

Inoculum Size as a Factor Limiting Success of Inoculation for Biodegradation

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A study was conducted to determine the role of inoculum size of a bacterium introduced into nonsterile lake water in the biodegradation of a synthetic chemical. The test species was a strain of *Pseudomonas cepacia* able to grow on and mineralize 10 ng to 30 µg of *p*-nitrophenol (PNP) per ml in salts solution. When introduced into water from Beebe Lake at densities of 330 cells per ml, *P. cepacia* did not mineralize 1.0 µg of PNP per ml. However, PNP was mineralized in lake water inoculated with 3.3×10^4 to 3.6×10^5 *P. cepacia* cells per ml. In lake water containing 1.0 µg of PNP per ml, a *P. cepacia* population of 230 or 120 cells per ml declined until no cells were detectable at 13 h, but when the initial density was 4.3×10^4 cells per ml, sufficient survivors remained after the initial decline to multiply at the expense of PNP. The decline in bacterial abundance coincided with multiplication of protozoa. Cycloheximide and nystatin killed the protozoa and allowed the bacterium to multiply and mineralize 1.0 µg of PNP, even when the initial *P. cepacia* density was 230 or 360 cells per ml. The lake water contained few lytic bacteria. The addition of KH_2PO_4 or NH_4NO_3 permitted biodegradation of PNP at low cell densities of *P. cepacia*. We suggest that a species able to degrade a synthetic chemical in culture may fail to bring about the same transformation in natural waters, because small populations added as inocula may be eliminated by protozoan grazing or may fail to survive because of nutrient deficiencies.

Fresh and marine waters, sewage, and soils possess highly diverse microbial communities that exhibit many degradative capacities, and species within these communities destroy many organic chemicals. Nevertheless, many synthetic compounds persist in these environments even though the molecules are biodegradable, and hence, inoculation with species possessing the appropriate catabolic properties has been proposed as a means of enhancing the decomposition of these chemicals.

Much of the research that has been done on inoculation of bacteria to enhance biodegradation has centered on pesticides, oil, or specific hydrocarbon constituents of oil. The inoculation of soil with a mixture containing *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* has been reported to enhance the destruction of parathion (1); addition of a pentachlorophenol-degrading *Flavobacterium* sp. to soil samples led to the mineralization of this chemical (2), and introduction of a tetramethylthiuram disulfide-degrading strain of *P. aeruginosa* into soil under laboratory conditions resulted in degradation of this fungicide (9). Inoculation of samples of natural waters has also been reported to enhance mineralization of *p*-nitrophenol (PNP) (17) and benzoate (11). In contrast, other reports show that addition of microorganisms to environmental samples fails to enhance biodegradation. Lehtomäki and Niemelä (6) found that inocula of oil-degrading bacteria did not have a significant effect on oil biodegradation in contaminated soils. Similarly, a mixture of hydrocarbon-degrading bacteria added to a seawater microcosm did not stimulate the decomposition of crude oil (13), and a PNP-utilizing *Pseudomonas* sp. did not mineralize PNP in samples of lake water (4).

One possible reason for the failure of inoculation of contaminated sites with species able to degrade the chemical in culture is the inoculum size. A bacterium in axenic culture

in rich medium is able to proliferate extensively even from a small inoculum. However, the low cell density that realistically can be introduced into large volumes of chemically polluted waters or soils may not replicate enough to bring about extensive biodegradation because other species inhabiting those environments may compete for the limited supply of inorganic nutrients or may prey on or parasitize the introduced population of bacteria before it becomes sufficiently large to bring about appreciable destruction of the polluting chemical. Hence, a study was conducted to determine the importance of inoculum size in limiting the success of inocula added to lake water to bring about biodegradation. The test chemical was PNP.

MATERIALS AND METHODS

Isolation of bacteria. A bacterium that was designated strain MR101 was isolated from Kendaia clay loam soil by enrichment culture in a medium previously described (17). When the yellow color of PNP had disappeared, the enrichment culture was transferred twice to fresh medium and then was plated on the same medium (30 µg of PNP per ml) solidified with 1.5% agar. A colony that caused disappearance of the yellow color of PNP was selected. The isolate was checked for purity and identified by standard methods, including API 20E strips (Analytab Products, Plainview, N.Y.).

An antibiotic-resistant mutant was obtained by transferring the culture serially in 0.3% Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) containing 50, 100, 200, or 500 µg of streptomycin (Sigma Chemical Co., St. Louis, Mo.) per ml. Colonies growing on TSB agar with the highest concentration of streptomycin were transferred to TSB containing 500 µg of streptomycin per ml and increasing concentrations of kasugamycin (Sigma). An isolate resistant to 500 µg of streptomycin and 100 µg of kasugamycin per ml was transferred three times serially in

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TSB. Counts of the culture on TSB agar with and without antibiotics gave similar values, indicating the stability of the antibiotic resistance. This antibiotic-resistant isolate was used only in studies in which bacterial abundance in nonsterile lake water was determined.

Environmental samples. Lake water samples were collected at different times of the year from Beebe Lake, Ithaca, N.Y., and used within 2 h of collection. The pH of the water ranged from 6.5 to 7.5. Untreated sewage was collected from the primary settling tank of the Ithaca, N.Y., treatment plant and used within 2 h of collection. The sewage samples were passed through Whatman no. 40 filter paper to remove the suspended matter. The pH of the samples ranged from 7.0 to 7.4. For some experiments, the lake water or sewage was sterilized by passage through 0.2- μm -pore-size membrane filters (Sybron Corp., Rochester, N.Y.).

Microbial counts. Bacteria were counted by the spread-plate technique. The medium used for counting the isolate in nonsterile lake water was TSB agar containing 500 μg of streptomycin, 100 μg of kasugamycin, and 250 μg of cycloheximide per ml. The plates were incubated at 30°C for 48 h before counting. TSB (half strength) agar without antibiotics was used for counts of inoculated sterile lake water and buffer. The ability of the bacteria to survive was tested by adding the organisms to duplicate 250-ml Erlenmeyer flasks containing 50 ml of lake water, buffer, or filter-sterilized lake water. The counts were made on triplicate plates incubated at 30°C for 48 h.

The trophic forms of protozoa were counted by direct microscopic examination of duplicate samples at $\times 400$ magnification. For each sample, five transects of a cover glass (22 by 22 mm) were counted. Lytic organisms were enumerated by the double-layer technique (14).

Preparation of the inoculum. The bacteria were grown at 30°C on a rotary shaker operating at 80 rpm and in a medium composed of the inorganic-salts solution described above and 30 μg of PNP per ml. When the culture reached the late logarithmic phase of growth, usually in 18 to 24 h, the cells were collected by centrifugation at $20,000 \times g$ for 20 min at 4°C, washed with fresh salts solution, and suspended in sterilized distilled water.

All glassware was soaked overnight in concentrated sulfuric acid containing 15 g of Nochromix (Godax Laboratories, New York, N.Y.) per liter and then rinsed twice with deionized distilled water.

Measurement of mineralization. Samples (50 ml) of salts solution, lake water, or sewage were added to 250-ml Erlenmeyer flasks sealed with Teflon-lined stoppers. Unless otherwise noted, washed cells were added to an initial density of 3.3×10^4 cells per ml. All treatments were in duplicate. The flasks were incubated at 30°C on a rotary shaker operating at 80 rpm. At intervals, samples from each flask were removed, acidified with 3 drops of concentrated sulfuric acid, and bubbled with forced air for 3 to 5 min to drive off radioactive CO_2 . The samples were mixed with Liquiscint scintillation fluid (National Diagnostics, Highland Park, N.J.), and the radioactivity remaining in the solution was determined with a Beckman liquid scintillation counter (model LS 7500; Beckman Instruments, Inc., Fullerton, Calif.). Full details of this method have been described previously (11).

[2,6- ^{14}C]PNP (30 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. In experiments in which the PNP concentration was 2 or 10 ng/ml, only labeled compound was added. In experiments in which the concentration of PNP was above 10 ng/ml, the radioactive chemical

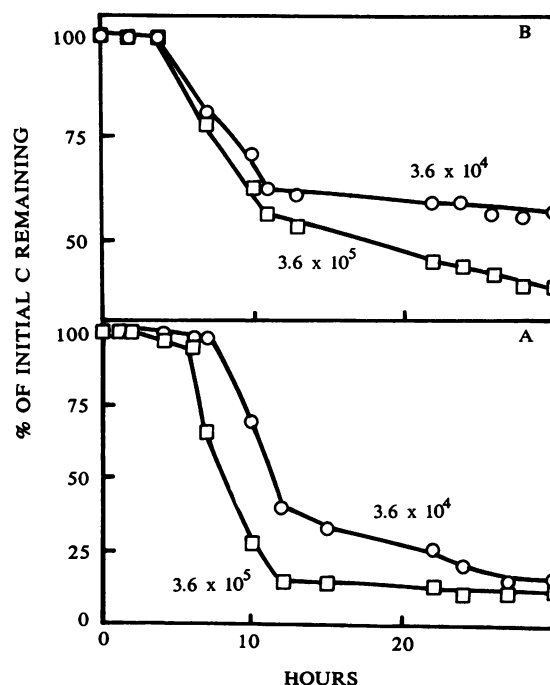


FIG. 1. Effect of inoculum size of *P. cepacia* on the mineralization of 1.0 μg (A) and 1.0 ng (B) of PNP per ml in nonsterile lake water.

was added to give 1,000 to 2,000 dpm/ml, and unlabeled compound was added to the desired concentration.

In each test of mineralization, the activity of the isolate was verified by showing that the bacterium inoculated into the inorganic-salts solution containing the test concentration of PNP metabolized the compound. No evidence was found for PNP volatilization, sorption by glassware, or photooxidation during the test periods when the same concentrations of labeled and unlabeled compound were added to uninoculated, nonsterile lake water.

For some experiments, cycloheximide (Sigma) was added to lake water in a 20% solution of methanol in water, and nystatin (Sigma) was added as a freshly prepared methanolic solution.

RESULTS

Isolate MR101 is a gram-negative, oxidase-positive, catalase-positive, aerobic, motile rod that liquefies gelatin. On the basis of these traits and the results of tests with API 20E strips, the bacterium was identified as a strain of *Pseudomonas cepacia*. The bacterium grew in the salts solution containing 10 ng to 30 μg of PNP per ml and mineralized the nitro compound.

When added at an initial density of 3.6×10^4 or 3.6×10^5 cells per ml, the bacterium readily mineralized 1.0 μg of PNP per ml in nonsterile Beebe lake water, and at least 80% of the added PNP was mineralized (Fig. 1A). Bacteria at the same densities also mineralized 1.0 ng of PNP per ml in nonsterile lake water, but the extent of mineralization at these concentrations depended on the inoculum size (Fig. 1B). The differences in extent of mineralization are not the result of differences in the amount of carbon incorporated into the cells, a fact indicated by the amounts of ^{14}C removed by passage of the liquids through 0.22- μm -pore-size filters.

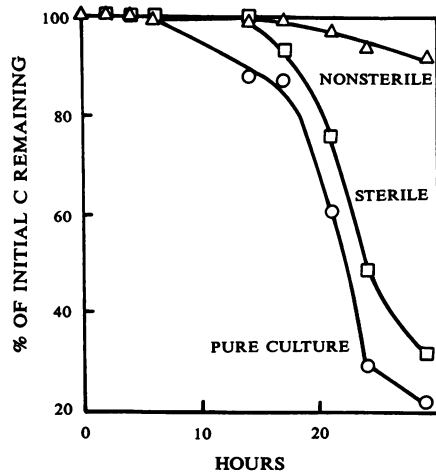


FIG. 2. Mineralization of 1.0 μg of PNP per ml by 330 *P. cepacia* cells per ml inoculated into salts solution and sterile and nonsterile lake water.

On the other hand, when nonsterile lake water containing 1.0 μg of PNP per ml was inoculated with 330 cells of *P. cepacia* per ml, mineralization was not evident after 40 h. Similarly, an inoculum of 230 cells per ml added to another sample of lake water mineralized less than 5% of 30 μg of PNP per ml in 50 h. Rapid mineralization of PNP at these concentrations was evident if the lake water received larger inocula.

To determine why the test bacterium at low cell densities failed to mineralize PNP in nonsterile lake water, samples of filter-sterilized lake water, nonsterile lake water, and salts solution were amended with 1.0 μg of PNP per ml. *P. cepacia* was added at an initial density of 330 cells per ml. After a period in which C loss was not detected, mineralization was rapid in salts solution and sterile lake water (Fig. 2). However, little PNP was mineralized in 29 h in nonsterile lake water following inoculation.

Because competition with indigenous bacteria for inorganic nutrients might have prevented rapid mineralization by *P. cepacia* inoculated into nonsterile lake water at a low cell density, a study was conducted to ascertain the effect of specific inorganic nutrients on mineralization. Nonsterile lake water was amended with 1.0 μg of PNP and 790 cells of *P. cepacia* per ml. The water also was amended with 100 μg of KH_2PO_4 , 100 μg of NH_4NO_3 , or 10 μg of CaCl_2 per ml or with nothing. Mineralization was evident in water amended with KH_2PO_4 or NH_4NO_3 , but CaCl_2 had no effect (Fig. 3).

Because the inability of *P. cepacia* at low inoculum density to degrade PNP could be merely a consequence of the decline of the population, a study was conducted to test the survival of this organism in nonsterile and filter-sterilized lake water. The bacterium was added to phosphate buffer (0.03% KH_2PO_4 and 0.07% K_2HPO_4 ; pH 7.2), filter-sterilized lake water, and nonsterile lake water. The bacterium persisted in filter-sterilized lake water and in phosphate buffer in numbers in excess of 5×10^3 per ml (Fig. 4A). The population declined more markedly in nonsterile lake water and reached a low level.

P. cepacia was also added to nonsterile lake water amended with 1.0 μg of PNP per ml at initial densities of 4.3×10^4 , 230, and 120 cells per ml. Under these conditions, the number of cells declined, and none could be detected at 13 h or later in water inoculated with either 230 or 120 cells per ml

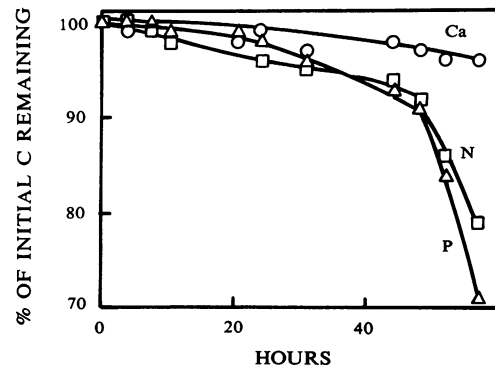


FIG. 3. Effect of added inorganic nutrients on mineralization of 1.0 μg of PNP per ml by *P. cepacia* in nonsterile lake water.

(Fig. 4B). At the highest inoculum density, the number of cells declined by almost 10-fold, but the remaining survivors persisted and started to increase in cell numbers at 15 h; the population reached 2.1×10^6 cells per ml after 30 h.

To determine the possible effect of eucaryotes on PNP

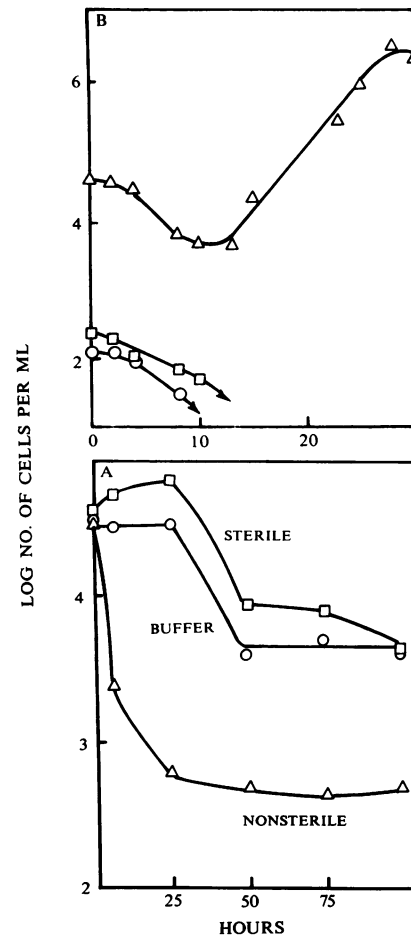


FIG. 4. Survival of *P. cepacia* in nonsterile lake water, filter-sterilized lake water, and phosphate buffer (A) and in nonsterile lake water amended with 1.0 μg of PNP per ml (B). The three curves in panel B depict the population changes following inoculation with the cell densities indicated at 0 h.

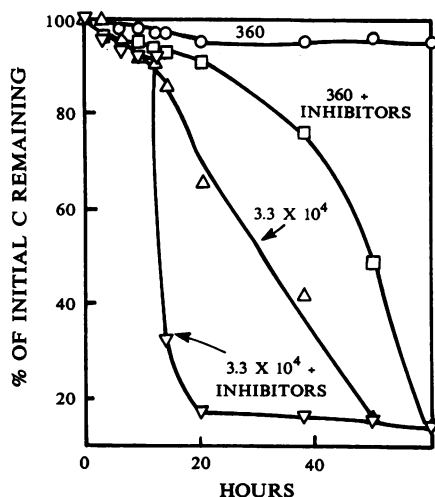


FIG. 5. Effect of eucaryotic inhibitors on mineralization of 1.0 μg of PNP per ml in nonsterile lake water inoculated with two *P. cepacia* cell densities.

mineralization by the test bacterium, 250 μg of cycloheximide and 30 μg of nystatin per ml were added to half of the samples of nonsterile lake water. The water samples were then inoculated with 3.3×10^4 or 360 *P. cepacia* cells per ml and amended with 1.0 μg of PNP per ml. The small inoculum mineralized PNP when cycloheximide and nystatin were present but not when these inhibitors were absent (Fig. 5). The large inoculum brought about mineralization in both the presence and absence of these two eucaryotic inhibitors, but mineralization was faster in lake water supplemented with the antibiotics.

To determine the possible role of protozoa in the elimination of the added organism in nonsterile lake water containing 1.0 μg of PNP per ml, the numbers of *P. cepacia* and indigenous protozoa were determined. Two inoculum sizes were tested. Even though PNP was present, the cell density arising from the small inoculum (230 cells per ml) declined when protozoa were not inhibited, and *P. cepacia* could not be detected at 21 h or thereafter (Fig. 6A). In water receiving the same inoculum size but with protozoa inhibited by addition of the two inhibitors, the inoculated bacterium multiplied after a lag period and reached a population of 3.7×10^3 cells per ml. In water inoculated with 1.3×10^5 *P. cepacia* per ml, the number of cells of this strain initially declined by almost 10-fold when protozoa were actively multiplying (in the absence of inhibitors), and then *P. cepacia* began to multiply (Fig. 6B). The number of protozoa decreased in the presence of the inhibitors, and none were detected by microscopic examination at 21 h or later in water samples inoculated with either low or high cell densities of the test bacterium.

Lytic bacteria were detected in lake water at a density of 130 cells per ml. Addition of a lytic isolate at an initial density of 630 cells per ml to a salts solution amended with 1 μg of PNP per ml and inoculated with 330 cells of *P. cepacia* had no effect on PNP mineralization, which was the same as that in a culture without the lytic bacteria.

DISCUSSION

The data show that a bacterium able to degrade PNP in pure culture is capable of destroying the nitro compound

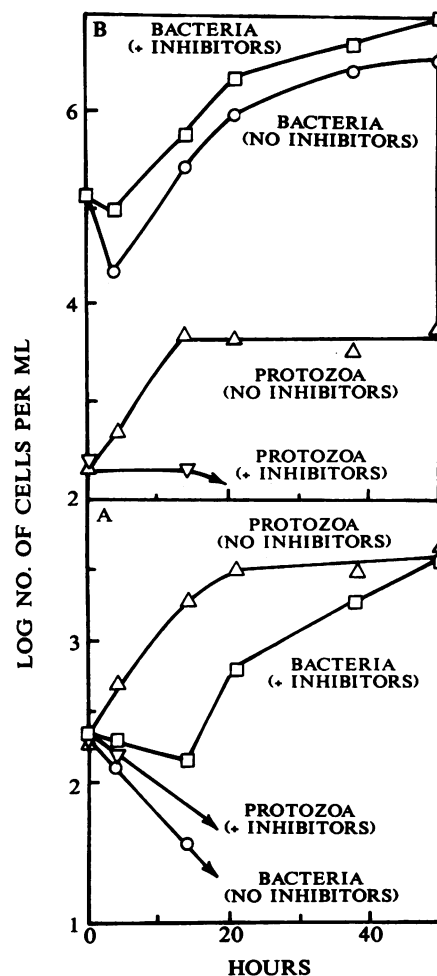


FIG. 6. Effect of eucaryotic inhibitors on the numbers of *P. cepacia* and indigenous protozoa in nonsterile lake water amended with 1.0 μg of PNP per ml. (A) Low inoculum density of *P. cepacia*. (B) High inoculum density.

when added to nonsterile lake water at relatively high cell densities but not at low ones. *Corynebacterium* sp. at high cell density also can mineralize PNP at 1.0 $\mu\text{g}/\text{ml}$ in nonsterile lake water (17), and a *Flavobacterium* sp. at a density of 5.5×10^7 cells per ml can degrade pentachlorophenol in fresh water (7). Edgehill and Finn (3) also found that the rate of disappearance of pentachlorophenol brought about by an *Arthrobacter* sp. was related to the inoculum size.

The failure of the small inoculum to mineralize PNP in lake water could be a result of the failure of the bacterium to survive. Because more cells were added with the large inoculum, a marked population decline might not result in the total elimination of viable cells. The results demonstrate that survival was indeed affected by inoculum size. Thus, when *P. cepacia* was added to lake water containing 1.0 μg of PNP per ml at initial densities of 120 and 230 cells per ml, the population declined to a density below the detection limit. In contrast, when *P. cepacia* was added at a high cell density, the population also declined, but the survivors subsequently were able to multiply. Although antibacterial toxins are present in some fresh waters (5), the decline is not a result of the presence of such toxins because the organism persisted in filter-sterilized lake water and also was able,

when inoculated into such filtered water at low cell density, to destroy the test compound. Toxins as well as bacteriophages should pass through such filters, so neither of these agents is implicated.

Protozoa are present in fresh waters as well as in sewage, and their influence on PNP metabolism was readily evident. Thus, low inoculum densities did not result in PNP mineralization when protozoa were active, but suppression of these grazers by eucaryotic inhibitors permitted the transformation to proceed. Population estimates also revealed the disappearance of *P. cepacia* added at low densities to lake water containing active protozoa, but the decline was small and followed by multiplication of *P. cepacia* when the predators were inhibited. The role of protozoa in controlling bacterial populations in natural waters is well documented (8), and protozoa are also known to delay the initiation of biodegradation of synthetic chemicals and to affect the populations of bacteria acting on such compounds (15, 16).

The ability of a bacterium to maintain itself in the face of grazing pressure is probably a balance between predation and growth rate of the prey, and it has been suggested that protozoa may eliminate slow-growing members of bacterial communities (10). In the presence of an organic nutrient that one bacterial species but few other members of the microbial community can use, such as PNP, the bacterium carrying out the transformation presumably is able to multiply. Nevertheless, the growth rate would be affected by the concentration of inorganic nutrients; therefore, it is not surprising that *P. cepacia* at low densities was able to destroy PNP when the lake water was supplemented with N or P. It has been reported that the addition of inorganic nutrients substantially reduces the acclimation period prior to biodegradation in fresh water (12). Since mineralization of PNP by a small inoculum was evident in filter-sterilized water, it is likely that the supply of inorganic nutrients in nonsterile water was reduced by members of the indigenous microbial community as they used natural organic materials in the water.

It is probably often not feasible to inoculate large volumes of natural waters with sufficient bacterial cells to provide a high initial cell density. Should the inoculated organism fail to survive some initial biotic or abiotic stress because of inadequate initial numbers, it may not bring about the biodegradative process for which it was introduced into the particular environment. Hence, additional attention should be given to devising means by which species introduced into natural or modified environments can successfully destroy chemicals that they have the capacity to metabolize in culture.

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