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Predation and Inhibitors in Lake Water Affect the Success of Inoculation To Enhance Biodegradation of Organic Chemicals

Baqar R. Zaidi,[†] Yoshitaka Murakami,[‡] and Martin Alexander*

Institute for Comparative and Environmental Toxicology and Department of Agronomy, Cornell University, Ithaca, New York 14853

■ *p*-Nitrophenol (PNP) at 50, 75, and 100 μg/L was mineralized extensively in lake water inoculated with *Corynebacterium* sp. but not in uninoculated lake water. The bacterium mineralized a far lower percentage of PNP at 26 μg/L than at the higher concentrations. Cycloheximide did not affect mineralization in lake water containing the three higher concentrations, but the extent of mineralization and *Corynebacterium* sp. numbers at 26 μg of PNP/L were reduced by this eucaryotic inhibitor. Dialysis of lake water did not influence the rate of mineralization of 1.0 mg/L, but it reduced the acclimation period, stimulated *Corynebacterium* sp., and usually enhanced the rate of its mineralization of PNP at 10 and 26 μg/L. An inhibitor of PNP metabolism and *Corynebacterium* sp. could be removed from lake water by a cation-exchange resin. A mixed culture mineralized PNP at 26 μg/L faster than *Corynebacterium* sp. The data show that predation or inhibitors may markedly affect bacteria inoculated into natural waters to promote biodegradation of low concentrations of organic chemicals.

Introduction

In many laboratory studies of the microbial transformation of organic compounds, the concentrations of chemicals used are higher than those found in natural waters, soils, and sediments. In those investigations, it was assumed that if a chemical is mineralized at higher concentrations, it would be transformed at the low concentrations found in natural environments. Evidence is accumulating that the occurrence of mineralization of organic compounds is a function of concentration. In some instances, a chemical that is mineralized in natural waters at high concentrations is not mineralized at low concentrations (*1*). Conversely, a compound mineralized at low concentrations may not be transformed at high but presumably still not toxic levels (*2*, *3*).

The inability of many microorganisms to degrade organic chemicals at low concentrations may pose a problem in attempts to develop microbial inoculants to destroy organic pollutants in nature. In many of these investigations, the isolates were obtained by use of substrate concentrations far greater than those found in most polluted environments (*4*, *5*). No attempt was made to determine whether the inoculant would be active at low levels of the organic compound supporting growth.

The present study was designed to investigate why an inoculant that grows on and destroys an organic chemical at high concentrations may function poorly at low concentrations of the same substrate. The test compound was *p*-nitrophenol (PNP).

Materials and Methods

Corynebacterium sp. was obtained from a sample of lake water in which mineralization of 1.0 mg of PNP/L was occurring, and it was purified on an agar medium containing inorganic salts and 1.0 mg of PNP/L. An antibiotic-resistant derivative of the culture, which can mineralize PNP, was obtained by inoculating the original culture into Trypticase (0.3%) soy broth containing 50 mg of streptomycin/L. The culture that developed was inoculated into Trypticase soy broth containing 100 mg of streptomycin and 10 mg of kasugamycin/L, and the culture that appeared was inoculated into Trypticase broth containg 50 mg of streptomycin, 10 mg of kasugamycin, and 10 mg of spectinomycin/L. Cell suspensions derived from these cultures were plated on Trypticase soy agar containing 100 mg of streptomycin, 10 mg of kasugamycin, and 25 mg of spectinomycin/L. The population size of this bacterium in lake water was determined in triplicate by the drop plate technique (*6*) on 0.3% Trypticase soy agar containing the three antibiotics at the indicated concentrations. The plates were incubated at 29 °C for 48–72 h before counting. Colonies of indigenous lake-water bacteria did not appear on plates of the medium containing the three antibiotics, but cells of the antibiotic-resistant *Corynebacterium* were quantitatively recovered.

Samples of water collected from Beebe and Cayuga Lakes, Ithaca, NY, were used within 1 h of collection. Some samples of lake water were sterilized by passage through 0.2-μm pore-size membrane filters (Sybron Corp., Rochester, NY).

[†] Present address: Department of Marine Sciences, University of Puerto Rico, Mayaguez, PR 00708.
[‡] Present address: Osaka Gas Co., Ltd., 5-11-61 Torishima, Konohana-ku, Osaka, Japan.

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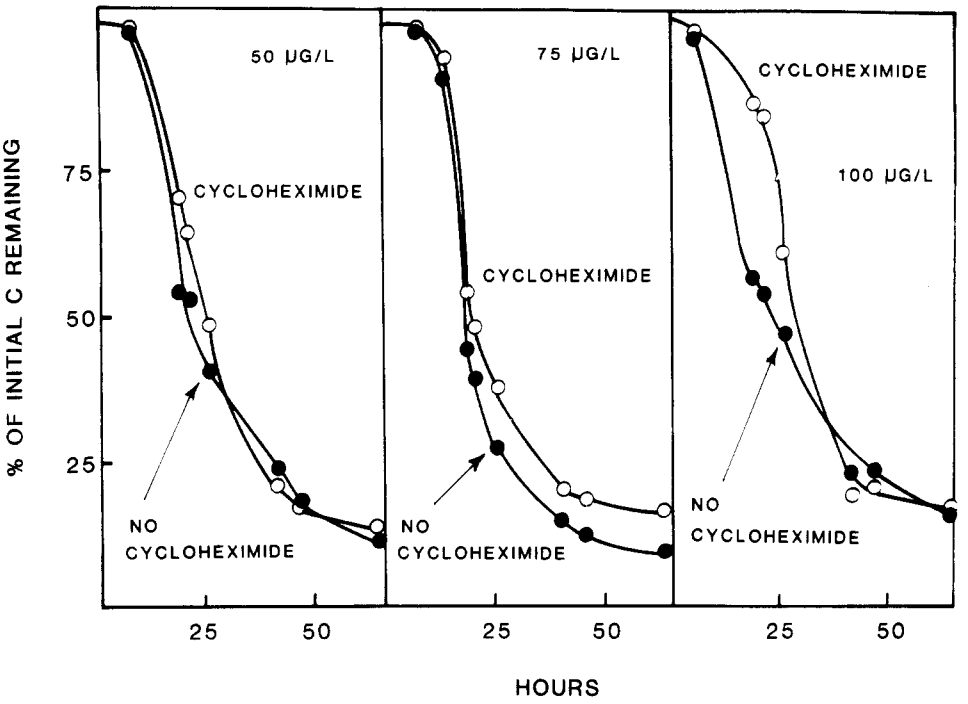

Figure 1. Mineralization of PNP at 50, 75, and 100 µg/L by *Corynebacterium* sp. added to lake water amended and not amended with 250 mg of cycloheximide/L.

To measure mineralization, duplicate 25-mL samples of inorganic salts solution or lake water were placed in Teflon-lined screw-capped 200-mL bottles. Washed cells grown for 24 h in salts solution amended with 10 mg of PNP/L were added to give initial densities of approximately 10^4–10^5 cells/mL. The patterns of mineralization were the same with an inoculum size of 10^3 cells/mL and with higher cell densities, but the larger inocula were used to reduce the lag phase prior to detectable mineralization. Because it is the convention in microbiology, cell counts are given as number per milliliter. The salts solution contained 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 10 mg of $FeCl_3$, and 0.1 g each of NH_4NO_3, $CaCl_2 \cdot 7H_2O$, and $MgSO_4$ per liter of distilled water. In a single experiment, all bottles received similar amounts of ^{14}C-labeled compounds, but the radioactivity in different experiments varied from 100 to 2900 dpm/mL. The final substrate concentration was varied by adding different quantities of unlabeled compounds. The liquids were incubated at 29 °C on a rotary shaker operating at 120 rpm. At regular intervals, 1.0-mL portions were transferred from the bottles to 9-mL plastic vials, and the liquids were acidified with 0.2 mL of 1 M H_2SO_4. Compressed air was bubbled through the liquid for 5 min to drive off CO_2, and 7 mL of Liquiscint scintillation fluid (National Diagnostics, Inc., Somerville, NJ) then was added to 1.0 mL of each of the acidified samples. The radioactivity was counted with a liquid scintillation counter (Model LS 7500; Beckman Instruments, Inc., Irvine, CA). This procedure has been shown to measure mineralization of PNP, since the loss of ^{14}C from labeled PNP added to the liquid is parallel to the formation of $^{14}CO_2$ (7).

For some studies, filter-sterilized lake water was dialyzed against distilled water for 24 h using dialysis tubing having a 1000 molecular weight cutoff. The liquids were sterilized by filtration after dialysis, but asepsis was not maintained during dialysis. To concentrate the inhibitor(s), 100-mL portions of filter-sterilized lake water were added to a column containing 5.0 g of sterilized cation-exchange gel (CM-Sephadex C-25; Pharmacia Fine Chemicals, Piscataway, NJ). After the slurry was allowed to equilibrate for 24 h, the void volume of sterilized lake water was collected, 0.5 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.0 was then passed through the column, and the fractions collected were pooled and freeze-dried.

To enrich for microorganisms acting on low concentrations of PNP, 25 mL of lake water containing 10 µg of labeled PNP/L was incubated at 29 °C on a rotary shaker, and disappearance of the radioactivity from the liquid was measured. After 10 days, at which time 90% of the C was mineralized, the sample was centrifuged at 5000*g*, and the cells removed were washed twice with filter-sterilized lake water and then resuspended in 25 mL of sterile lake water. A 0.1-mL portion of this suspension was added to 25 mL of sterilized lake water containing 10 µg of PNP/L. This procedure was repeated five times.

Results

PNP at 50, 75, and 100 µg/L was not mineralized in lake water in 7 days. However, *Corynebacterium* sp. added to lake water mineralized the chemical in the presence or absence of 250 mg of cycloheximide/L (Figure 1). An analysis of variance of the data indicated that cycloheximide did not have a statistically significant effect. Cycloheximide is an inhibitor of protozoa, fungi, and other eucaryotic organisms.

However, only 35% of PNP C was mineralized when the bacterium was inoculated into Cayuga Lake water containing 26 µg of PNP/L (Figure 2). Under these conditions, the cell number initially increased somewhat but then declined. Addition of this bacterium to lake water amended with 250 mg of cycloheximide/L resulted in rapid degradation of 26 µg of PNP/L. About 75% of the PNP C was mineralized in 70 h, and the cell number increased to approximately 10^6 cells/mL in this period if the inhibitor was present. No mineralization of PNP was observed in uninoculated lake water in 7 days.

A study was then conducted to ascertain the effects of two eucaryotic inhibitors, cycloheximide (250 mg/L) and nystatin (30 mg/L), on the growth and degradation of PNP by *Corynebacterium* sp. in inorganic salts solution containing 26 µg of PNP/L. Measurements of mineralization

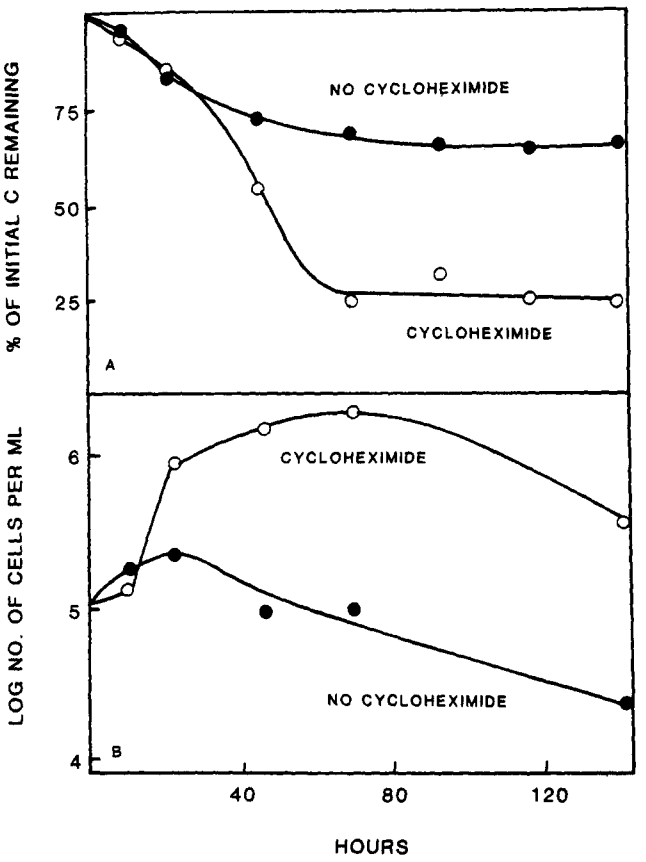

Figure 2. PNP mineralization (A) and growth of *Corynebacterium* sp. (B) in lake water containing 26 μg of PNP/L in the presence and absence of cycloheximide.

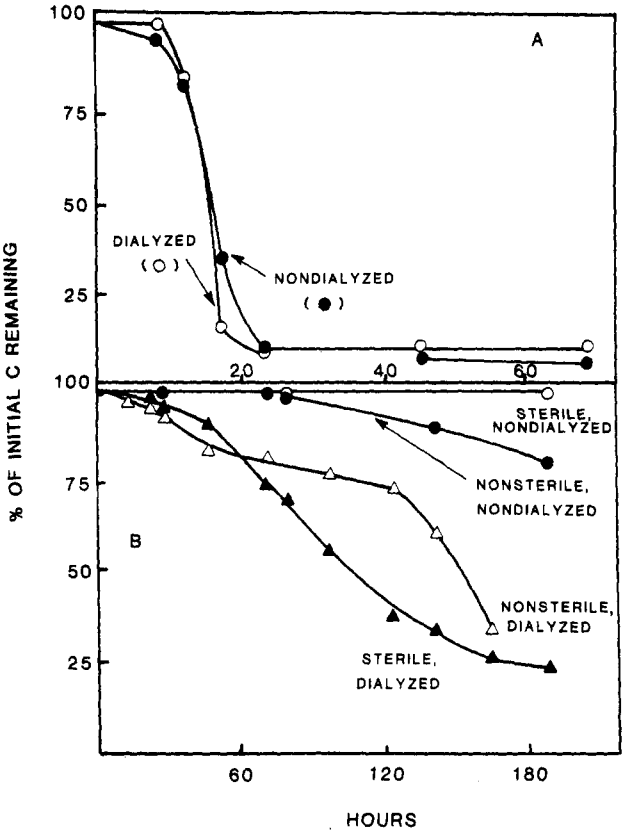

Figure 3. Mineralization by *Corynebacterium* sp. inoculated into (A) dialyzed and nondialyzed nonsterile lake water containing 1 mg of PNP/L and (B) dialyzed sterile and nonsterile lake water and nondialyzed sterile and nonsterile lake water containing 10 μg of PNP/L.

at 3, 6, 10, 12, 19, 22, 28, 46, and 70 h and counts at 0, 6, 12, 18, 28, and 46 h showed that the inhibitors had no statistically significant effects on either increases in cell number or the biodegradative activity of the population.

To determine whether the decline in number of *Corynebacterium* sp. and its slow degradation of low concentrations of PNP resulted from the presence of an inhibitor in lake water, samples of nonsterile Cayuga Lake water were dialyzed against distilled water for 24 h. *Corynebacterium* sp. was inoculated into the nonsterile dialyzed (pH 6.0) and nondialyzed (pH 8.1) samples of lake water amended with 1 mg of PNP/L. No mineralization was evident in 2 days without the inoculum. However, addition of the bacterium to both dialyzed and nondialyzed lake water resulted in 90% mineralization of PNP in 23 h (Figure 3A). Thus, the possible presence of an inhibitor did not have a detectable effect on degradation of this concentration of PNP in inoculated lake water.

The influence of dialysis of lake water on mineralization of 10 μg of PNP/L by *Corynebacterium* sp. was also measured. In this instance, samples of both nonsterile and sterile lake water were tested to assess possible effects on both indigenous bacteria and the isolate. Mineralization in nonsterile lake water inoculated with the isolate began somewhat after 77 h in lake water that had not been dialyzed, but activity was not evident if the bacteria were inoculated into sterile lake water that had been dialyzed (Figure 3B). In contrast, mineralization in dialyzed lake water was rapid. The reason for the biphasic transformation in dialyzed nonsterile lake water inoculated with *Corynebacterium* sp. is unknown.

An experiment was conducted to determine whether the enhanced degradation of low concentrations of PNP in dialyzed lake water resulted from the change in the pH of lake water. A sample of lake water was used in which the pH fell after 24 h of dialysis from 8.0 to 6.0. The pH of the dialyzed lake water was brought to 8.0 by addition 0.5 M Tris buffer. No degradation of PNP at 26 μg/L was observed in nondialyzed, uninoculated lake water in 5 days. However, when *Corynebacterium* sp. was inoculated in sterile and nonsterile dialyzed Cayuga Lake water at pH 8.0 containing 26 μg PNP/L, about 90% of the chemical was mineralized in 25 h (Figure 4A). In contrast, only 16% was mineralized in nondialyzed, nonsterile lake water in 43 h. Following its inoculation into dialyzed sterile and nonsterile lake water, the cell number of *Corynebacterium* sp. rapidly increased, whereas there was an initial decline following inoculation of the nondialyzed nonsterile lake water (Figure 4B). Similarly, the bacterium rapidly degraded 10 μg of PNP/L in dialyzed samples of sterile and nonsterile lake water, but the conversion was slow in nondialyzed, nonsterile lake water inoculated with the bacterium; under these conditions, the cell number increased in dialyzed portions of sterile and nonsterile lake water following inoculation, whereas a rapid decline was noted following inoculation of nondialyzed lake water (data not shown).

In sterile lake water amended with 26 μg of PNP/L and 40 mg of a freeze-dried material obtained by treating lake water with a cation-exchange gel, *Corynebacterium* sp. very slowly mineralized PNP, and about 15% of the chemical was mineralized in 117 h (Figure 5). Mineralization by the isolate was far more rapid and extensive in the void volume of the sterile lake water not amended with the freeze-dried material.

Addition of the freeze-dried, low molecular weight, cationic fraction at 400 mg/L to sterile lake water prevented degradation of the chemical. The cell number decreased from an initial value of 3.7×10^4 to less than 100 per milliliter within 1 day in the void volume amended

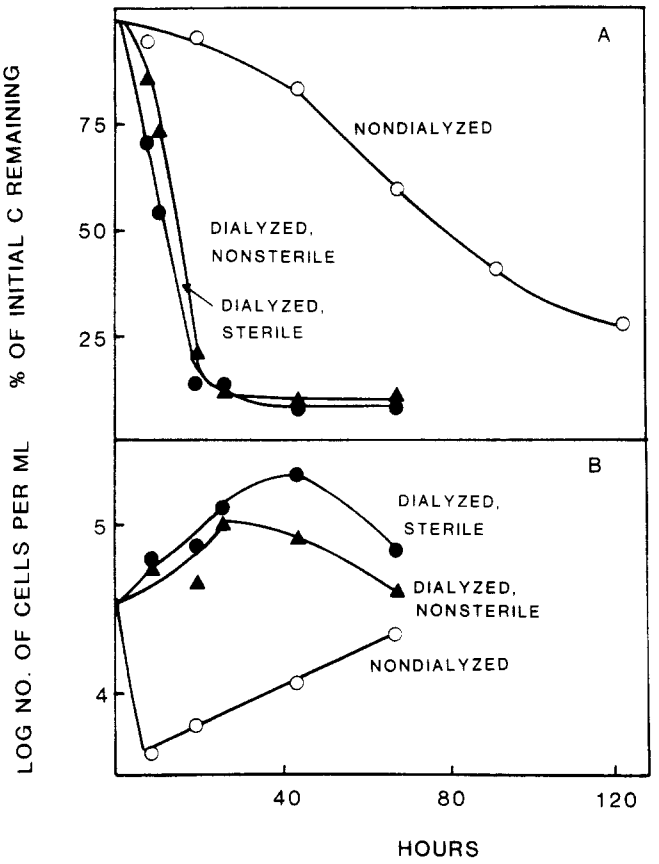

Figure 4. PNP mineralization (A) and growth (B) of *Corynebacterium* sp. in dialyzed sterile and nonsterile lake water and in nondialyzed, nonsterile lake water containing 26 μg of PNP/L.

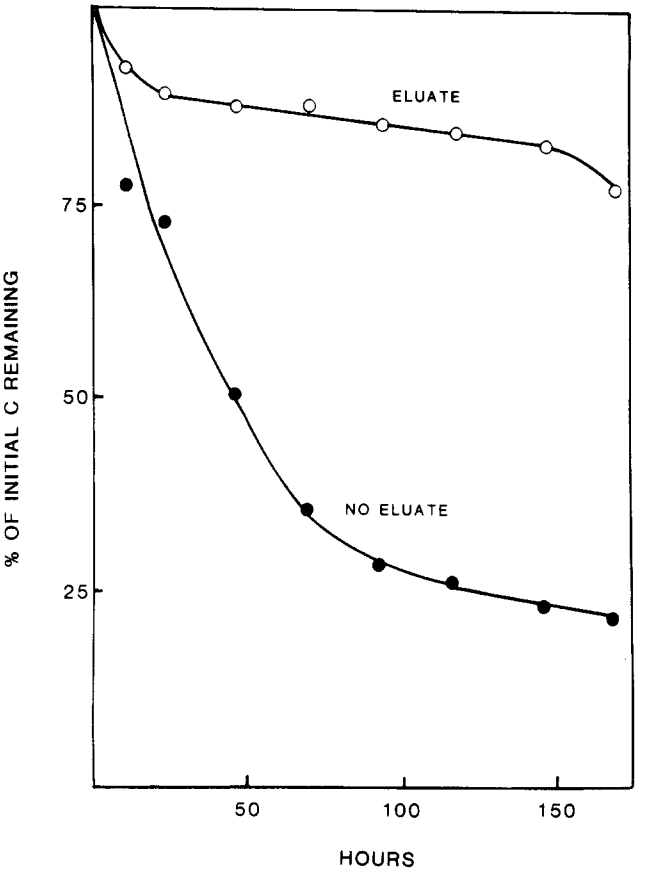

Figure 5. Degradation of 26 μg of PNP/L in lake water with and without 40 mg of dried material eluted from a cation-exchange resin.

with 26 μg of PNP and 400 mg of the freeze-dried fraction per liter, whereas the cell numbers rose to 5.5×10^5 cells/mL in the void volume of sterile lake water not containing the inhibitor (data not shown).

Because *Corynebacterium* sp. as grown in the previous experiments did not degrade the low concentrations of PNP, enrichments were made for microorganisms able to degrade 10 μg of PNP/L. This enrichment culture and *Corynebacterium* sp. were inoculated into sterile lake water containing 10 μg of PNP/L. After 10–15 days, 100-μL portions of the enrichment and the *Corynebacterium* sp. culture were transferred to 25-mL samples of freshly collected nonsterile and sterile lake water, respectively. After repeating this procedure five times, 100-μL portions of the enrichments and *Corynebacterium* sp. culture were added to freshly collected lake water amended with 26 and 100 μg of PNP/L. The enrichment culture and *Corynebacterium* sp. degraded PNP at both concentrations (Figure 6). However, the enrichment culture destroyed the lower concentration more quickly than *Corynebacterium* sp.

Discussion

The purpose of this study was to establish some of the factors limiting the success of inoculation to stimulate biodegradation at low chemical concentrations. Thus, *Corynebacterium* sp. extensively mineralized 50, 75, and 100 μg of PNP/L when inoculated into lake water, but mineralization was far less at low substrate concentrations. A slow rate of destruction of organic compounds at low concentration has been reported for other bacteria (*1*, *8*).

Cycloheximide, an inhibitor of eucaryotes, did not affect the degradation of PNP at the higher levels, but it resulted in greater activity at the low PNP concentrations. In lake waters containing PNP at 26 μg/L, the abundance of *Corynebacterium* sp. increased slightly initially and then declined, but the cell number increased markedly if cycloheximide was present. This enhanced degradation and growth of the bacterium is not an effect of the inhibitor on the bacterium, as indicated by the absence of an effect in pure culture, but the effect may be associated with an influence of cycloheximide on bacterial grazing by protozoa in the lake water. At levels of the carbon source below the K_s value (i.e., the concentration at which growth occurs at half the maximum rate), the growth rate of bacteria increases with increasing concentrations of that carbon source (*9*). Hence, the cells lost to grazing at the higher PNP level may be replaced rapidly, so that suppression of protozoa does not have a detectable influence on mineralization. In contrast, protozoa may markedly reduce the numbers of the inoculum strain when that organism is growing slowly, so that suppression of the predator by a selective inhibitor enhances biodegradation (*10*).

The data indicate that the samples of lake water contained a low molecular weight, cationic inhibitor that suppressed mineralization. Although dialysis of lake water could promote biodegradation because of the removal of dissolved organic substrates used preferentially to PNP by the bacterium, the results indicate that the suppression of PNP mineralization results from the presence of an antibacterial compound(s). The view that the effect of dialysis results from the presence of an inhibitor is consistent with the findings that *Corynebacterium* sp. grew and readily mineralized PNP in dialyzed as compared to nondialyzed lake water and that a fraction obtained from the lake water markedly slowed the transformation. Antimicrobial compounds are known to occur in marine waters (*11*, *12*) and soils (*13*). Inhibitory agents also have been found in Cayuga Lake water (*14*). The results of the

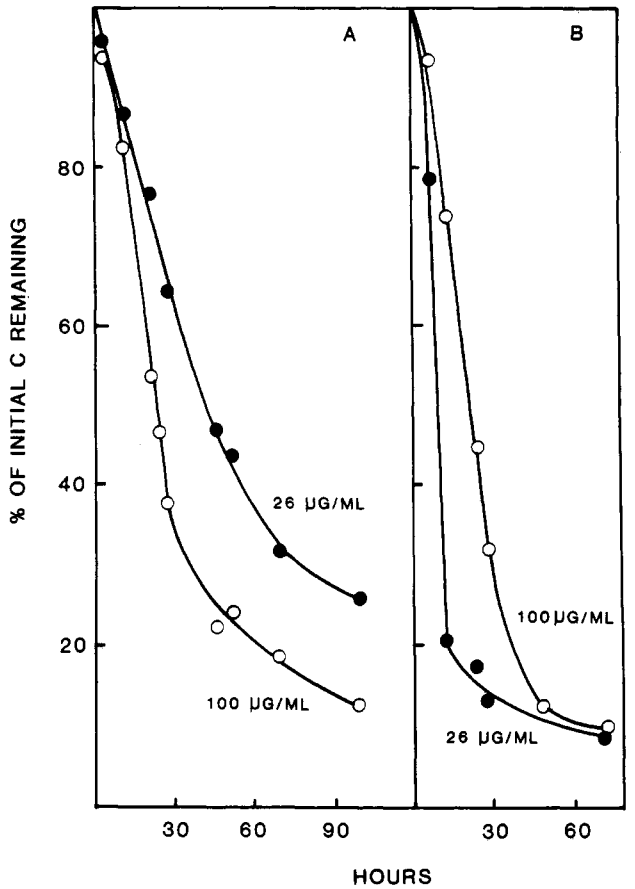

Figure 6. Degradation of 100 and 26 µg of PNP/L by (A) *Corynebacterium* sp. and (B) an enrichment culture.

present study show that the toxicant in Cayuga Lake water did not detectably affect the degradation of PNP at high concentrations by *Corynebacterium* sp., but it did influence the transformation at low concentrations of the chemical. It is not clear why the inhibitor is more effective at low levels of the substrate being transformed.

The results show that the inoculation of bacteria into polluted environments to destroy organic contaminants may have limited success if the chemical is at low concentrations or if predators or inhibitors are present that are harmful to the added organism. However, it is evident that microorganisms differ in their ability to mineralize low concentrations of organic compounds, so that species may be found that actively transform even the low levels of pollutants. A species that is able to grow faster than another presumably would be less affected at the same rate of protozoan grazing. It should also be possible to isolate biodegrading species with high tolerances to inhibitors present in natural or polluted environments. Other constraints in addition to the three shown here—low substrate concentration, inhibitors, and presumably eucaryotic predators—and those demonstrated previously (*7*, *15*) undoubtedly influence the outcome of attempts to use microbial inoculation to promote the destruction of unwanted chemicals. Further work is thus required to define those constraints and to find means to overcome them or minimize their impact.

Registry No. *p*-Nitrophenol, 100-02-7; cycloheximide, 66-81-9.

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