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Accelerated Parathion Degradation in Soil Inoculated With Acclimated Bacteria Under Field Conditions

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Abstract. The feasibility of decontaminating soil at parathion spillage or disposal sites by inoculation with a highly acclimated culture of parathion-degrading bacteria was demonstrated under *in situ* field conditions. The acclimated culture (AC), capable of utilizing parathion as a sole carbon and energy source, was inoculated into Yolo silt loam soil in which parathion was applied at rates up to 5000 kg/ha. The AC was shown to be capable of completely degrading parathion in soil containing up to 1250 kg/ha of parathion within 35 days. A slower rate of parathion degradation by the AC was observed when the pesticide was applied as the commercial 46.5% emulsifiable concentrate than when applied as the 98% technical grade. The ability of the AC to degrade parathion deteriorated at application rates greater than 1250 kg/ha. The AC may have been adversely affected by the accumulation of the parathion hydrolytic products, *p*-nitrophenol and ionic diethyl thiophosphate, which were tentatively identified in soil samples.

Concern for the persistence of synthetic toxicants in nature has primarily focused on means of controlling their release and dispersion. Little interest has developed for means of decontamination other than controlled waste treatment processes. Daughton (1976) has reviewed the literature on the use of acclimated microorganisms as a possible means for enhancing the dissipation of pollutants. The accelerated degradation of high levels of pesticide by microbial inoculation to non-sterilized laboratory soil was demonstrated for the first time by Daughton and Hsieh (1977a).

We have further investigated the use of microbial inocula in accelerating the degradation of pollutants, using the highly toxic organophosphorus insecticide parathion [*O,O*-diethyl-*O*-(4-nitrophenyl)phosphorothioate] as the model compound. Organophosphorus pesticides are generally considered to be rela-

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tively nonpersistent since they are usually degraded quite rapidly when applied at rates necessary for insect control (Chisholm *et al.* 1955, Lichtenstein and Schulz 1964). However, a recent study has shown parathion to be very persistent in soil when present at the high concentrations resulting from spillage or disposal (Wolfe *et al.* 1973). The heavy annual usage of parathion of almost one-half million kg in California alone (Pesticide Use Report 1977) poses not only the high probability of spills but also the problem of safely disposing of the large amounts of excess wastes such as that generated by the used pesticide containers.

The long-term hazards associated with spillage or disposal of parathion has led us to investigate the feasibility of detoxifying high parathion levels in soils under *in situ* field conditions by the addition of a highly acclimated bacterial culture. The ability of this culture to greatly accelerate the degradation of up to 5000 ppm parathion in non-sterilized laboratory soil has previously been reported (Daughton and Hsieh 1977a). Only a few researchers have investigated the use of microbial inocula for decontamination under *in situ* field conditions, and they have all focused on oil degradation (Miget 1973, Stewart 1975).

Materials and Methods

Reagents

Parathion (PTN), as technical grade, 98% purity, and as commercial 46.4% parathion emulsifiable concentrate (PTN-EC), were kindly supplied by Stauffer Chemical Co., Richmond, CA. The potassium salt of diethyl thiophosphate (DETP) was obtained from American Cyanamid Co., Wayne, NJ. Diethyl hydrogen phosphate (DEP) and *p*-nitrophenol (NP) were obtained from Eastman Chemical Co., Rochester, NY. Ethereal ethanolic diazomethane was prepared from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., San Leandro, CA) using an Aldrich Diazomethane Generator (Daughton *et al.* 1976). (Caution: Diazomethane is highly explosive and toxic.)

Acclimated Culture

The enrichment, description and maintenance of the highly acclimated bacteria have been detailed (Daughton and Hsieh 1977b). The acclimated culture (AC) was shown to contain a strain of *Pseudomonas stutzeri* capable of rapidly hydrolyzing PTN to DETP and NP; the resultant NP was utilized as a sole carbon and energy source by the other member of the AC, a strain of *Pseudomonas aeruginosa*.

The AC was grown in minimal salts medium (containing in grams per liter: K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; and $FeCl_3 \cdot 6H_2O$, 0.007) with a continuous feed of parathion as a sole carbon and energy source (Daughton and Hsieh 1977b). The AC was harvested by centrifugation and resuspended in 50 m*M* K_2HPO_4 (pH 7.2) for inoculation into soil.

Field Plot

The study was conducted from September to early November in a field plot located in the agricultural research fields of the University of California, Davis. The soil was classified as Yolo silt loam and has been previously characterized (Table 1) (Andrews 1972). Soil moisture to a depth of 4 in.

Table 1. Characteristics of Yolo Silty Loam Soil as analyzed by the Department of Soil and Plant Nutrition, University of California at Davis, 1972

Depth From surface	Silt	Clay	Sand	Organic carbon	Total nitrogen
0 to 2 in.	46.4%	24.9%	28.7%	1.38%	0.125%
2 to 8 in.	46.8%	27.1%	26.1%	1.24%	0.092%

was monitored throughout the study. The water content in the top 4 in. of soil was 7% (wt/wt) at the beginning of the study. During the study, total precipitation was approximately 3.6 cm, soil temperature at a depth of 1 in. ranged from 7° to 33°C, and air temperature ranged from 6° to 36°C.

"Columns" of soil were isolated *in situ* by driving 4 in. lengths of 1.25 in. o.d. polyvinyl chloride (PVC) pipes into the soil plot; the pipes were spaced 2 in. apart. The open-ended pipe allowed the soil columns to transpire and maintain approximately the same water and temperature regimes as the surrounding soil. Since PTN does not migrate easily in soil, it could be recovered quantitatively, when present, from the confined soil columns. When inoculated, soil columns received 5 ml of the AC suspension at a rate of dry cell mass equivalent to 1.25 kg/ha; control soil columns received 5 ml of the 50 m*M* phosphate buffer.

Analysis

Soil was sampled by removing the PVC pipe containing the intact soil column and placing the entire sample into a 250-ml screw-capped polyethylene centrifuge bottle for extraction. After the addition of 5 ml of water to each sample, PTN was extracted with 200 ml of acetone/hexane (1:1 v/v) by shaking for two min in the centrifuge bottle; the resultant emulsion was broken by centrifugation. The top layer was removed and washed with water. The organic phase that remained after washing was adjusted to an appropriate volume and analyzed for PTN by gas-liquid chromatography; no interferences were incurred. Recoveries from soil were greater than 90% for PTN application rates of 10 kg/ha and above.

For experiment 5, soil samples each received 50 ml of water and were acidified with HCl to a pH value of 3.0 prior to extraction with 200 ml of acetone:hexane (1:1 v/v). The organic phase contained NP and PTN. The organic layer was washed with HCl-acidified water (pH 3.0) followed by extraction with 50 ml of aqueous 1% Na_2CO_3. The organic phase was adjusted to an appropriate volume for PTN analysis. The Na_2CO_3 extract was membrane-filtered (0.45 μm pore diameter) to remove soil colloids and adjusted to pH 7.5; NP was determined by measuring the absorbance at 410 nm on a Spectronic 20 colorimeter (Bausch and Lomb).

DETP and DEP were extracted from the appropriate soil columns of experiment 5 with two 50-ml portions of water. After centrifuging the samples, the aqueous extracts were decanted and pooled. A 5-ml portion of the pooled extract was membrane-filtered (0.45 μm pore diameter) into a 50-ml glass centrifuge tube and acidified with 5*N* HCl to a pH value of less than 1.0. Sodium chloride was added to saturation, a 5-ml portion of ethyl acetate was added, and the mixture was vortexed for one min. The organic layer was transferred to a 25-ml volumetric flask. The ethyl acetate extraction was repeated and the organic phases were pooled. Ten drops of methanol were added to the pooled organic extracts followed by the dropwise addition of excessive diazomethane as determined by a persistent yellow color. Unreacted diazomethane was eliminated after two min. by the addition of 1% acetic acid in hexane. The extract was then diluted to 25 ml with hexane for analysis of the methyl esters of DETP and DEP by gas-liquid chromatography (Daughton 1976, Daughton *et al.* 1976). DETP in soil was tentatively identified as the methyl ester by cochromatography with methylated authentic material and detection with a phosphorus thermionic detector.

A Packard Becker model 417 gas-liquid chromatograph equipped with a phosphorus thermionic detector was used for the analysis of PTN and the methylated esters of DETP and DEP. A glass column, 1 m × 2 mm i.d., packed with 10% Apiezon N on 60/80 mesh Gas Chrom Q was

employed for PTN analysis. A glass column, 1.8 m × 2 mm i.d., packed with equal mixtures of 15% QF-1 and 10% DC-200 on 80/100 mesh Gas Chrom Q was used for the analysis of methylated DETP and DEP

Experiment 1

A trial experiment was conducted, to determine if the AC could accelerate PTN degradation in soil under actual field conditions. Each soil column received technical PTN dissolved in acetone at a rate of 75 kg/ha of active ingredient (a.i.). After evaporation of the acetone, the AC was inoculated to half of the soil columns; the other columns served as controls. Each of these groups was further divided into two groups; one group periodically received water to maintain soil moisture above 20% and the other received no additional water. The latter group reached a water content of 13% with the initial addition of 5 ml of buffer, but after 15 days the water content had decreased to less than 8%. Duplicate samples from all four groups were taken after inoculation and/or addition of PTN on days 0, 3, 7, 11, and 15 for PTN determination. The percentages of PTN remaining on days 3, 7, 11, and 15 were calculated on the basis of the average amount recovered from all soil columns on day 0, which was normalized to 100%. In addition, the viability and activity of the AC in soil was confirmed by re-isolating the culture after 10 days of soil incubation.

Experiment 2

This experiment was designed to determine the effect of exogenous organic matter in the soil on the ability of the AC to degrade PTN in the field. The experiment involved amending soil columns with 0.5 g of milled rice straw incorporated by mixing to a depth of one in. Technical PTN was again applied at 75 kg/ha. Sample columns were removed 0, 3, 7, 11, and 15 days after initiation; the percentage of PTN remaining was calculated on the basis of the average amount of PTN recovered from all soil columns on day 0, which was normalized to 100%. In this experiment, and those that followed, all soil columns received water daily to maintain water content above 20% and were periodically mixed to a depth of one inch.

Experiment 3

This experiment determined the duration that the AC could retain its PTN-degrading activity in the field when PTN was not present. The AC was added to two groups of 30 soil columns each; one group was amended with 0.5 g of rice straw per soil column, and the other group received no amendment. On the same day as AC inoculation, PTN at a rate of 75 kg/ha was added to six of the soil columns from each group. After three days, another set of six soil columns from each group was fortified with 75 kg/ha PTN; this procedure was repeated for sets of six soil columns from both groups at 8, 14, and 21 days after AC inoculation. The retention of the ability of the AC to degrade PTN in each set was determined by extracting two soil columns from each group of six columns on 0, 5, and 10 days after fortification with PTN. Controls for both rice straw-amended and non-amended soils received PTN at a rate of 75 kg/ha, but they were not inoculated with the AC; these were extracted in duplicate 0, 5, 10, and 21 days after PTN addition.

Experiment 4

The promising results of Experiments 1-3 led to a field test to determine the range of PTN application rates that could be effectively degraded by the AC. Both technical PTN and PTN-EC were tested at rates of 250, 1250, 2500, and 5000 kg(a.i.)/ha; technical PTN was applied as an acetone solution, and PTN-EC was applied as neat material. After PTN fortification, the soil columns were

allowed to equilibrate for 14 hr prior to day 0 sampling. The AC was then inoculated to one-half of the remaining soil columns; the other half served as noninoculated controls. Duplicate samples were extracted on days 0 and 21. Soil groups receiving 1250 and 2500 kg/ha had additional duplicate samples extracted on day 35. The percentages of parathion remaining in soils on days 21 and 35 were calculated on the basis of the amount recovered on day 0 for each application rate.

Experiment 5

A final experiment was attempted, in order to quantify the soil concentrations of the AC-mediated parathion hydrolysis products NP and DETP. The presence of ionic diethyl phosphate, which would indicate desulfuration of PTN and/or DETP, was also investigated. Soil columns received technical PTN at a rate of either 1250 or 2500 kg/ha. One-half of the soil columns at both application rates were inoculated with the AC; the remaining columns served as controls receiving only buffer. On days 0, 3, 9, and 15 after inoculation, two pairs of duplicate samples were taken from each of the four groups for analysis. On each sampling day, one duplicate pair from each group was analyzed for PTN and NP and the other was analyzed for DETP and DEP.

Results and Discussion

Experiment 1

The average amount of PTN recovered from Exp. 1 soils on day 0 was greater than 90% of that applied. The percentage of PTN remaining in the soil versus time is shown for different treatments (Figure 1). After 15 days, at least 80% of the PTN remained in the non-inoculated control soils. These high recoveries indicated that little PTN disappeared, due to indigenous microbial activity, chemical hydrolysis, volatilization, leaching, and time-dependent sorption to soil colloids. Soil inoculated with the AC, but receiving no additional water also showed little PTN loss. In contrast, less than 60% of the PTN was recoverable three days after application to AC-inoculated soil in which the water content was maintained above 20%; less than 30% of the applied PTN remained in this inoculated soil after 15 days. These results indicate that the AC was capable of accelerating PTN dissipation in the field, but a requirement for a minimum soil water content above normal field levels existed for the AC to function effectively. Therefore, in all of the following experiments, soil water content was maintained above 20% by periodic additions.

Another objective of this investigation was to re-isolate the AC from inoculated soils after 10 days incubation to verify that its activity was associated with viability. Soil inoculated with AC yielded high counts of nonfilamentous colonies, most of which were motile rods when isolated 10 days after inoculation and plated on Bacto Pseudomonas Agar F (Difco Laboratories, Detroit). These microorganisms were capable of rapidly hydrolyzing 100 ppm PTN in minimal salts medium in batch culture; the resultant NP was subsequently degraded and growth of gram negative rod-shaped bacteria ensued. This culture could also be maintained in the PTN chemostat described by Daughton and Hsieh (1977b). In contrast, cultures derived from the non-inoculated soil showed no PTN-degrading activity.

The soils in Exp. 1 that received the periodic additions of water became

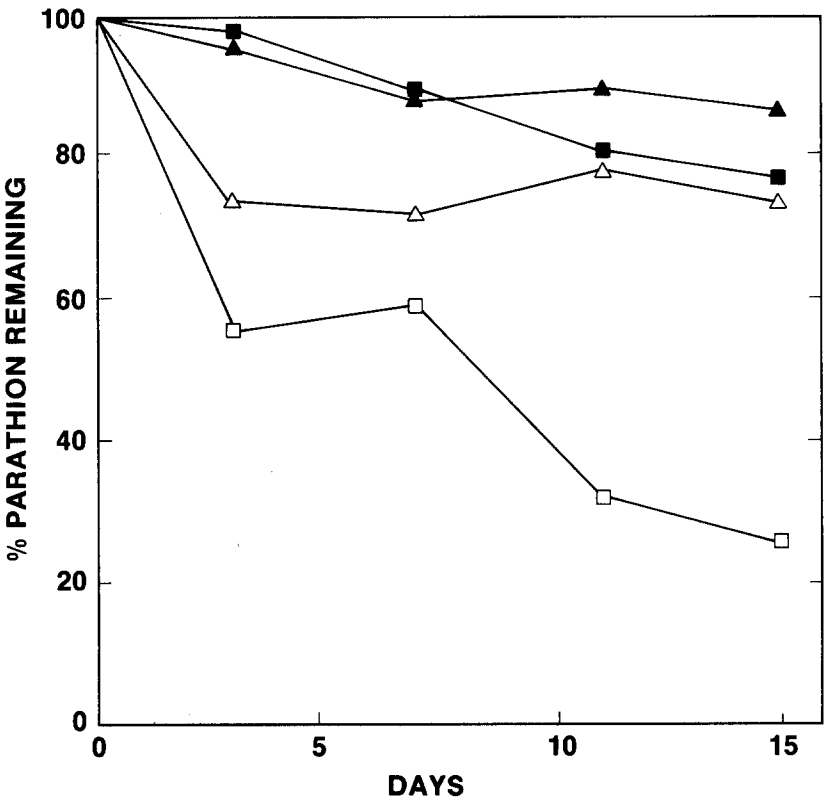

Fig. 1. Parathion remaining (% of initial 75 kg/ha) versus days of incubation in soil with and without the acclimated culture (AC).

Soil Treatments:

△ inoculated with AC; water not added.

▲ control, receiving no AC; water not added.

□ inoculated with AC; water content maintained at 20%.

■ control, receiving no AC; water content maintained at 20%.

highly compacted. This soil aggregation may have hampered degradation of PTN by the AC within the inoculated soil by limiting oxygen diffusion and/or PTN availability. The soils in the following experiments (all of which were maintained above a 20% moisture content) were, therefore, periodically mixed with a spatula to a depth of one inch to disrupt this aggregation.

Experiment 2

The levels of PTN remaining in Exp. 2 soils versus time are shown in Figure 2. The AC accelerated PTN degradation much more efficiently in these periodically mixed soils than in the unmixed soils of Exp. 1; less than 30% of the PTN remained three days after application to inoculated soil, and only traces

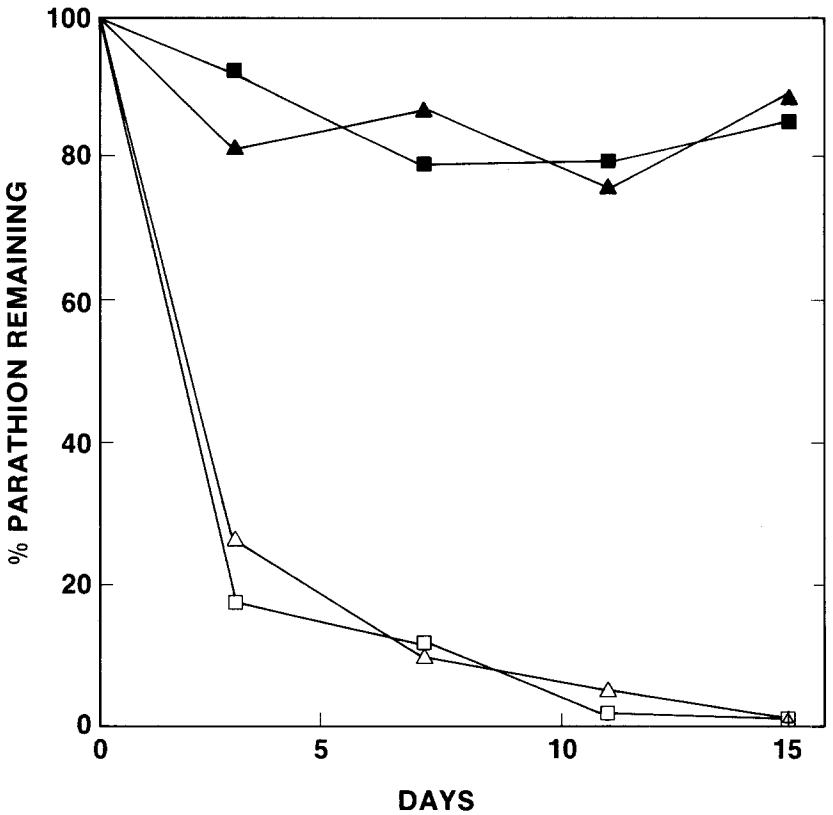

Fig. 2. Parathion remaining (% of initial 75 kg/ha) versus days of incubation in soil with and without the acclimated culture (AC).

Soil Treatments:

△ inoculated with AC.

▲ control, receiving no AC.

□ inoculated with AC, amended with rice straw.

■ control, receiving no AC, amended with rice straw.

All soils were maintained at a moisture content of 20% by the daily addition of water, and periodically mixed to a depth of one inch.

were recovered after 15 days. PTN in non-inoculated soils continued to be persistent regardless of the mixing; at least 80% of the insecticide was recoverable 15 days after application. In actual practice, soil contaminated with PTN may have to be periodically plowed to increase aeration and homogeneity for optimum PTN degradation by the AC.

Amending the soil with rice straw had no significant effect on the loss of PTN from either AC-inoculated or non-inoculated soils (Figure 2). Organic compounds in the soil may slow the loss of PTN by being preferentially metabolized by the AC or by being utilized by the indigenous microbiota, which would then compete with the AC for other nutrients; results indicated that rice straw apparently does not have these effects.

Experiment 3

The retention of the PTN-decontamination ability of the AC under field conditions in the absence of the pesticide was examined (Exp. 3). The results are shown in the first five graphs of Figure 3. The percentage of PTN remaining was calculated on the basis of the average amount of the 75 kg/ha PTN recovered from the samples of all five groups that were extracted on the day of PTN fortification (91 ± 2.9% at the 95% confidence interval), which was normalized to 100% remaining. Control soils receiving PTN, but no AC, showed recoveries of PTN greater than 75% after 21 days (not shown in Figure 3); this indicated all PTN losses due to effects other than those mediated by the AC.

The results of Exp. 3 indicated that the AC maintained most of its PTN-degrading activity in the absence of PTN for up to three weeks only in soils that had been amended with milled rice straw. The AC in soils not amended with rice straw began to lose effectiveness eight days after inoculation. A complete

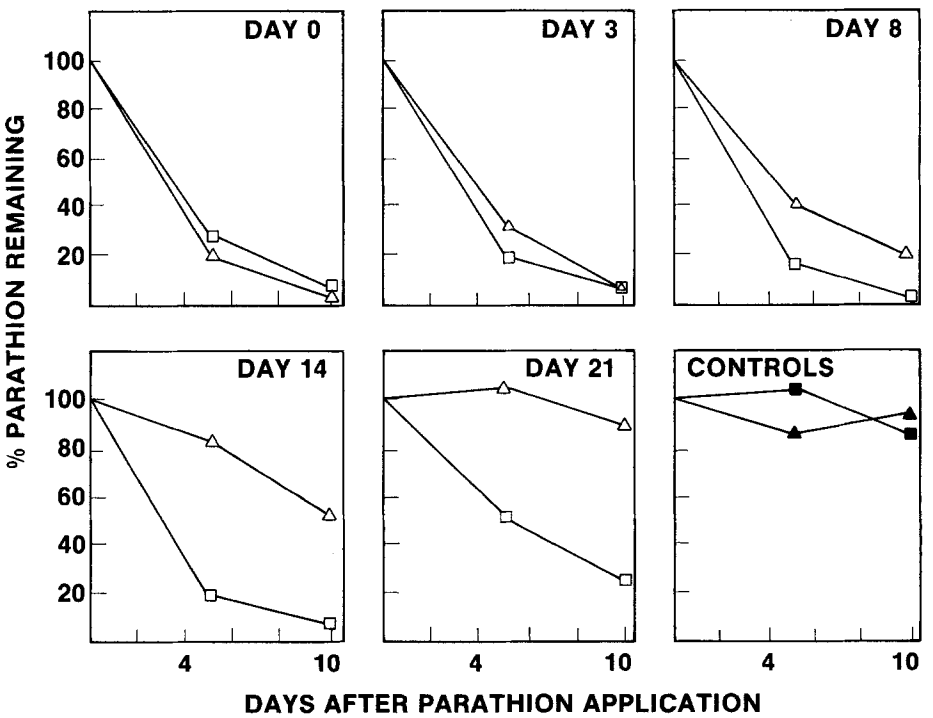

Fig. 3. Functional longevity of the acclimated culture (AC) in the field. Parathion remaining (% of initial 75 kg/ha) in soil versus days after parathion fortification. Each of the graph labels, Day 0, 3, 8, 14, or 21, indicates the day of incubation of the AC in soil prior to fortification with parathion.
Soil Treatments:
△ inoculated with AC.
▲ control, no AC added.
□ inoculated with AC, amended with rice straw.
■ control, no AC added, amended with rice straw.

loss of effectiveness by the AC in non-amended soil is indicated by the lack of accelerated disappearance of PTN when fortified to soils 21 days after inoculation. Amending the soil with rice straw, usually a poor carbon and energy source for the pseudomonad AC, may have enhanced survivability of the bacteria by providing microsites in which the AC could be protected from environmental stresses such as predation of fluctuations in temperature and moisture. The addition of straw along with the AC may allow for the establishment of toxic wastes disposal sites capable of receiving periodic parathion wastes with a minimal need for additional inoculations.

Experiment 4

The results of Exp. 4 (Figure 4) demonstrated the range of PTN applications that were effectively degraded by the AC. At all application levels, AC-

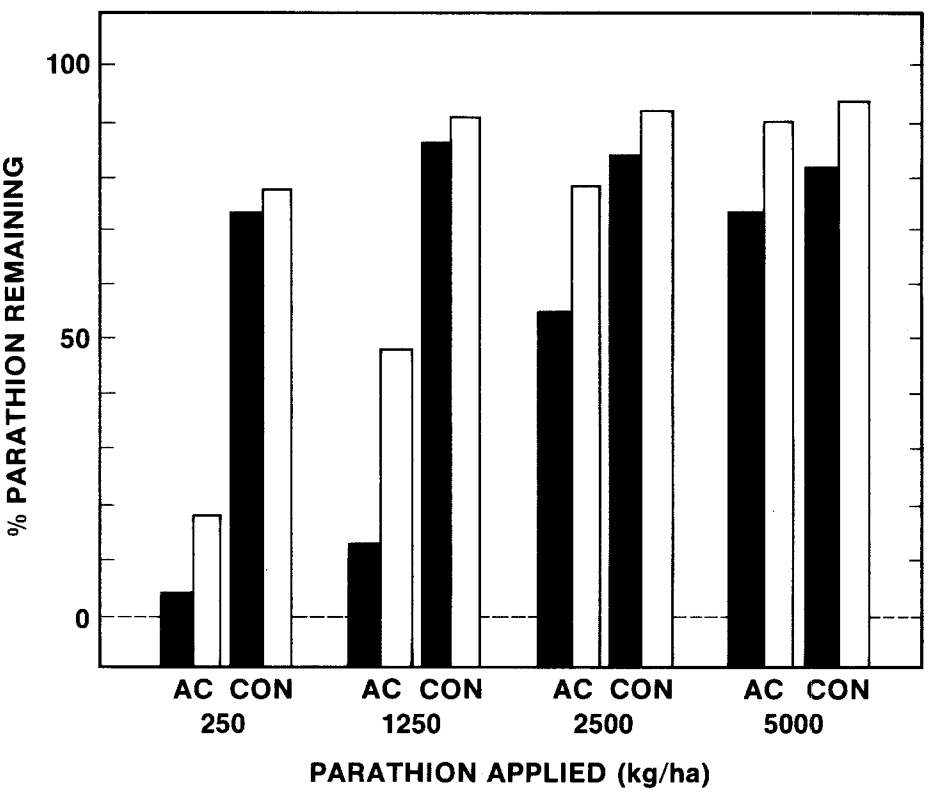

Fig. 4. Dissipation of high levels of technical parathion and parathion EC in inoculated and non-inoculated soils after 21 days.

White Bars = parathion emulsifiable concentrate (EC).

Black Bars = technical parathion.

AC = soil inoculated with the acclimated culture.

CON = controls, soil not inoculated.

inoculated soils showed greater PTN losses after 21 days than the non-inoculated control soils. The actual amount of PTN degraded after 21 days by the AC (parathion lost from AC-inoculated soil minus the loss from non-inoculated soil) at each application rate is shown in Figure 5. The AC was less capable of degrading PTN when applied as EC than when applied as technical grade at application rates greater than 250 kg/ha. This difference in degradation rates may be due to (i) a preferred metabolism by the AC for the detergents and/or xylenes of the EC, (ii) possible toxicity of these diluents toward the AC, or (iii) enhanced migration of PTN by the diluents, possibly allowing for increased sequestering of PTN by soil colloids.

The ability of the AC to degrade PTN deteriorated in the two application rates greater than 1250 kg/ha, thus indicating the possible toxicity of PTN or its metabolites toward the AC at these application levels (Figure 5). Soon after the initiation of this experiment, a transient yellow tinge was observed in those soils that had received technical PTN at rates of 2500 and 5000 kg/ha. The yellow color presumably indicated the presence of substantial amounts of the PTN-hydrolytic product NP which possesses known toxicity to bacteria (Daughton 1976).

In contrast to the percentage levels of parathion remaining after 21 days (Figure 4), the percentage levels of the pesticide remaining after 35 days in soils

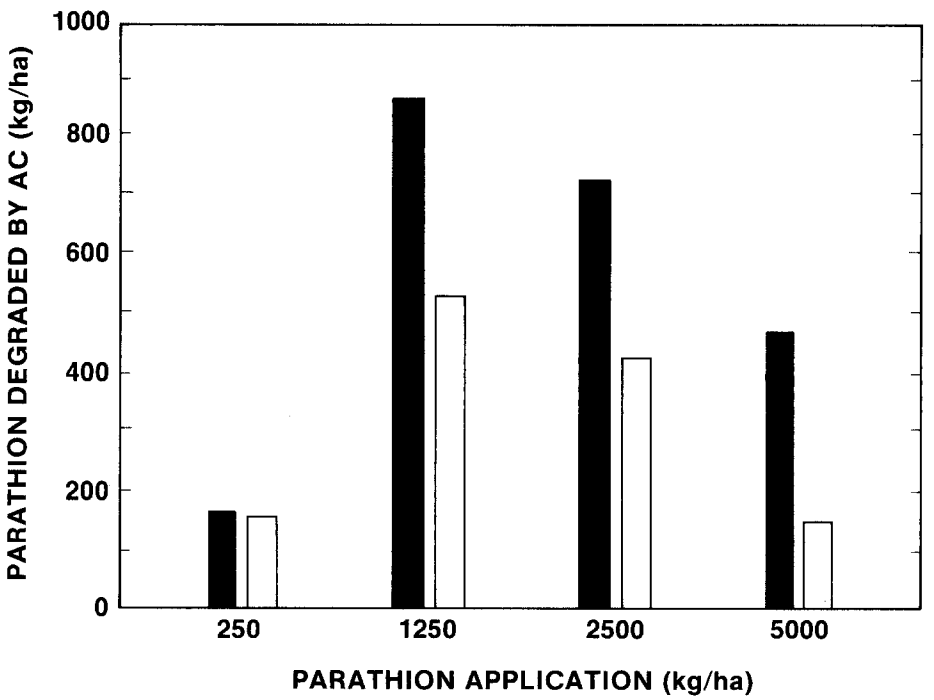

Fig. 5. The amount of parathion (kg/ha) degraded by the acclimated culture after 21 days incubation in soils receiving high levels of parathion.
White Bars = parathion emulsifiable concentrate.
Black Bars = technical parathion.

that received parathion at rates of 1250 and 2500 kg/ha are presented in Figure 6. The net amounts of PTN degraded by AC in 35 days (PTN lost from AC-inoculated soil minus the loss from non-inoculated soil) were 1000 kg/ha and 668 kg/ha for technical PTN and PTN-EC, respectively at the 1250 kg/ha application rate, and 750 kg/ha and 500 kg/ha for technical PTN and PTN-EC, respectively at the 2500 kg/ha application rate. The rate of PTN dissipation occurring after day 21 was essentially the same in both AC-inoculated and non-inoculated soils that received 2500 kg/ha technical PTN. These results indicate a nearly complete cessation of activity of the AC in further degrading PTN after 21 days at the 2500 kg/ha application rate.

Experiment 5

The AC-mediated production in soil of the PTN hydrolytic products DETP and NP was examined. Ionic diethyl phosphate was not recovered, presumably because little or no desulfuration of PTN or DETP occurred. Recovery of DETP in amounts stoichiometric with quantities of hydrolyzed PTN was not observed, probably due to the rapid leaching of this anion from the soil columns

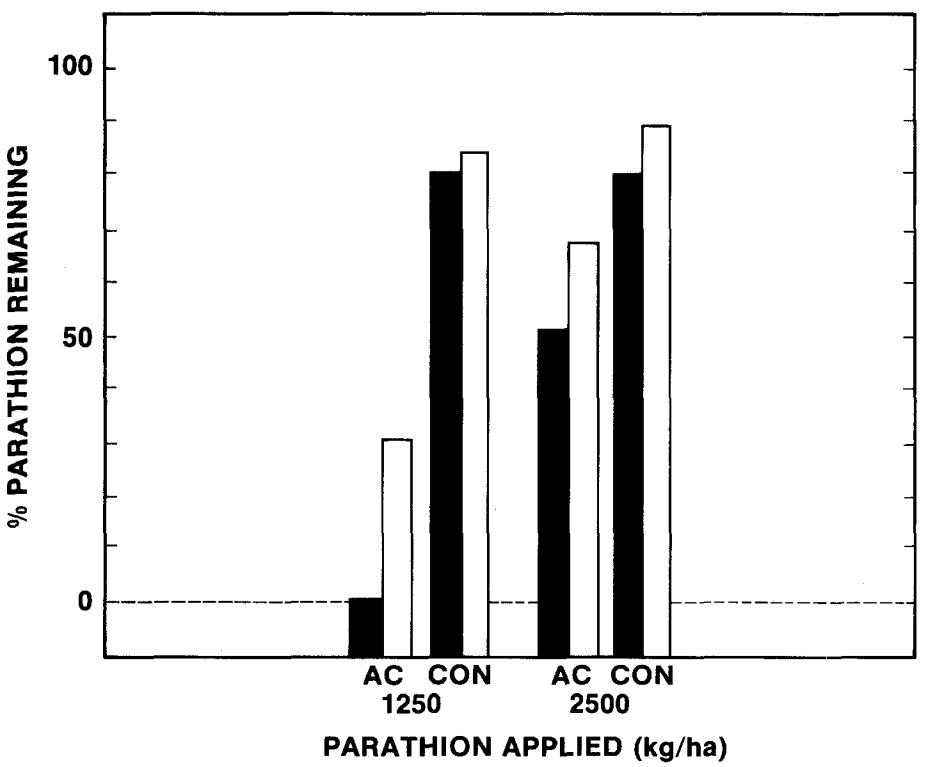

Fig. 6. Dissipation of high levels of technical parathion and parathion EC in inoculated and non-inoculated soils after 35 days. (Key as in Figure 4).

(Daughton *et al.* in press). Similarly, NP was not recovered in amounts stoichiometric with the quanities of hydrolyzed PTN since NP was probably used as a carbon and energy source by the AC and because of its mobility through the soil. Higher peak levels of both DETP and NP occurred in the AC-inoculated soil that received 2500 kg/ha technical PTN as compared to the inoculated soil that received only 1250 kg/ha PTN (Figure 7 a and b). The rate of PTN dissipation was initially much more rapid in the inoculated 2500 kg/ha soil

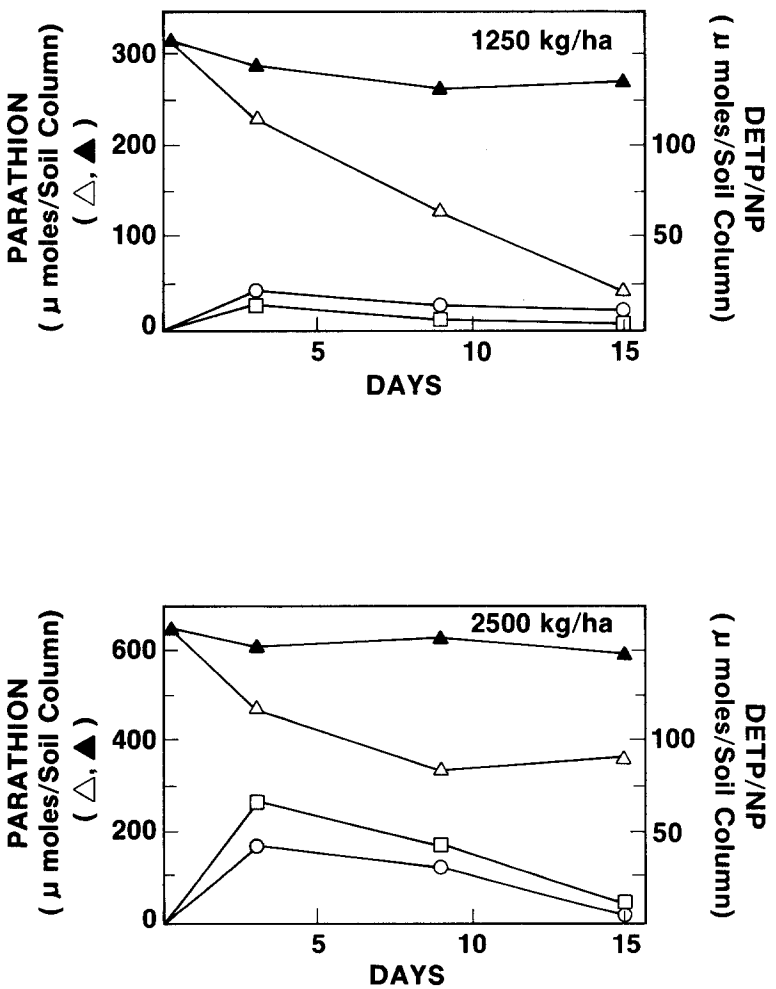

Fig. 7. Dissipation of parathion applied at 1250 kg/ha (a) and 2500 kg/ha (b) and the appearance of the hydrolytic products in soil inoculated with the acclimated culture (AC).

△ parathion in inoculated soils.
○ DETP in inoculated soils.
□ NP in inoculated soils.
▲ parathion in non-inoculated soils.
(values are not corrected for losses in controls)

than in the inoculated 1250 kg/ha soil. However, this initial rate began to decrease after the third day, and after nine days, no additional loss of PTN in the 2500 kg/ha soil was observed. The slowing of the PTN dissipation rate in the 2500 kg/ha soil may have been related to the relatively high levels of NP and DETP. Since both of these PTN hydrolytic products can be toxic to the AC (Daughton and Hsieh 1977b), their rapid production in the 2500 kg/ha soil may have overwhelmed the inoculum, resulting in the loss of ability to degrade PTN. The slower initial degradation rate of PTN in the 1250 kg/ha soil appears to have limited the accumulation of these hydrolytic products.

The results of Experiments 4 and 5 indicate that very high levels of PTN may limit the effectiveness of the AC in soil. Soil containing PTN levels above 1250 kg/ha may require more than one inoculation or mixing with noncontaminated soil to lower PTN levels prior to inoculation. The results presented herein suggest the possible efficacy of using specifically acclimated microorganisms as agents for decontaminating native environments.

The positive results of Experiments 1 and 2 are the first ever obtained for actual *in situ* accelerated pesticide decontamination under field conditions by inoculation with acclimated microorganisms. To our knowledge, the recovery of DETP in Experiment 5 is the first ever reported for an ionic dialkyl thiophosphate from an environmental sample. Daughton and Hsieh (1977b) and Cook *et al.* (1978) have summarized the results of several studies of the properties and metabolism of ionic dialkyl phosphates and thiophosphates. A potential exists for environmental contamination by these compounds through their hydrolytic release from the widely used organophosphorus insecticides. The ultimate fate of simple ionic alkyl phosphorus compounds in the environment should be investigated.

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