used a highly organic soil (36%) in comparison with our study where the organic matter content of the soil was 2.5%. It may be that similar studies of ethophosphos degradation in an organic soil would magnify the differences in the degradation rate between fresh and aged ethophosphos residues.

A variety of soil factors, such as pH and organic matter content, and environmental factors, such as soil temperature and moisture, can significantly influence the success or failure of bioremediation. Degradation of ethophosphos by *P. putida* epI was rapid at 20 and 35°C (Fig. 3). The degrading ability of *P. putida* epI in soil was somewhat reduced at 5°C, but even at this low temperature, degradation was complete within 13 days. The EDI was equally successful in degrading ethophosphos when soil moisture was maintained at 40 or 60% of the soil WHC, equivalent approximately to ethoprophos when soil moisture was maintained at 40 days. The EDI was equally successful in degrading ethophosphos at a similar rate in the other two soils with pH 6.8 and 8.3 (Fig. 5). The faster degradation of ethophosphos observed in the uninoculated Sheep Pens samples relative to the corresponding samples from Thessaloniki may be attributed to the higher organic matter content of the latter (3.5%), which increases ethophosphos persistence by increasing adsorption (Jones and Norris, 1998). These observations further illustrate the significance of pH in the development of enhanced biodegradation in soil, as recorded with other soil-applied pesticides (Read, 1986; Suett et al., 1996).

The effect of organic matter content on the degrading-ability of *P. putida* epI was confirmed in a study with three different soils (Fig. 6). Degradation of ethophosphos was more rapid in Soakwaters subsoil with low organic matter (0.3%) and in Sheep Pens soil with an intermediate amount of organic matter (2.3%) than in the relatively high organic matter soil from Brimstone farm (8.5%). The faster rates of degradation achieved by *P. putida* epI in the Soakwaters subsoil were expected because of the very low adsorption capacity and, hence, increased bioavailability of the pesticide in the subsoil. In addition, the limited size and diversity of the native microflora would offer a less competitive environment for the introduced bacteria. In a similar way, a reduced rate of degradation was expected in the soil from Brimstone farm (OM 8.5%), since the high sorption capacity of this soil will reduce the amount of pesticide in the solution phase and, therefore, reduce its bioavailability. In similar studies, Greer and Shelton (1992) observed reduced rates of degradation in a soil with high organic matter content inoculated with a 2,4-D-degrading isolate. They attributed this to the decreased amounts of soluble pesticide available for metabolism by the degrading bacteria. Ethophosphos is less water-soluble and more strongly adsorbed to soil organic matter than 2,4-D; hence, more pronounced effects may be expected (Smelt et al., 1987). A somewhat faster degradation of ethophosphos observed in the uninoculated control samples of the Soakwaters subsoil may, in part, be attributed to its alkaline pH (8.1), which favors chemical hydrolysis (Jones and Norris, 1998). In the earlier pH study (Fig. 5), the relatively slow degradation of ethophosphos observed in the uninoculated soil sample from the Thessaloniki site, which had similarly high pH (8.3) to the Soakwaters subsoil, may further illustrate the protective effect of soil organic matter, since this soil had a much greater organic content (3.5%).

The effects of pH on degradation ability were studied in soils from Northern Greece, Sheep Pens, HRI and Thessaloniki, Greece which were of similar texture and organic matter content, but varied widely in pH (Table 1). *P. putida* epI failed to degrade ethophosphos in the soil from Northern Greece which had the lower pH (5.4). However, ethophosphos degraded at a similar rapid rate in the other two soils with pH 6.8 and 8.3 (Fig. 5). The faster degradation of ethophosphos observed in the uninoculated Sheep Pens samples relative to the corresponding samples from Thessaloniki may be attributed to the higher organic matter content of the latter (3.5%), which increases ethophosphos persistence by increasing adsorption (Jones and Norris, 1998). These observations further illustrate the significance of pH in the development of enhanced biodegradation in soil, as recorded with other soil-applied pesticides (Read, 1986; Suett et al., 1996).
5. Conclusions

A *P. putida* strain was able to rapidly remove fresh and aged ethoprophos residues when it was introduced into soils at densities higher than $10^6$ cells g$^{-1}$. The ability of this strain to degrade ethoprophos even in adverse environmental conditions (low moisture, low temperature), and in a wide range of soils with different properties (pH, organic matter), suggests that it may be potentially useful for the decontamination of sites where spillage has occurred. Its inability to degrade ethoprophos at low pH could be overcome by adjusting the pH of the soil to values favorable for the bacteria. All the present experiments were made under ideal laboratory conditions where the microbial inoculum was uniformly mixed with the soil. Such uniformity would be impossible to achieve in the field, thus limiting the possibilities of bioaugmentation in practice. Further studies should be conducted under field conditions, focusing on issues, such as distribution of residues, ageing of residues, and survival of the degrading ability of the bacterium in the absence of ethoprophos.

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