Accelerated Parathion Degradation in Soil by Inoculation with Parathion-Utilizing Bacteria

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The use of microorganisms for the decontamination of pollutants in the environment is only a recently acknowledged feasibility, even though sewage and certain industrial liquid wastes are routinely treated biologically. Nearly all of the research has been directed toward the use of microbial preparations for the cleanup of oil spills (BERNER et al., 1975; MIGET, 1973; STEWART, 1975); several oil-degrading microbial preparations are commercially available (ANONYMOUS, 1970; ATLAS and BARTHA, 1973). Potential problems involved with the use of microbial inocula in the environment have been discussed in reference to oil cleanup (COBERT et al., 1973; ZOBELL, 1973).

Only in the past decade have investigators shown that soil undesirably contaminated with a pesticide could possibly be decontaminated by inoculation with specifically adapted microorganisms. This idea perhaps was first embodied in the experiments of AUDUS (1951), who inadvertantly showed that bacteria, acclimated to utilize the herbicide 2,4-D, could acelerate its disappearance from soil when applied as an inoculum. Probably not for another 15 years did anyone attempt to use acclimated microorganisms for the purposeful detoxication of pesticides in soil.

MACRAE and ALEXANDER (1965) attempted to protect alfalfa seeds from the herbicide 4-(2,4-DB) by inoculation with a heavy suspension of 4-(2,4-DB)-utilizing Flavobacterium sp. prior to planting. ANDERSON et al. (1970) inoculated soils containing 1 ppm DDT with spore suspensions of a DDT-degrading fungus. BOYCE THOMPSON INSTITUTE researchers (1971) inoculated soil containing the herbicide Bromacil with Penicillium paraherquei which could degrade 20 ppm Bromacil in liquid culture. LEHTOMÄKI and NIEMELÄ (1975) added large inocula of axenic oil-degrading bacterial cultures to oil-contaminated soils. All of these studies were under laboratory conditions, and results were negative for non-sterile soils inoculated with the various microorganisms.

On the other hand, there have been positive results from the inoculation of non-sterile laboratory soil. KEARNEY et al. (1969) showed accelerated DDT disappearance from flooded soils inoculated with DDT-acclimated Enterobacter aerogenes. CLARK AND WRIGHT (1970) showed that the phytotoxicity of isopropyl N-phenylcarbamate (IPC)-fortified soils was decreased by inoculation with IPC-utilizing Arthrobacter sp. SETHUNATHAN (1973) reported that 26.5 ppm parathion in flooded soils could be hydrolyzed at a rate

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of 82% per day after inoculation with a parathion-hydrolyzing enrichment culture; this work has since been repeated (RAJARAM and SETHUNATHAN, 1975).

Perhaps the most thorough and successful experiments were those of MCCLURE (1972). Several phenylcarbamate herbicides were added to non-sterilized soil flats under greenhouse conditions, at rates up to 15 kg/ha. When a mixed bacterial culture capable of growth on IPC as a sole carbon source was applied to the soils, final plant yields were increased sometimes by 2000% over uninoculated controls. Of practical significance, in the absence of the herbicide, the functional activity of the acclimated cells in non-sterile soil decreased exponentially, losing all effectiveness in 2.5 months.

Only a few experiments have reported the use of microbial inocula under *in situ* environmental conditions and they have all concerned oil degradation (MIGET, 1973; STEWART, 1975).

Our results, reported here, concern the use of a highly acclimated parathion-utilizing bacterial culture for accelerating the degradation of large concentrations of parathion in soil, such as those found at disposal and spill sites. High concentrations of organophosphorus insecticides in field soils have been shown to be quite persistent (STAIFF *et al.*, 1975; WOLFE *et al.*, 1973), and the effectiveness of microbial inocula toward degrading insecticide soil concentrations greater than 50 ppm has not been investigated.

We have previously reported on the characteristics and maintenance (in continuous culture) of a parathion-utilizing culture (DAUGHTON and HSIEH, 1976a,b,c). This culture was recently shown to contain a bacterium (*Pseudomonas stutzeri*) capable of rapidly hydrolyzing parathion to ionic diethyl thiophosphate and *p*-nitrophenol; the resultant *p*-nitrophenol was utilized as a sole carbon and energy source by acclimated *P. aeruginosa*.

METHODS AND MATERIALS

Sewage samples were collected from an aeration tank of the University of California, Davis sewage treatment plant which also received agricultural runoff. From these samples a mixed acclimated bacterial culture (AC) was selected and enriched for growth on parathion as a sole carbon source (MUNNECKE and HSIEH, 1974). A "non-acclimated" culture (NAC) consisted of a sewage sample inoculum grown on yeast extract with no exposure to parathion.

Yolo silt loam (paste pH = 7.35) was obtained from the one to six inch surface layer and seived through a 2 mm wire mesh, mixed to assure uniformity, and 10 g aliquots were placed into conical Pyrex screw-cap centrifuge tubes. Fortification with the proper amount of parathion as technical parathion (PTN) (98% Stauffer Chem. Co., Richmond, Calif.) or as parathion emulsifiable concentrate (PTN-EC) (46.4%, Stauffer Chem. Co., Richmond, Calif.) was done with either neat material or with a solution of 0.5-1.0 ml of hexane. The PTN was then evenly dispersed by using a vortex mixer. Other subsequent treatments included autoclaving (20 min. for 2 successive days), amendment with yeast extract or glucose, inoculation with the acclimated culture (at most 0.1 mg dry cell mass

per soil sample) or the nonacclimated culture (control), flooding with water (15 ml) or bringing to 40% saturation (3 ml) and finally mixing to assure homogeneity. All samples were then incubated at room temperature (20-24 C) and ambient water content.

PTN was extracted from the samples with acetone/hexane (59:41) after addition of 10 ml of water (non-flooded samples) by shaking for one minute in the incubation centrifuge tube. The resultant emulsion was broken by centrifugation. The top layer was removed and washed with water and the organic phase adjusted to an appropriate volume and analyzed by flame-ionization gas-chromatography; no interferences were incurred. Quantitation of PTN was by interpolation from peak heights of standards.

RESULTS AND DISCUSSION

Since our PTN-utilizing culture was enriched for and maintained in liquid culture, we first determined whether the AC could function (i.e., degrade PTN) in the soil environment. Samples were fortified to 300 ppm (wt/dry wt soil) with PTN and subjected to various treatments. The percent PTN remaining in duplicate samples was determined after 0, 4, 7, and 11 days of incubation. Figures 1 and 2 show results from non-flooded and flooded soils respectively. The percent PTN remaining on days 4, 7, and 11 were calculated on the basis of the average amount of PTN recovered from all soil samples on day 0, which were then normalized to 100%.

Results in Fig. 1 indicate that 86% of the PTN was recoverable 11 days after fortification to non-autoclaved, non-flooded soil. The high recovery indicates that little PTN disappeared due to autochthonous microbiota, chemical hydrolysis, volatilization and time-dependent adsorption to soil colloids. In comparison, less than 15% of the PTN was recoverable only 4 days after PTN fortification and inoculation with the AC to either autoclaved or non-autoclaved soil; only traces remained 11 days after fortification. Amendment with yeast extract or glucose only delayed the onset of degradation by the AC, indicating possible diauxic growth. To our knowledge, this is the first report of accelerated PTN degradation in non-flooded soil by inoculation with adapted microorganisms. In flooded soil, Fig. 2, the same treatments all yielded PTN recoveries over 90% after 11 days incubation. The absence of any effect by the AC in flooded soils is expected since the culture was enriched and maintained in an oxygen-rich environment.

Another experiment was designed to determine the time period during which the AC could retain its PTN-degrading ability in non-flooded soil when PTN was not present as a growth and energy substrate.

The AC (1 ml) was inoculated to 10 g samples of autoclaved or non-autoclaved soil in centrifuge tubes which were either amended or non-amended to 0.5% with yeast extract. After inoculation (day 0), groups of six samples were fortified to 300 ppm PTN on days 0, 3, 8, 14, and 21 for each of the four treatments: amended and non-amended soils which were either autoclaved or non-autoclaved. In effect, for each of the four treatments, there were five groups of six soil samples each which had had the AC incubating in the

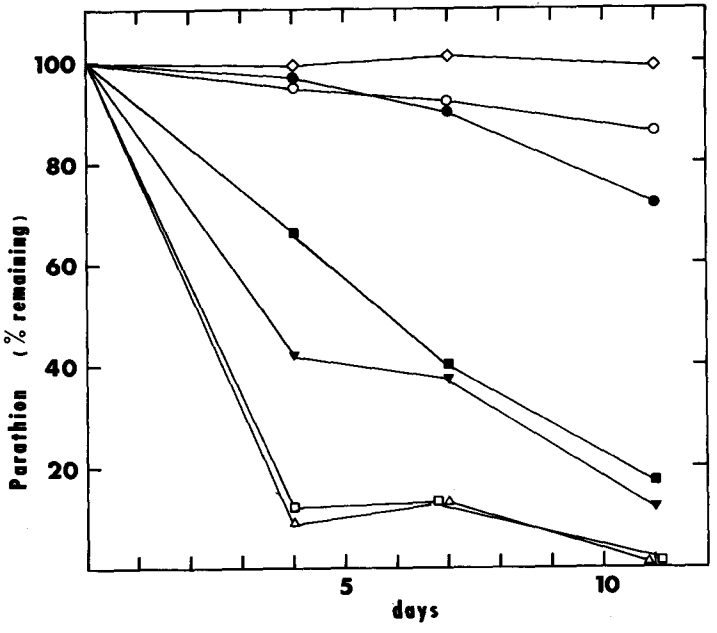

FIGURE 1

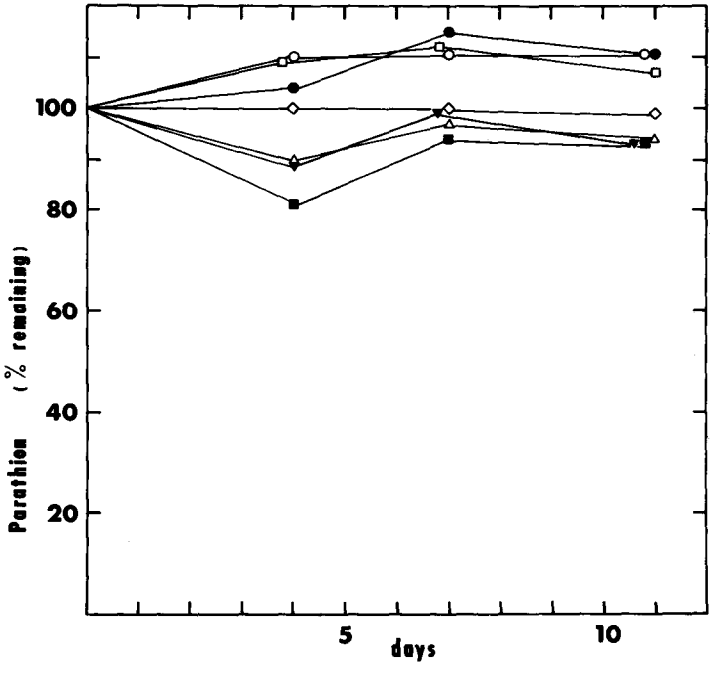

FIGURE 2

Parathion remaining (% of initial 300 ppm) in non-flooded (Fig. 1) and flooded (Fig. 2) laboratory soils versus days of incubation with and without the acclimated culture (AC).

SOIL TREATMENTS: ● inoculated with AC, autoclaved, PTN added; ○ PTN added to non-sterilized soil; △ PTN added to non-sterilized soil, inoculated with AC; ▼ PTN added to non-sterilized soil, amended with yeast extract, inoculated with AC; ■ PTN added to non-sterilized soil, amended with glucose, inoculated with AC; □ autoclaved, PTN added, inoculated with AC; ◇ same as ● , but water only (no soil).

absence of PTN for either 0, 3, 8, 14, or 21 days, a total of 120 samples. The retention of PTN degradative activity of the AC in each of the five groups was determined by extracting the PTN remaining in duplicate samples 0, 4, and 10 days after fortification with PTN. One of the four controls (d), consisting of autoclaved water (no soil) with 300 ppm PTN, was run in parallel with each of the five groups. The other three controls, analyzed in triplicate on days 0, 4, 10, and 23 after PTN fortification to 300 ppm, were: (a) non-sterilized soil amended with yeast extract, (b) non-sterilized soil, amended with yeast extract and inoculated with the NAC, and (c) minimal salts (no soil), inoculated with the NAC.

The retention of degradative activity in the absence of PTN is shown in the first five graphs of Fig. 3. The percent PTN remaining was calculated on the basis of the average amount of PTN recovered on day 0 (91.3% $\pm$ 2.3, 95% confidence interval) after PTN fortification for all samples of the five groups, which were then normalized to 100%.

The results indicate that the AC, when inoculated to autoclaved soil, maintained full activity for up to 3 weeks in the absence of PTN, regardless of amendment with yeast extract; more than 80% of the initial 300 ppm PTN was degraded within 4 days. The same was true for non-autoclaved soil when inoculated with the AC and amended with yeast extract. All of the controls, including the soils inoculated with the NAC showed only minor losses of PTN throughout the experiment. Recoveries for controls (a), (b) and (c) were 77, 90 and 90% respectively on day 23 (not shown on graph), accounting for losses of PTN due to all background effects. Notably, if the non-autoclaved soil were not amended with yeast extract, the AC showed a lesser rate of PTN degradation than did the other three treatments. After 2 weeks of incubation in the absence of PTN, the AC in non-autoclaved soil was less able to degrade PTN; more than 30% of the PTN was recoverable after 10 days incubation. After 3 weeks incubation in the absence of PTN in non-autoclaved soil, more than 60% of the PTN was recoverable at the end of the 10 day incubation. This phenomenon could perhaps be explained by assuming that the acclimated cells, when forced to compete with the autochthonous microbiota for low levels of carbon sources, begin to die or lose their ability to synthesize enzymes required for PTN metabolism.

Finally, we determined the range of PTN and PTN-EC concentrations in the soil which the AC could effectively degrade.

All soil samples were amended with 0.5% yeast extract and were not autoclaved. After PTN fortification, the samples were mixed and allowed to equilibrate for 12 hours prior to inoculation. Four PTN concentrations in the soil were tested: 300, 1000, 5000, and 10000 ppm. In each of these four groups, half were inoculated with the AC and half served as controls inoculated with the NAC. Within each of these groups, half were fortified with technical PTN and half with PTN-EC. Each of these treatments was performed in groups of six; one set of triplicates was extracted on day zero (all percent recoveries were greater than 90%), and the second group of triplicates was extracted after 3 weeks of incubation.

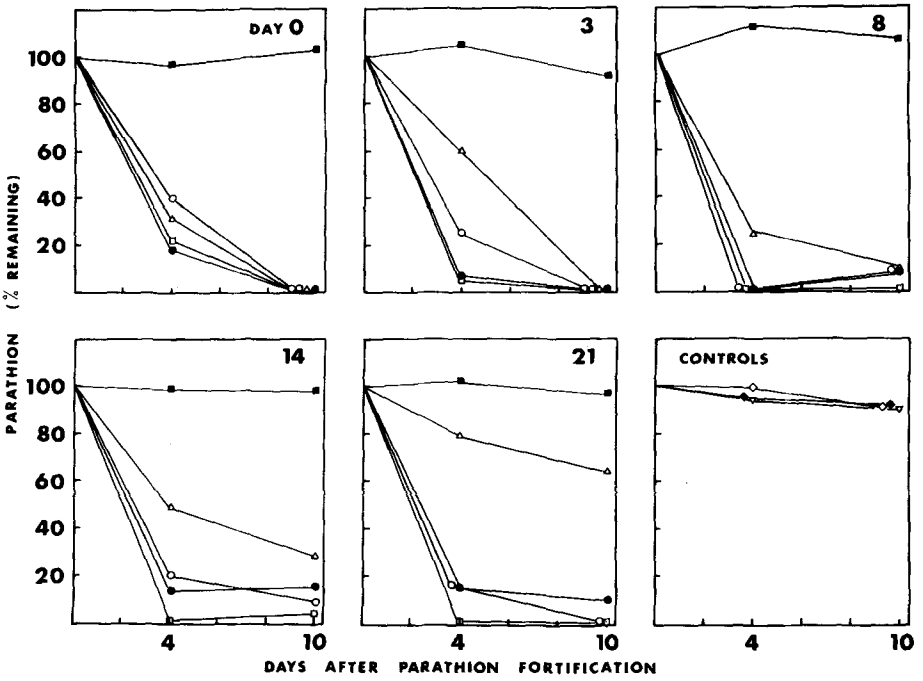

FIGURE 3

Functional longevity of acclimated culture in soil.

Parathion remaining (% of initial 300 ppm) in non-flooded laboratory soil versus days after parathion fortification. Each of the graph labels, Day 0, 3, 8, 14 or 21, indicates the days of incubation with the acclimated culture (AC) prior to fortification with parathion (PTN).

TREATMENTS:

- ○ autoclaved soil, inoculated with AC.
- △ non-sterilized soil, inoculated with AC.
- □ autoclaved soil, inoculated with AC, amended with yeast extract.
- ● non-sterilized soil, inoculated with AC, amended with yeast extract.

CONTROLS:

(a) ▽ non-sterilized soil, amended with yeast extract, PTN added.

(b) ◆ non-sterilized soil, amended with yeast extract, PTN added, inoculated with a non-acclimated culture.

(c) ◇ minimal salts (no soil), PTN added, inoculated with a non-acclimated culture.

(d) ■ water (no soil), autoclaved, PTN added.

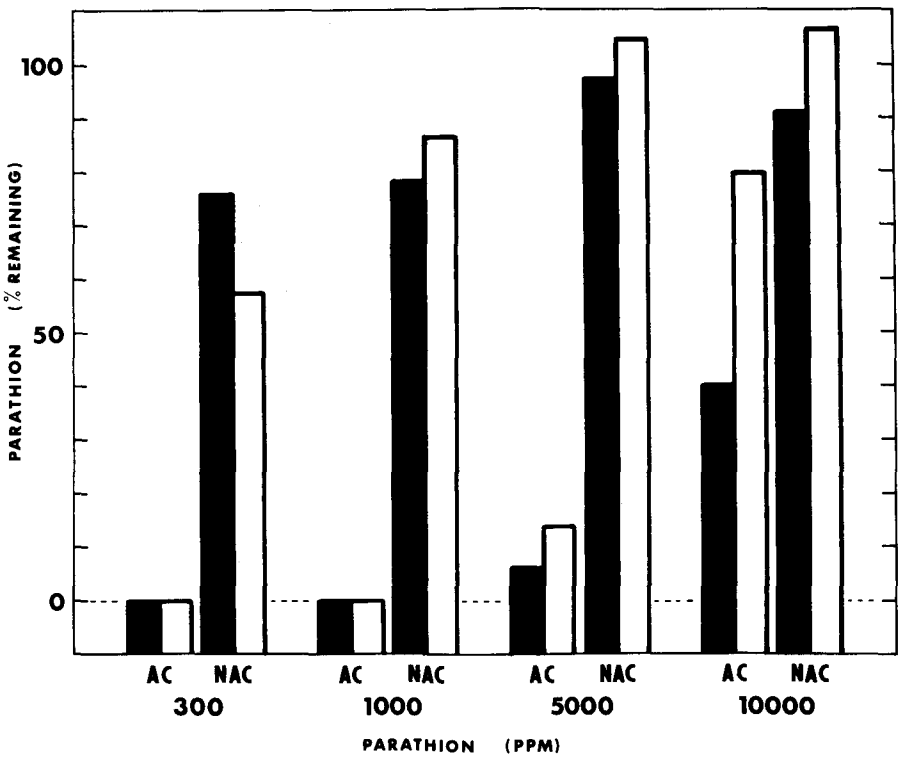

FIGURE 4

Effectiveness of the acclimated and non-acclimated cultures toward degradation of high concentrations of parathion and parathion EC in laboratory soil.

Parathion remaining (% after 3 weeks incubation) versus initial concentration of parathion in non-flooded soil (ppm).

White Bars = parathion emulsifiable concentrate.

Black Bars = technical parathion.

AC = inoculated with the acclimated culture.

NAC = inoculated with the non-acclimated culture.

The results shown in Fig. 4 indicate that within each of the treatments, the AC was much more effective than the NAC. Less than 10% of the PTN remained after 3 weeks in soil fortified to 5000 ppm with PTN when inoculated with the AC. In contrast, when soil fortified to 5000 ppm was inoculated with the NAC, 97% of the PTN was recovered after 3 weeks. Even at 10000 ppm, less than 40% of the PTN remained when treated with the AC. Apparently, the AC was capable of degrading nearly 100% of a PTN concentration as high as 5000 ppm within 3 weeks, but at 10000 ppm, the amount of metabolism of PTN applied as PTN-EC was drastically reduced; this was probably due to toxicity of the detergents and/or xylenes of the formulated PTN.

In conclusion, the PTN-acclimated microorganisms were extremely effective in rapidly degrading concentrations of PTN of at least 5000 ppm in non-flooded soil within 3 weeks under laboratory conditions. These acclimated cells could maintain their PTN-degrading ability in non-sterilized soil for at least 8 to 14 days under laboratory conditions, while their effectiveness after 3 weeks in non-sterilized soil without exposure to PTN was greatly diminished. These results warrant a study under field conditions to determine if the AC can accelerate PTN degradation and maintain activity under fluctuating temperature extremes and water content.

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