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Reasons for Possible Failure of Inoculation to Enhance Biodegradation

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Pseudomonas **strains capable of mineralizing 2,4-dichlorophenol (DCP) and *p*-nitrophenol (PNP) in culture media were isolated from soil. One DCP-metabolizing strain mineralized 1.0 and 10 μg of DCP but not 2.0 to 300 ng/ml in culture. When added to lake water containing 10 μg of DCP per ml, the bacterium did not mineralize the compound, and only after 6 days did it cause the degradation of 1.0 μg of DCP per ml. The organism did not grow or metabolize DCP when inoculated into sterile lake water, but it multiplied in sterile lake water amended with glucose or with DCP and supplemental nutrients. Its population density declined and DCP was not mineralized when the pseudomonad was added to nonsterile sewage, but the bacterium grew in sterile DCP-amended sewage, although not causing appreciable mineralization of the test compound. Addition of the bacterium to nonsterile soil did not result in the mineralization of 10 μg of DCP per g, although mineralization was evident if the inoculum was added to sterile soil. A second DCP-utilizing pseudomonad failed to mineralize DCP when added to the surface of sterile soil, although activity was evident if the inoculum was mixed with the soil. A pseudomonad able to mineralize 5.0 μg of PNP per ml in culture did not mineralize the compound in sterile or nonsterile lake water. The bacterium destroyed PNP in sterile sewage and enhanced PNP mineralization in nonsterile sewage. When added to the surface of sterile soil, the bacterium mineralized little of the PNP present at 5.0 μg/g, but it was active if mixed well with the sterile soil. It is suggested that microorganisms able to degrade organic pollutants in culture sometimes may fail to function when inoculated into natural environments because the concentration in nature may be too low to support growth or because the organisms may be susceptible to toxins or predators in the environment, may use other organic compounds in preference to the pollutant, or may be unable to move through soil to sites containing the chemical.**

Many synthetic organic compounds that are mineralizable persist in sewage, natural waters, and soils for some time before a population of microorganisms becomes sufficiently large or active to bring about their destruction. One way to enhance destruction of these chemicals is to inoculate the environment with microorganisms known to metabolize the chemicals readily. It is also possible that genetically engineered microorganisms may be developed to bring about the rapid destruction of compounds that are slowly destroyed or that are not known to be mineralized in nature, and these newly constructed organisms would then be added to natural ecosystems after their growth in the laboratory or in large fermentors. Nevertheless, because abiotic stresses in natural environments are often different from those in the laboratory and because the introduced species may face intense competition, predation, or parasitism in nature, the inoculated organism may not bring about the desired biodegradation after its addition to sewage, natural waters, or soils.

Both successes and failures have been reported when species capable of destroying synthetic organic compounds in culture are added to samples of natural environments. MacRae and Alexander (13) found that a 4-(2,4-dichlorophenoxy)butyrate-utilizing *Flavobacterium* sp. did not destroy the herbicide when added to soil containing the chemical. A strain of *Mucor alternans* able to metabolize DDT [1,1,1-trichloro-2,2-bis-(4-chlorophenyl)ethane] in culture did not act on the insecticide after addition of the fungus to soil (2). Oil-degrading bacteria added to samples of soil contaminated with light fuel oil or heavy waste oil had no significant effect on the oil (12).

In contrast, there are reports that additions of microorganisms to samples of natural environments enhance biodegradation. For example, *Arthrobacter* sp. capable of utilizing isopropyl *N*-phenylcarbamate in culture was also able to degrade the herbicide in soil (7), and a strain of *Pseudomonas cepacia* able to grow on 2,4,5-trichlorophenoxyacetic acid also degraded the pesticide in soil (11). The detoxication of DDT in flooded soils was shown to be enhanced by a DDT-utilizing bacterium (10). Success has also been reported after inoculation in the field. Thus, Barles et al. (3) observed that a mixed culture containing a parathion-utilizing strain of *Pseudomonas stutzeri* facilitated the decontamination of field plots containing the insecticide.

The reasons for the inability of microorganisms added to natural environments or samples of these environments to bring about reactions they effect in culture are unknown. Because the use of naturally occurring or genetically engineered microorganisms represents a potentially promising means of destroying polluting chemicals in sewage, natural waters, or soils, a study was initiated to determine some of the reasons for the failure of microbial inocula to bring about degradative reactions in nature that they can catalyze in culture.

MATERIALS AND METHODS

Isolation of bacteria. The enrichment culture technique was used to isolate bacteria from soil. The enrichment medium contained 10 μg of 2,4-dichlorophenol (DCP) or 5.0 μg of *p*-nitrophenol (PNP) (Eastman Chemical Co., Rochester, N.Y.) per ml and 0.1 g of NH_4NO_3, 0.8 g of K_2HPO_4, 0.2 g

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of KH_2PO_4, 0.1 g of $CaCl_2 \cdot 7H_2O$, 0.1 g of $MgSO_4$, and 10 mg of $FeCl_3$ per liter of distilled water. We determined utilization of PNP by centrifuging portions of the enrichments at 8,500 × *g* for 12 min and examining changes in absorbancy of the solution at 400 nm by the method of Alexander and Lustigman (1). When the absorbancy of the supernatant fluid had declined, portions of the enrichments were streaked on a medium containing 1.5% (wt/wt) Bacto-Agar (Difco Laboratories, Detroit, Mich.), the inorganic salts, and 5 μg of PNP per ml. We incubated the plates at 30°C for 3 to 7 days, and transferred isolated colonies to tubes containing 20 ml of a solution containing 5 μg of PNP per ml and the inorganic salts to determine which strains used PNP as determined spectrophotometrically. We isolated bacteria growing on DCP as a sole carbon source by plating the enrichment cultures onto solid enrichment medium containing 2% $CaCO_3$ and 10 μg of DCP per ml. Colonies that produced zones of clearing on the agar were picked and purified.

To determine whether the organisms lost their ability to metabolize DCP and PNP when grown in the absence of these compounds, we grew *Pseudomonas* sp. strain 1 and *Pseudomonas* sp. strain 3 in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for four serial cultures. The cultures were then plated on agar containing the inorganic salts and 10 μg of DCP or 5.0 μg of PNP per ml. Of 10 colonies picked from each plate, all from DCP-containing agar rapidly mineralized 10 μg of DCP per ml when tested in the salt solution amended with the chemical. No colonies from the PNP-containing plates mineralized PNP.

An antibiotic-resistant strain derived from *Pseudomonas* sp. strain 1 was obtained by growing the bacterium in Trypticase soy broth and plating a thick cell suspension on Trypticase soy agar containing 100 μg of streptomycin (Sigma Chemical Co., St. Louis, Mo.) per ml. Colonies appearing on this medium were picked, and the isolates were grown in Trypticase soy broth containing 100 μg of streptomycin per ml. Thick cell suspensions derived from these cultures were plated on Trypticase soy agar containing 100 μg of streptomycin and 200 μg of erythromycin (Sigma) per ml. Colonies that appeared were transferred to Trypticase soy broth containing 200 μg of erythromycin per ml and increasing concentrations of streptomycin. Cultures growing in the broth containing 200 μg of erythromycin and 500 μg of streptomycin per ml were serially transferred in the same medium supplemented with increasing levels of spectinomycin (GIBCO Laboratories, Grand Island, N.Y.). The strain thus obtained was resistant to 200 μg of erythromycin, 500 μg of streptomycin, and 50 μg of spectinomycin per ml. Using the same procedures, we obtained a strain derived from *Pseudomonas* sp. strain 3 that was resistant to 200 μg of erythromycin, 100 μg of streptomycin, and 50 μg of rifampin (Sigma) per ml. The isolates were designated *Pseudomonas* sp. strains 1A and 3A, respectively. After three serial subcultures of the antibiotic-resistant isolates in Trypticase soy broth, counts of *Pseudomonas* sp. strains 1A and 3A on Trypticase soy agar with and without the appropriate antibiotics gave similar values, indicating that resistance to the inhibitors was stable. These strains also used DCP and PNP in liquid culture. The antibiotic-resistant isolates were only used in studies in which densities of the test bacteria in sewage were determined.

Environmental samples. Air-dry Canandaigua silt loam (4.7% organic matter; pH 6.7) was passed through a 2-mm (pore size) sieve and used for tests of mineralization. It had a moisture content of 27% on a dry-weight basis and a pH of 6.7 when tested in a 1:1 (wt/vol) water suspension. When sterilized soil was required, 10-g portions were exposed to 2.5 Mrads of irradiation from a ^{60}Co source.

Samples of raw sewage collected from the primary settling tank of the Ithaca, N.Y., treatment plant were used within 1 h of collection. Sewage was sterilized either by autoclaving or by sequential passage through Whatman Inc. (Clifton, N.J.) no. 42 filters, 3-μm (pore size) membrane filters (Fisher Scientific Co., Pittsburgh, Pa.), and 0.2-μm membrane filter units (Fisher).

Lake water was collected from Cayuga Lake, Ithaca, N.Y., at a distance of ca. 6 to 9 m from the shore. If necessary, the lake water was sterilized by passage through 0.2-μm membrane filter units.

Bacterial counts. Bacteria were enumerated by the spread-plate technique. The medium for counting *Pseudomonas* sp. strain 1 in nonsterile sewage was Trypticase soy agar containing 500 μg of streptomycin, 250 μg of erythromycin, 50 μg of spectinomycin, and 250 μg of cycloheximide per ml. The plates were incubated at 30°C for 24 to 48 h before counting.

We determined the ability of *Pseudomonas* sp. strain 3 to survive when added to the surface of sterile soil by using 50-ml Erlenmeyer flasks containing 10 g of air-dried, gamma-irradiated soil. Sterile, distilled, deionized water (1.9 ml) was aseptically mixed with the soil, and 0.1 ml of a washed cell suspension was added to the surface of the soil. The flasks were stoppered with air-tight Suba seals (Thomas Scientific, Philadelphia, Pa.) to prevent evaporation and incubated at 20 to 22°C. Triplicate flasks were sampled on days 0, 1, 2, 4, 7, 9, and 12, and counts were made on Trypticase soy agar amended with 10 μg of PNP per ml. The plates were incubated at 30°C and counted after 24 h.

Measurement of mineralization. Samples (50 or 100 ml) of sewage, lake water, or inorganic salt solution were placed in 250-, 500-, or 1,000-ml Erlenmeyer flasks, and washed cells from a 24-h-old culture (grown in the salt solution amended with 5 or 10 μg of DCP or PNP per ml) were added to give initial densities of 10^4 to 10^5 cells per ml, unless otherwise noted. The labeled chemicals were added to give 350 to 600 dpm/ml. At the lower concentrations of DCP, only the labeled compound was added. Unless otherwise stated, all treatments were in duplicate. Two analyses usually were performed per flask at each sampling time.

The samples were analyzed by procedures similar to those of Subba-Rao et al. (19). At intervals, 2.5-ml portions of liquid were removed and placed in tubes containing 2 drops of concentrated H_2SO_4. The acidified samples were bubbled for 10 min with air to remove final traces of CO_2. Two milliliters of the acidified liquid was added to 8 ml of Liquiscint solution (National Diagnostics, Somerville, N.J.). The scintillation vials were agitated and then left undisturbed for at least 6 h. The radioactivity of the samples was measured with an LS7500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

To test whether loss of the chemicals was a result of volatilization, sorption to glassware, or photo-oxidation, we added filter-sterilized labeled and unlabeled DCP or PNP to filter-sterilized or autoclaved sewage or filter-sterilized lake water in all mineralization studies.

In studies of mineralization in soil, 2.0 or 6.0 g of air-dried, sterile soil in 50-ml Erlenmeyer flasks was brought to 20% moisture (wt/wt) by mixing well with a sterile solution of labeled and unlabeled DCP or PNP to give ca. 500 to 600 dpm/g of soil. The following day, 10^4 to 10^5 cells per g of soil was either added to the surface or mixed into the soil with a

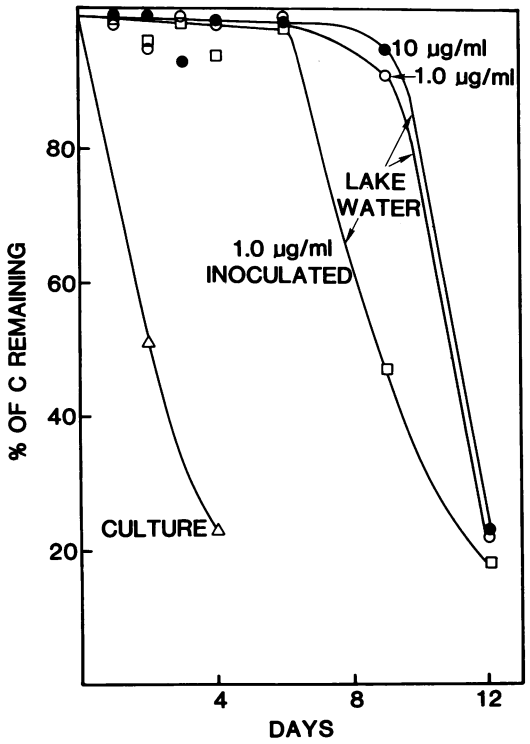

FIG. 1. Mineralization of DCP in culture (10 µg/ml) and in lake water inoculated with *Pseudomonas* sp. strain 1 and in uninoculated lake water.

vortex mixer and a sterile spatula. The flasks were immediately sealed with rubber serum stoppers, from which were suspended polypropylene cups, each containing folded Whatman no. 42 filter paper. In some instances, a 1-dram (ca. 3.7-ml) glass vial containing the filter paper was placed inside the flask, care being taken that the contents of the vial did not enter the soil. At intervals, 2.0-ml portions of 2 N H_2SO_4 were injected through the septum directly onto the soil in triplicate flasks, and then 0.2 or 0.5 ml of CO_2-Met (Amersham Corp., Arlington Heights, Ill.) or 0.5 ml of 0.33 N KOH was injected through the septum onto the filter paper. The flasks were left undisturbed for 6 to 8 h, and the papers were then removed and placed in 12 ml of Liquiscint solution (National Diagnostics). The vials were then shaken and counted 6 to 24 h later. In tests in which ^{14}C-labeled bicarbonate was added to soil samples before acidification, more than 95% of the ^{14}C was recovered as CO_2 by this method. Less than 1% of added dichlorophenol was found in the CO_2-trapping solution.

p-Nitro[U-^{14}C]phenol (24.05 mCi/mmol; 98% radiopurity) was provided by A. W. Bourquin, U.S. Environmental Protection Agency, Gulf Breeze, Fla. 2,4-Dichloro[U-^{14}C]phenol was obtained from Amersham Corp.

Statistical analyses. When all points were considered, a nested two-way analysis of variance was used. A one-way analysis of variance was used to compare treatments at a single time interval. We compared individual means by using the least significant difference of their means at the 95% level of confidence. When individual points were analyzed, the Student *t* test at the 95% level of confidence was used.

RESULTS

Culture. The isolates were identified by standard methods (6, 18) as members of the genus *Pseudomonas*. The two isolates mineralizing DCP were designated strains 1 and 2, and the bacterium degrading PNP was designated strain 3.

To test the ability of strain 1 to metabolize different concentrations of DCP, we added labeled and unlabeled substrate to 100 or 400 ml of the salt solution contained in 250- or 1,000-ml Erlenmeyer flasks to give ca. 500 dpm/ml, except that ca. 60 dpm/ml was used at the lowest substrate concentration. The flasks were incubated at 20 to 22°C without shaking. Compared with uninoculated flasks, the bacterium did not mineralize DCP in 33 days at concentrations of 2.0, 6.0, 20, 100, and 300 ng/ml. The substrate loss in sterile medium, which, for example, was 20% in 33 days at 300 ng/ml, could be a result of its volatilization. In contrast, DCP was mineralized at 10 µg/ml, 68% of the C being lost from solution in 4 days. The reaction was slower at 1.0 µg/ml, but about 40% of the C had disappeared in 4 days as a result of bacterial activity.

Lake water. *Pseudomonas* sp. strain 1 mineralized 10 µg of DCP per ml in culture, and 73% of the C was mineralized in 4 days (Fig. 1). The compound at 1.0 and 10 µg/ml also disappeared in nonsterile lake water. If the bacterium was added to nonsterile lake water containing 1.0 µg of DCP per ml, mineralization was not detected in 6 days, although the bacterium enhanced mineralization in the subsequent period. At 10 µg of DCP per ml, the inoculum did not enhance mineralization in lake water, and no mineralization was observed in sterile lake water containing 10 µg of DCP per ml whether or not the bacterium was added (data not shown).

In 9 days, *Pseudomonas* sp. strain 1 at an initial density of 10^4 to 10^5 cells per ml failed to mineralize much of the DCP added to filter-sterilized lake water at 10 µg/ml (Fig. 2). However, it readily metabolized glucose added at the same concentration to lake water. Nevertheless, if the water was amended with yeast nitrogen base to a final concentration of 0.02% and inorganic salts, 57% of the carbon of DCP was mineralized in 9 days. Bacterial counts were made of inoculated lake water with Trypticase soy agar containing 10 µg of DCP per ml. In unamended and DCP-supplemented lake water, the population initially declined and then maintained itself at low numbers (Fig. 2B). If lake water received only glucose or was supplemented with yeast nitrogen base and inorganic salts, the extent of growth was appreciable.

Pseudomonas sp. strain 3 readily mineralized 5.0 µg of PNP per ml in culture (Fig. 3). The compound also disappeared from uninoculated lake water after 7 days. However, inoculation of nonsterile lake water with the bacterium did not enhance the mineralization of 5.0 µg of PNP per ml. Moreover, when the bacterium was added to sterile lake water, it had no activity on the compound.

Sewage. Mineralization of 10 µg of DCP per ml appeared to be biphasic in nonsterile sewage, about 10% of the C being mineralized in 1 day before the process stopped (Fig. 4A). Mineralization then began again on day 6. Essentially the same biphasic pattern occurred if the sewage was inoculated with *Pseudomonas* sp. strain 1, and the differences between inoculated and uninoculated treatments were not statistically significant. In sterilized sewage containing 10 µg of DCP per ml, little or no loss of ^{14}C occurred.

At 2.0 ng/ml, DCP disappeared from sewage at a slow rate (Fig. 4B). Inoculation with the pseudomonad did not give a statistically significant increase in that rate. Inoculation of sterilized sewage containing 2.0 ng of DCP per ml had little or no effect on mineralization. The inoculum readily metabolized 10 µg of DCP per ml in culture medium, however, and more than 70% of the C was lost from solution in 5 days.

Pseudomonas sp. strain 1A was inoculated into sterile and nonsterile sewage with or without 10 μg of DCP per ml. The bacterium grew when added to sterile sewage, and the extent of growth was not affected by the presence of DCP (Fig. 5). In nonsterile sewage with or without DCP, density declined from 1.6×10^6 to about 10^4 cells per ml in 12 days. The differences in counts in nonsterile sewage with and without amendment were not statistically significant. Tests of the ability of the antibiotic-resistant mutant to mineralize DCP in sterile sewage indicated that 96% of the ^{14}C remained in solution at 9 days under the test conditions, indicating that the inoculum did not mineralize the substrate.

Sterile and nonsterile sewage were amended with 5.0 μg of PNP per ml. Half of each set of flasks were inoculated with *Pseudomonas* sp. strain 3A at an initial density of 2.5×10^4 cells per ml. In addition, the bacterium was inoculated into the synthetic medium. Disappearance of the substrate was measured spectrophotometrically. No substrate loss was detected in sterile, uninoculated sewage. However, the bacterium readily destroyed the chemical in culture and when inoculated into sterile sewage (Fig. 6). This compound was only slowly mineralized in raw sewage, but the process was markedly enhanced after introduction of the pseudomonad into sewage.

Soil. Nonsterile and sterile soil were amended with 10 μg of DCP per g, and half the samples of sterile and nonsterile soil were inoculated with *Pseudomonas* sp. strain 1. Less than 1.5% of the substrate C was detected among the volatile products emanating from sterile, uninoculated soil in 12 days. In nonsterile soil that did not receive the inoculum, little mineralization was detected in 12 days (Fig. 7). Inoculation of nonsterile soil did not produce a statistically significant increase in mineralization. The variation in points shown in the figure for this treatment results from differences among replicate flasks. Mineralization was marked in sterile soil that received the inoculum.

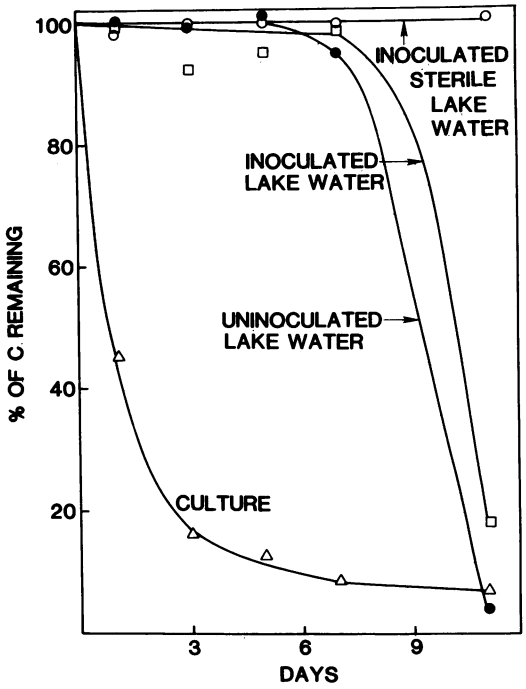

FIG. 3. Mineralization of PNP (5.0 μg/ml) in uninoculated lake water and in culture and nonsterile and sterile lake water inoculated with *Pseudomonas* sp. strain 3.

Pseudomonas sp. strain 3 was inoculated onto the surface of sterile soil amended with 5.0 μg of PNP per g. At the same time, the bacterium was added to the surface of another set of PNP-amended soils and then thoroughly mixed into the soil. Each treatment was in triplicate. In soil in which

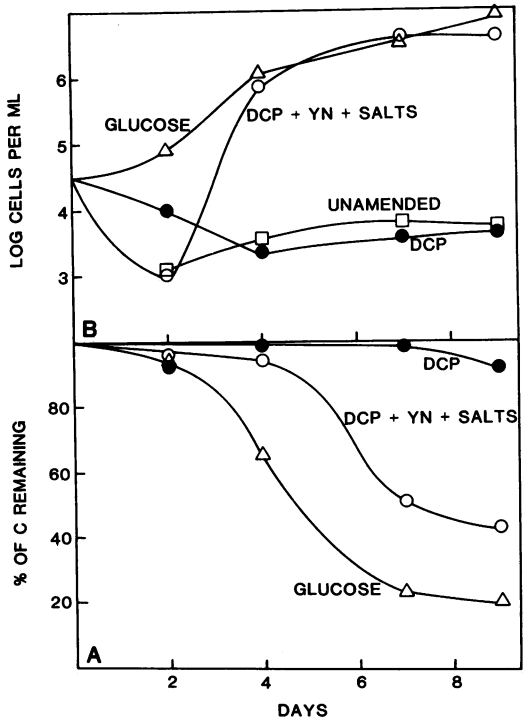

FIG. 2. Mineralization of DCP and glucose (A) and population density of *Pseudomonas* sp. strain 1 (B) after inoculation of sterilized lake water amended with DCP, glucose, or a mixture of DCP, yeast nitrogen base (YN), and inorganic salts.

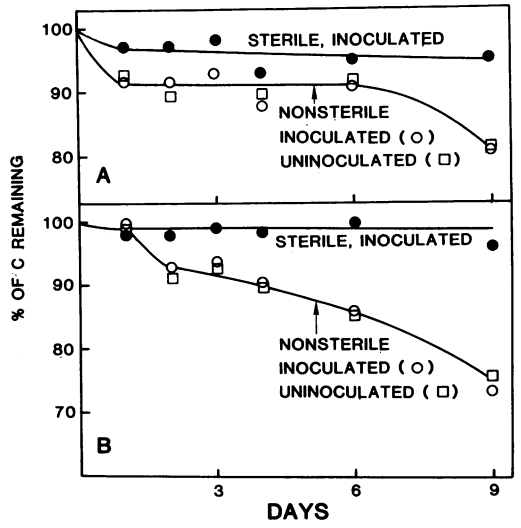

FIG. 4. Mineralization of 10 μg (A) and 2.0 ng (B) of DCP per ml in nonsterile sewage and in nonsterile and sterile sewage inoculated with *Pseudomonas* sp. strain 1.

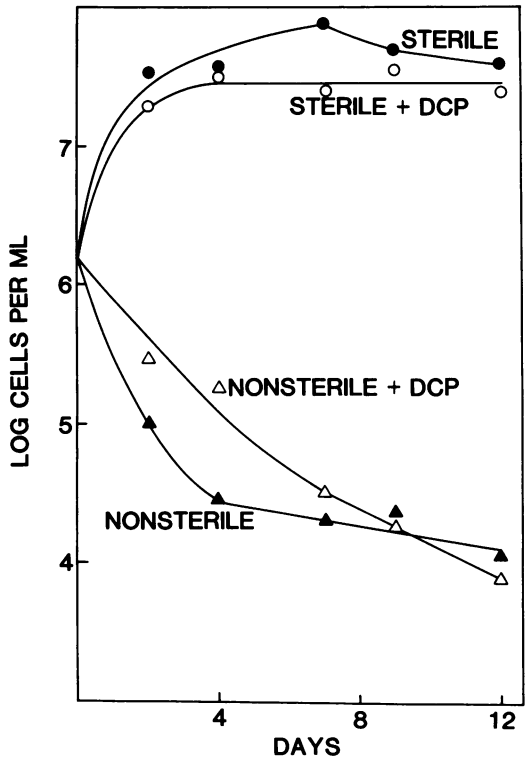

FIG. 5. Population changes of *Pseudomonas* sp. strain 1A inoculated into sterile and nonsterile sewage amended with DCP (10 μg/ml) or into unamended sewage.

inoculum was well mixed, mineralization was rapid (Fig. 8). At the end of 12 days, about 40% of the substrate was mineralized. In soil in which inoculum was applied to the surface and not mixed, only 6% of the PNP was mineralized

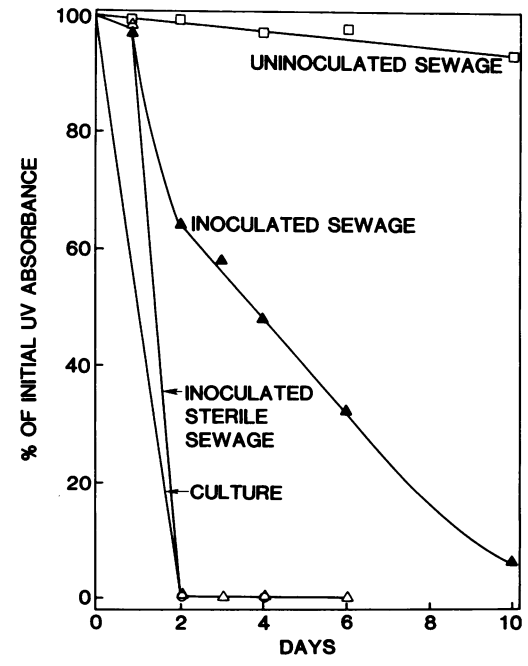

FIG. 6. Decomposition of PNP (5.0 μg/ml) in sewage and in culture medium, nonsterile sewage, and sterile sewage inoculated with *Pseudomonas* sp. strain 3A.

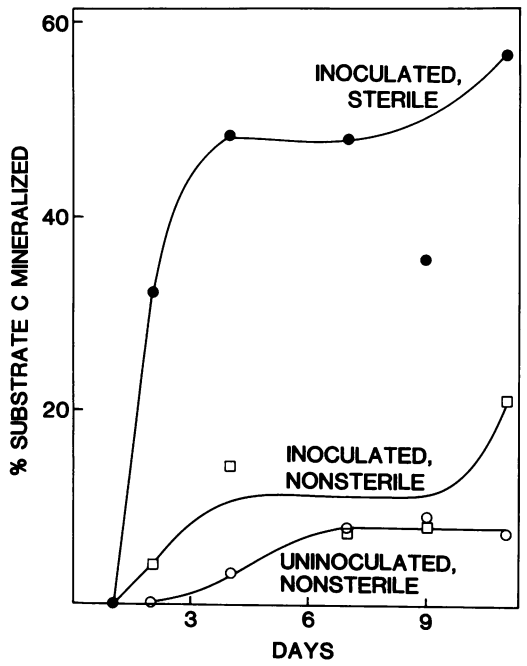

FIG. 7. Mineralization of DCP (10 μg/g) in nonsterile soil and in sterile and nonsterile soil inoculated with *Pseudomonas* sp. strain 1.

in 12 days. The error associated with the treatment was less than 1% of the mean. No $^{14}CO_2$ was released from uninoculated soil. The differences among the three treatments were statistically significant. After 21 days, one of the replicate flasks was shaken vigorously. In the flask that was left

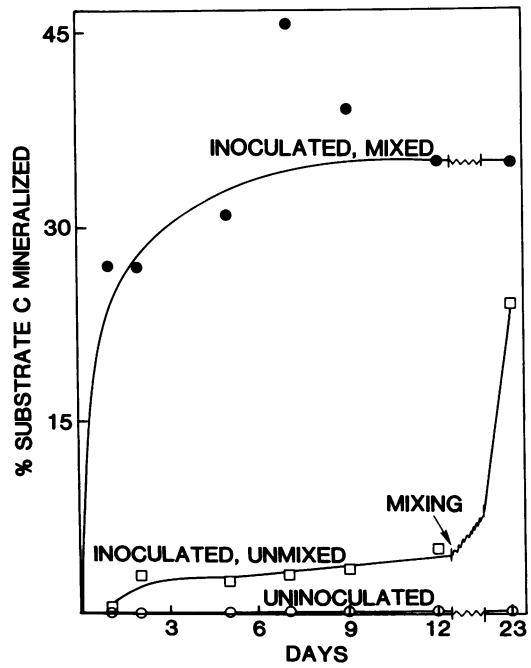

FIG. 8. Mineralization of PNP (5.0 μg/g) in sterile soil that received no inoculum or was inoculated at the surface with *Pseudomonas* sp. strain 3 and was either mixed or left unmixed. The arrow designates the times when one flask of the previously unmixed soil was shaken vigorously.

unmixed, there was no more mineralization at 23 days than at 12 days, whereas in the flask that was mixed, mineralization was markedly enhanced (Fig. 8).

Counts were made of *Pseudomonas* sp. strain 3 that had been inoculated onto the surface of sterile soil in another experiment. From an initial density of 1.8×10^6 cells per g, the population grew to 4.8×10^6 cells per g at 4 days and 8.2×10^7 cells per g at 9 days. Thus, the bacteria grew on the organic carbon present in soil and must have had sufficient moisture even at the soil surface to permit multiplication.

Pseudomonas sp. strain 2 was added to the surface of sterile soil that had been amended with 10 μg of DCP per g of soil. After 9 days, no mineralization was detected. In contrast, if the cells were mixed with sterile soil when they were initially added, 20% of the C was mineralized in 9 days.

DISCUSSION

The capacity of a microorganism to grow by using a particular organic compound as a carbon source does not mean that inoculation of that organism into a natural environment will cause biodegradation of the compound. The ability to metabolize the chemical is a necessary but not a sufficient condition for the organism to effect the transformation. The data presented herein suggest several reasons for the failure of such inocula to do in nature what they can do in axenic culture. These reasons are as follows. (i) The concentration of the compound in nature may be too low to support growth of the inoculated species. (ii) The natural environment may contain substances inhibiting growth or activity of the added organism. (iii) The growth rate of the organism on the low ambient concentration of the chemical of interest may be slower than the rate of predation, for example, by protozoa, so that the activity of grazers reduces the cell density of the inoculum species. (iv) The added bacterium may use other organic substrates in the natural environment rather than the pollutant whose destruction is desired. (v) The organism may fail to move through the pores of soil to sites containing the organic compound. The failure of inocula to function, as shown in the present study, does not mean that inoculation is not a feasible method for destroying organic pollutants. This study was not designed to show that the approach of using inocula to destroy pollutants is not feasible; rather, the purpose was to characterize the pitfalls that should be avoided to make inoculation a useful procedure for eliminating organic toxicants from natural ecosystems.

It is not surprising that an organism may fail to metabolize low concentrations of a test chemical. For example, marine bacteria have been reported not to grow at low substrate concentrations (9), and a pseudomonad able to grow at glucose levels of 18 ng/ml or higher had almost no effect on lower levels of the sugar (5). Although larger populations of nongrowing cells or species growing on organic substrates other than the test compound may mineralize the chemical of interest, a bacterium in small numbers whose sole selective advantage in an environment is its ability to multiply at the expense of a particular substrate may not proliferate if that substrate is below some threshold level (17a).

The inability of *Pseudomonas* sp. strain 3 to enhance mineralization of PNP in nonsterile lake water or to carry out the degradation of PNP in sterile lake water could result from the presence of toxins in the water or its preferential utilization of other substrates. The growth of *Pseudomonas* sp. strain 1 in sterile sewage without its destruction of added DCP lends credence to the view that other substrates can be used in preference to the pollutant, although presumably the inoculum strain has a selective advantage when it is the only organism present that is able to use a synthetic organic molecule. The capacity of the latter bacterium to grow and metabolize glucose but not DCP in sterile lake water, except if the DCP-containing samples received supplemental nutrients, possibly results from the presence of inhibitors whose actions are reversed by the nutrients or from the need for additional nutrients for the metabolism of DCP but not glucose. The presence of antimicrobial agents in natural waters has been described (4, 17), and Gispen and Gan (8) reported that filterable, heat-labile inhibitors of *Shigella* sp. and *Salmonella* sp. were present in lake water. Samples of water from the lake used in the present study have also been found to contain low-molecular-weight compounds toxic to several bacterial species added to the water (T. M. Klein and M. Alexander, unpublished data). The beneficial effect of added nutrients has been described before. Thus, Wang et al. (20) reported that the mineralization of isopropyl *N*-phenylcarbamate by inhabitants of lake water was enhanced by organic nutrients, and Rubin and Alexander (16) found that the addition of inorganic nutrients, arginine, or yeast extract enhanced the rate of phenol mineralization in fresh waters.

Pseudomonas sp. strain 1 declined in abundance in nonsterile sewage, and it failed to mineralize DCP. The decline is probably not a result of competition with indigenous bacteria for a carbon source because DCP was present. The decline is not a result of starvation, moreover, because the bacterium does not die readily in buffer (unpublished data). It is more likely that the introduced bacteria are adversely affected by protozoa. These predators not only feed actively on bacteria in sewage, but they can reduce the abundance of an added bacterium to densities below those believed to represent the threshold for protozoan grazing (15).

Neither DCP-degrading nor PNP-degrading pseudomonads had a significant effect on the test compounds when the bacteria were added to the surface of sterile soil, although both were active if mixed thoroughly into the soil. The failure to mineralize PNP was not a result of the inability of the organism to survive, because some cells were still active when the soil was later mixed. It is likely that the organisms did not move through the soil to mineralize the chemical at a distance from the point of inoculation and that only the compound near the points of inoculation was destroyed. The inability of bacteria to move vertically through soil is not surprising. For example, the vertical movement of *Pseudomonas putida* and *Rhizobium japonicum* was found to be restricted unless transporting agents such as percolating water or a burrowing earthworm were present (14). In nature, moreover, mixing of soil with an inoculum to an extent sufficient to bring all of the chemical in proximity to the inoculum is probably often not economically feasible, except if the soil is made into a slurry with water.

Although this study was designed to seek reasons for the failure of inocula to enhance or bring about degradation of organic pollutants, the approach is feasible and worthwhile if means are found to overcome the ecological constraints on the inoculum strains. Some isolates will function without knowledge being available on why they act, but many others will fail to destroy the chemical in natural or polluted ecosystems. Success is evident here in the enhanced destruction of PNP in sewage by inoculation with *Pseudomonas* sp. strain 3, and reports exist of successes after inoculation of soil with bacteria metabolizing several pesti-

cides (3, 7, 10). However, an approach that will lead to a higher frequency of success is first establishing the reasons for failure and then finding organisms or practices not subject to the stresses causing those failures. This investigation will, we hope, aid in facilitating that approach.

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