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Explanations for the Acclimation Period Preceding the Mineralization of Organic Chemicals in Aquatic Environments

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A study was conducted of possible reasons for acclimation of microbial communities to the mineralization of organic compounds in lake water and sewage. The acclimation period for the mineralization of 2 ng of *p*-nitrophenol (PNP) or 2,4-dichlorophenoxyacetic acid per ml of sewage was eliminated when the sewage was incubated for 9 or 16 days, respectively, with no added substrate. The acclimation period for the mineralization of 2 ng but not 200 ng or 2 μg of PNP per ml was eliminated when the compound was added to lake water that had been first incubated in the laboratory. Mineralization of PNP by *Flavobacterium* sp. was detected within 7 h at concentrations of 20 ng/ml to 2 μg/ml but only after 25 h at 2 ng/ml. PNP-utilizing organisms began to multiply logarithmically after 1 day in lake water amended with 2 μg of PNP per ml, but substrate disappearance was only detected at 8 days, at which time the numbers were approaching 10^5 cells per ml. The addition of inorganic nutrients reduced the length of the acclimation period from 6 to 3 days in sewage and from 6 days to 1 day in lake water. The prior degradation of natural organic materials in the sewage and lake water had no effect on the acclimation period for the mineralization of PNP, and naturally occurring inhibitors that might delay the mineralization were not present. The length of the acclimation phase for the mineralization of 2 ng of PNP per ml was shortened when the protozoa in sewage were suppressed by eucaryotic inhibitors, but it was unaffected or increased if the inhibitors were added to lake water. We suggest that the time for enzyme induction, mutation, diauxie, and the presence of toxins are not the causes of the acclimation period for PNP mineralization in aquatic environments but rather that the acclimation period largely reflects the time for multiplication of the initially small population of active organisms. That time may be further extended by a limiting supply of inorganic nutrients in lake water or by predation by protozoa in sewage.

The mineralization of many organic compounds in different environments is preceded by an acclimation period. The acclimation period, which is the length of time between the addition of a compound and the onset of its detectable mineralization, may be environmentally significant if the time for acclimation is long because the chemical may become widely disseminated and may affect susceptible species at distant sites before it is destroyed. Furthermore, the use of rate constants for mineralization to predict the concentration of the compound and the duration of exposure of potentially affected populations may be misleading if the acclimation period is not considered.

A number of mechanisms may account for the acclimation period. Several investigators have proposed that it is a result of the time needed for enzymes to be induced (19, 21) or for mutation or genetic exchange to occur (16, 26). The time for small populations of mineralizing organisms to become sufficiently large to bring about a detectable loss of the chemical has also been cited as an explanation for acclimation (17, 24). Other possible explanations include an insufficient supply of inorganic nutrients (11, 23), the preferential utilization of other organic compounds before the chemical of interest (10, 11), the time needed for the mineralizing species to acclimate to toxins or for inhibitors that are present in the environment to be destroyed, or predation by protozoa on the mineralizing organisms.

The purpose of this study was to examine various hypotheses that have been proposed for the acclimation period and to determine which of the postulated mechanisms accounts for the phenomenon in aquatic environments. *p*-Nitrophenol (PNP), which is mineralized after consistent acclimation periods in biodegradation tests (12), was used as the test chemical to evaluate the relative importance of the different mechanisms.

MATERIALS AND METHODS

^{14}C-labeled PNP (30 mCi/mmol) was obtained from ICN Pharmaceuticals Inc., Irvine, Calif., and ^{14}C-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) (28 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. PNP was labeled at the 2 and 6 positions, and the methylene carbon of 2,4-D was labeled. The concentration of both PNP and 2,4-D in tests with samples of sewage was 2 ng/ml, and unlabeled compound was not added. In other experiments, PNP concentrations ranged from 2 to 2,000 ng/ml; [^{14}C]PNP was added to give 500 to 1,500 dpm/ml, and unlabeled PNP was used to give the desired concentration. In some instances, only unlabeled PNP was added to solutions to give 2 μg/ml.

A bacterium capable of mineralizing PNP was isolated from lake water samples amended with 200 ng of PNP per ml. Samples of the enrichment were streaked onto agar medium containing 25 μg of PNP per ml, and colonies that caused the yellow-colored PNP to disappear were restreaked on PNP agar. The bacterium was maintained on agar medium containing 3.0 g of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) per liter.

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Acclimated communities of microorganisms were prepared by adding 2 ng of PNP per ml to fresh sewage and incubating for 11 days at 28°C or by adding 200 ng of PNP per ml to lake water and incubating for 10 days at 23°C. Fresh and acclimated communities of sewage and lake water microorganisms were collected by centrifugation at 12,100 × *g* for 15 min.

Fresh sewage was obtained from the primary settling tank of the sewage treatment system of Ithaca, N.Y. The sewage was filtered through filter paper (no. 41; Whatman Inc., Clifton, N.J.) and used within 2 h of collection. Lake water samples were collected from the top 10 cm of Beebe Lake, Ithaca, N.Y., and used within 2 h of collection.

The inorganic salts solution contained 26 mg of KH_2PO_4, 120 mg of Na_2HPO_4, 50 mg of $(NH_4)_2SO_4$, 10 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $MgSO_4 \cdot 7H_2O$, and 0.5 mg of $FeCl_3 \cdot H_2O$ per liter of deionized, distilled water.

Radiolabeled compounds were added in small portions of water to 50 ml of filtered sewage, lake water, or inorganic salts solution contained in 250-ml Erlenmeyer flasks. The flasks were incubated without shaking at 28°C for the studies of sewage and at 23°C for the experiments with lake water and salts solution. Samples from each flask were acidified, bubbled with air to drive off radioactive CO_2, and mixed with Liquiscint (National Diagnostics, Highland Park, N.J.). The amount of radioactivity remaining in the solution was determined with a liquid scintillation counter (model LS7500; Beckman Instruments, Inc., Fullerton, Calif.). Full details of this method have previously been described (20). The disappearance of 2 μg of unlabeled PNP per ml from some lake water samples was determined with a Spectronic 88 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at 410 nm.

Some sewage and lake water samples were sterilized by autoclaving for 20 min. Some sewage samples were filtered through 8.0- and then 3.0-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). To filter sterilize sewage, the filtrate from the 3.0-μm filter was passed through 0.8-, 0.45-, and finally 0.22-μm filters (Millipore). Filtered lake water was prepared by passing samples through type A/E glass fiber filters (Gelman Sciences, Inc., Ann Arbor, Mich.) before passage through a 3.0- or 0.22-μm filter (Millipore); the latter filters were used to filter sterilize the samples.

To inhibit protozoa in some environmental samples, cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was added in a 20% methanol solution in water, and nystatin (5,890 USP U/mg) (Sigma) was added as dry chemical. The final concentrations of cycloheximide and nystatin were 250 and 30 μg/ml, respectively.

The numbers of microorganisms that could grow on 2 μg of PNP per ml were estimated by the most-probable-number method with a computer program written by the authors using the equation of Cochran (4). Subsamples of lake water were diluted in autoclaved lake water, and PNP was added to give 2 μg/ml in flasks containing serial, 10-fold dilutions of the test samples. Twelve 2.5-ml portions from each dilution were then added to separate wells in sterile tissue culture plates (Thomas Scientific, Philadelphia, Pa.) and incubated for 3 weeks. PNP mineralization was evident by the disappearance of the yellow color of the substrate. Error bars (see Fig. 4) indicate the 95% confidence intervals of the most-probable-number estimates. The number of total viable protozoa in lake water was determined by microscopic examination of five microscopic fields at ×160.

The results represent the average of data from triplicate flasks of sewage and duplicate flasks of lake water. The length of the acclimation period was determined from the point of intersection of the horizontal line and the portion of the linearized curve reflecting active mineralization (18).

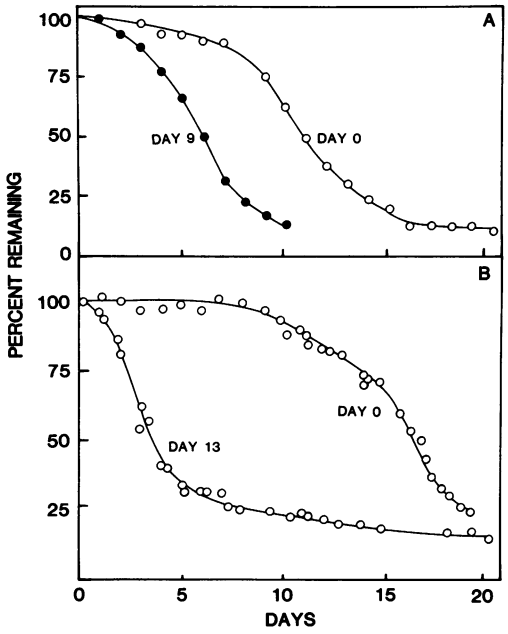

FIG. 1. Mineralization of 2 ng of PNP per ml added at day 0 or after 9 or 13 days to sewage (A) or lake water (B).

RESULTS

The mineralization of 2 ng of PNP per ml of sewage was initially slow, but the rate became rapid after 7 days (Fig. 1A). However, when PNP was added to sewage that had been incubated without amendment for 9 days, rapid mineralization began after 1 day. The acclimation period preceding detectable mineralization of 2 ng of PNP per ml of lake water was 9 days (Fig. 1B). However, little or no acclimation was evident in lake water that was incubated for 13 days before the introduction of the test compound. In other experiments, higher PNP concentrations were added to lake water samples that were freshly taken or that had been incubated in the laboratory for 10 days without amendment; under these conditions, preincubation of the lake water had no effect, and the acclimation periods were 9 and 8 days for 200 ng and 2 μg of PNP per ml, respectively. The mineralization of 2 ng of 2,4-D per ml was not evident initially in sewage, but after an acclimation period of 10 days, rapid mineralization occurred (Fig. 2). In contrast, an acclimation period was not observed when 2,4-D was added to sewage that had been incubated without amendment for 16 days.

A PNP-metabolizing bacterium was isolated from lake water. The organism is an aerobic, nonmotile, gram-negative rod that is oxidase and catalase positive and uses citrate as the sole carbon source. It does not hydrolyze arginine, decarboxylate lysine, or ornithine, produce H_2S, possess urease, liquefy gelatin, or metabolize tryptophan. These characteristics suggest that the organism belongs to the genus *Flavobacterium*.

Flasks containing 49 ml of the inorganic salts solution were inoculated with 1.0 ml of a stationary-phase culture of *Flavobacterium* sp. that had been grown in filter-sterilized lake water. When growth in the inorganic salts solution

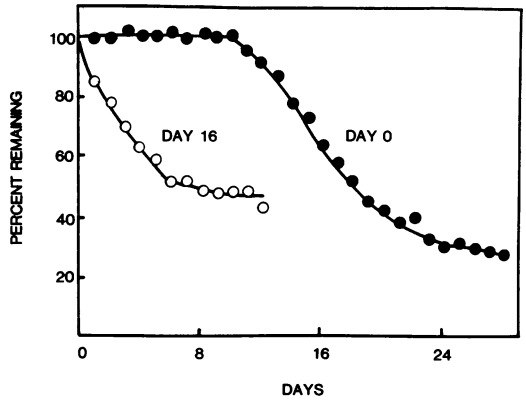

FIG. 2. Mineralization of 2 ng of 2,4-D per ml added to sewage at 0 or 16 days.

ceased, the solutions were amended with PNP at six concentrations. Mineralization of 20 to 2,000 ng of PNP per ml was detected within 7 h (Fig. 3). On the other hand, mineralization of 2 ng of PNP per ml was evident only after 25 h. Cell numbers were constant at approximately 10^5 cells per ml during the first 10 h in solutions containing 20 to 200 ng of PNP per ml and for the first 150 h in solutions with 2 ng/ml.

A study was conducted to determine whether the acclimation period reflects the time for a small population of mineralizing organisms to become sufficiently large to bring about detectable loss of the organic substrate. Lake water was amended with 2 μg of PNP per ml or received no amendment. The number of PNP-mineralizing organisms and PNP mineralization were determined. The initial density of PNP mineralizers was less than 10 cells per ml. Multiplication of these organisms was evident after 1 day whether PNP was added or not (Fig. 4). The number of PNP degraders was greater after 5 days in amended than in

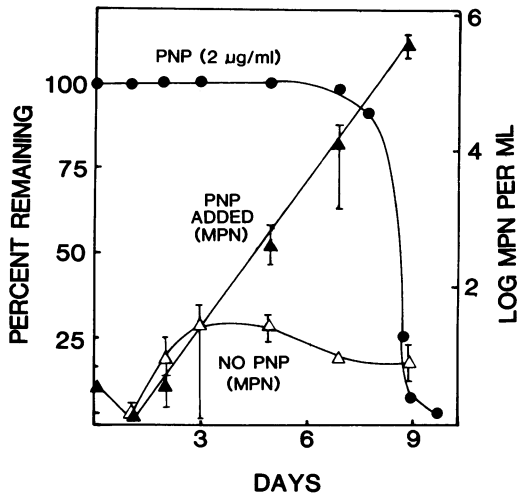

FIG. 4. Mineralization of 2 μg of PNP per ml of lake water and growth of organisms capable of mineralizing PNP. MPN, Most probable number.

unamended lake water. Growth of the organisms in PNP-amended lake water continued for the next 4 days, but the numbers declined in unamended lake water. Disappearance of PNP was observed as the number of PNP-degrading organisms approached 10^5 cells per ml.

Addition of 188 μM NH_4NO_3–690 μM K_2HPO_4–220 μM KH_2PO_4 to sewage decreased the length of the acclimation period for the mineralization of 2 ng of PNP per ml from 6 to 3 days (Fig. 5A). The addition of 11 mM KH_2PO_4–17 mM K_2HPO_4–0.41 mM $MgSO_4 \cdot 7H_2O$–68 μM $CaCl_2 \cdot 2H_2O$ to lake water containing 200 ng of PNP per ml reduced the acclimation period from 6 days to 1 day (Fig. 5B). In another experiment, the acclimation phase preceding the mineralization of 200 ng of PNP per ml of lake water was increased

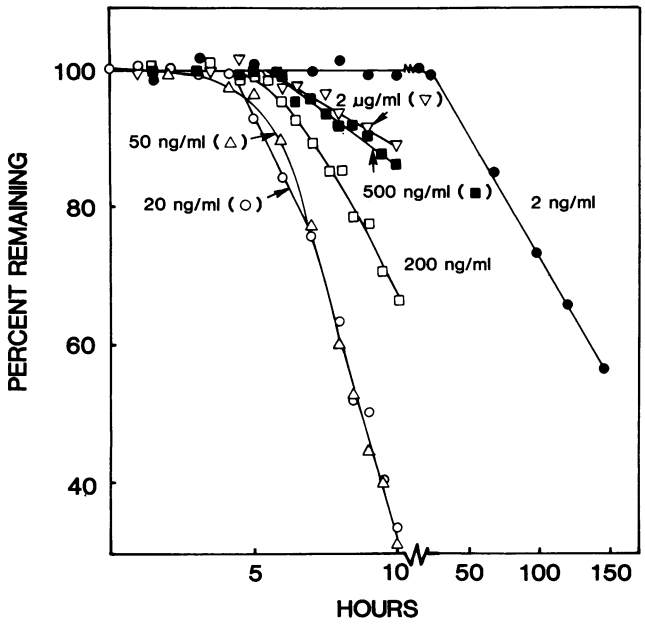

FIG. 3. Mineralization of various concentrations of PNP by *Flavobacterium* sp.

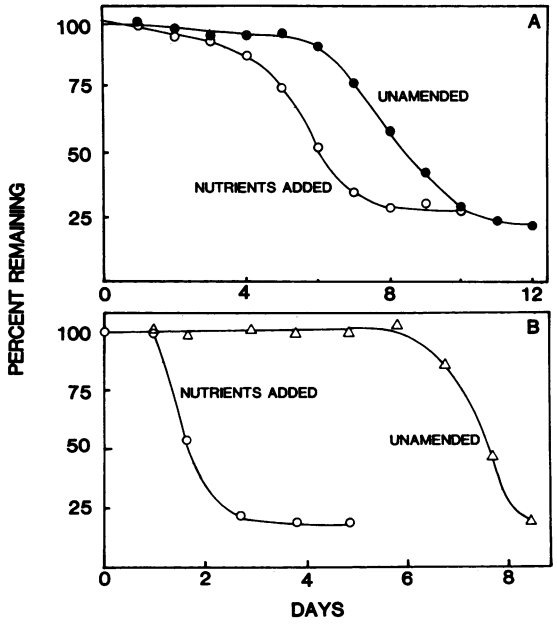

FIG. 5. Effect of the addition of inorganic nutrients on the acclimation period in sewage (A) and lake water (B).

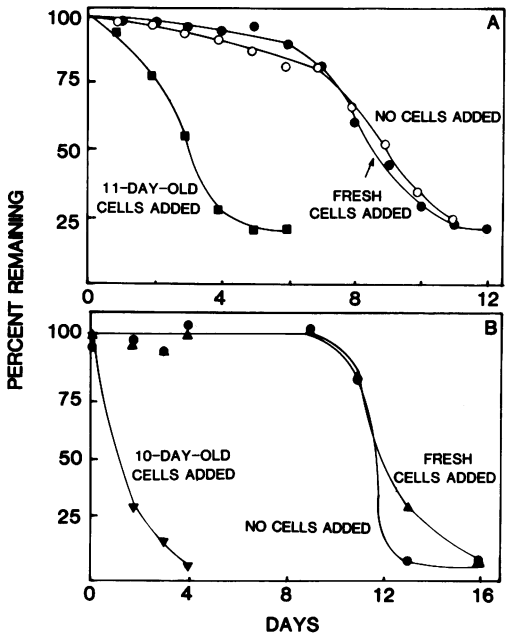

FIG. 6. Mineralization of PNP in samples of sewage (A) or lake water (B) receiving no additional cells, cells obtained from freshly taken samples, or cells obtained from samples incubated in the laboratory for 10 or 11 days. PNP was added to sewage and lake water to give final concentrations of 2 and 200 ng/ml, respectively.

from 17 to 23 days by the addition of 10 mM $KHCO_3$. The pH of the lake water was increased from 8.1 to 8.3 by the addition of $KHCO_3$. The length of the acclimation period for the mineralization of 200 ng of PNP per ml was reduced from 12 to 10 days by yeast nitrogen base (Difco Laboratories, Detroit, Mich.) added to lake water at 2 μg/ml and was increased from 9 to 13 days by the addition of 10 μg of glucose per ml.

The acclimation period preceding the mineralization of 2 ng of PNP per ml was 6 days when the microbial community from fresh sewage was added to filter-sterilized 7-day-old sewage. The acclimation time was the same when this community was added to filter-sterilized fresh sewage. This suggests that the acclimation phase is not the result of the preferential utilization of organic compounds other then PNP by the PNP-degrading populations. However, in another experiment, when the microbial community from fresh sewage was added to sewage that had been incubated for 16 days and then sterilized by filtration, the acclimation period was 5 days; in contrast, the acclimation period lasted 8 days when these organisms were added to fresh sewage that had been sterilized by filtration. In lake water, the acclimation period preceding rapid mineralization of 200 ng of PNP per ml was 10 days when the microbial community from fresh lake water was added to either fresh lake water that was sterilized by filtration or lake water that was incubated in the laboratory for 10 days before sterilization by filtration.

To determine whether acclimation resulted from the time required for the destruction of inhibitors that prevented the early initiation of mineralization, acclimated microbial communities were added to fresh sewage or lake water. Mineralization commenced with no delay when the microbial community from sewage that had been incubated with 2 ng of PNP per ml for 11 days was added to fresh unfiltered sewage (Fig. 6A). The time for acclimation in the samples with no added cells was 6 days; the acclimation period was the same when the sewage received the microflora of fresh sewage. In lake water, the acclimation period for the mineralization of 200 ng of PNP per ml was 10 days (Fig. 6B). The interval was the same when fresh filter-sterilized lake water was amended with cells collected from fresh lake water. In contrast, little or no acclimation was observed when fresh filter-sterilized lake water was amended with microorganisms collected from lake water that had been incubated with 200 ng of PNP per ml for 10 days.

To assess the effect of protozoan predation on the acclimation period, eucaryotic inhibitors were added to fresh samples of sewage that were supplemented with 2 ng of PNP per ml. In the absence of cycloheximide and nystatin, the acclimation period was 9 days (Fig. 7). In the presence of the two inhibitors, the acclimation time was reduced to 3 days. A comparable reduction in acclimation period occurred when the sewage was passed through 3-μm-pore-size filters and amended with 250 μg of cycloheximide per ml before being supplemented with PNP. In both tests, 90% of the carbon was mineralized in the absence of the inhibitors, and the figure was 40% when the inhibitors were present.

The acclimation period for the mineralization of 2 ng of PNP per ml was 10 days for lake water that was filtered through a 3-μm-pore-size filter and amended with cycloheximide or for lake water that was not so treated. For lake water samples amended with 2 μg of PNP per ml, mineralization did not occur within 10 days in the presence of cycloheximide and nystatin, whereas mineralization was detected in 7 days in the absence of inhibitors. The numbers of viable protozoa were reduced from 4.6×10^3/ml at day 0 to below detectable levels (fewer than 100/ml) after 1 day in the presence of inhibitors.

The possibility that eucaryotic organisms are required for PNP mineralization in lake water was tested by adding 250 μg of cycloheximide per ml, 1 mg of streptomycin per ml, or no inhibitor to 12-day-old lake water samples that were actively mineralizing 200 ng of PNP per ml. Cycloheximide did not affect the rate of mineralization, whereas the rate of mineralization was reduced in samples amended with the procaryotic inhibitor streptomycin. These results indicate that procaryotes are responsible for mineralization of PNP in lake water.

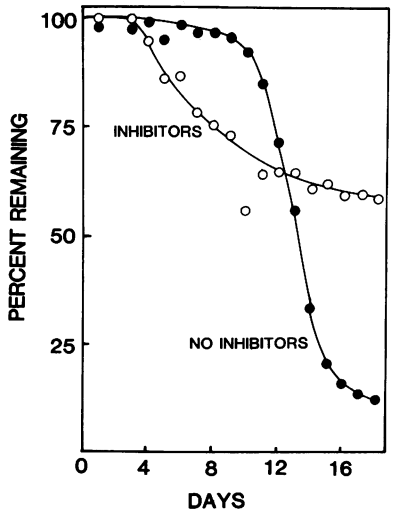

FIG. 7. Effect of eucaryotic inhibitors on the mineralization of PNP in sewage.

DISCUSSION

A number of explanations have been proposed for the acclimation period. It often has been suggested that acclimation reflects the time for induction of enzymes after exposure of the indigenous populations to the compound of interest. The time required for enzyme induction is usually minutes or hours (14), although the period for enzymes to become fully induced may be longer, especially at low substrate concentrations (3). Our studies indicate that the acclimation periods were much longer than the time required for induction. Other investigators have also concluded that induction is not a likely explanation because of the long time involved (8, 22). The relatively short time period for a noninduced *Flavobacterium* sp. culture to initiate PNP mineralization and the lack of an appreciable acclimation period for the microbial communities of unamended, aged sewage and lake water to mineralize 2 ng of PNP per ml also suggest that induction does not account for long acclimation periods.

Mutation or the appearance of new genotypes by genetic exchange may account for acclimation. In the defined microbial community used by Schmidt et al. (16), the creation of a new genotype by plasmid transfer occurred during the acclimation to chlorophenols. However, it has been argued that acclimation periods that have reproducible durations among replicates are not the result of mutations (6, 18). We observed that the acclimation period for mineralization of PNP in sewage was also highly reproducible, both among replicates and among experiments, suggesting that mutations are not implicated. In lake water, the acclimation period for mineralization of PNP was reproducible among replicates, although not always among experiments. The lack of reproducibility among experiments may have resulted from the fact that they were conducted at different times of year, since the time of year at which such samples are taken affects mineralization (9). The little or no acclimation preceding the mineralization of PNP or 2,4-D or both in aged sewage and lake water and the detection of PNP-mineralizing organisms in unamended lake water suggest that mutations are not necessary for PNP mineralization.

Diauxie has also been suggested as a reason for acclimation. Thus, Kuiper and Hanstveit (10) proposed that readily degradable organic substrates in seawater delay the acclimation of bacteria to 4-chlorophenol degradation. Such an explanation is not appropriate for PNP mineralization in sewage or lake water because the acclimation period had the same duration after the addition of fresh cells to unamended aged samples, in which the concentration of readily available organic matter was probably appreciably reduced, and in fresh sewage and lake water samples that presumably still contained such nutrients.

Growth of small populations to reach cell densities sufficiently high for mineralization to be detected also may be responsible for the apparent acclimation phase preceding PNP biodegradation. Our data show that although PNP mineralizers were initially present in lake water, loss of the 2 μg of PNP per ml was not detectable by measurements of carbon disappearance until about 10^5 PNP degraders per ml were present. Because each cell consumes small amounts of substrate, high cell densities would be required to give a readily detectable loss of substrate carbon in solutions with 2 μg of PNP per ml. However, the presence of toxins, the lack of an adequate supply of essential nutrients, or predation by protozoa may delay the increase in the numbers or activity of mineralizing organisms. Toxins may be responsible for acclimation if they prevent the growth of the mineralizing species until these organisms adapt to the inhibitors or the toxins are inactivated or disappear. Atlas and Bartha (2) suggested that part of the acclimation period for biodegradation of oil reflected the time for the loss by volatilization of toxic constituents of the oil. The observation that an acclimation period was not evident when indigenous PNP degraders were added to fresh sewage or lake water suggests that toxins that delayed the early initiation of mineralization were not present. Although the cells may have adapted to the toxin or developed high detoxifying activity during the incubation period, our observations that the acclimation period can be much shorter under other conditions (for example, when protozoa in sewage are inhibited or when inorganic nutrients are added to lake water) represent indirect evidence against a role for toxins.

The slight reduction in the acclimation period when inorganic nutrients were added to sewage suggests that nutrient limitation is not a major factor affecting acclimation in that environment. However, lakes often have limiting levels of nutrients for certain microbial activities (15), and Lewis et al. (11) showed that the addition of inorganic nutrients reduced the acclimation period preceding the mineralization of *p*-cresol in samples of fresh water. In the present study, the greatly diminished acclimation period for PNP mineralization when inorganic nutrients were added also suggests that such nutrient limitations are important in lake water. The acclimation period was not reduced by added bicarbonate, although the lack of CO_2 has been reported to cause a lag phase in the growth of low densities of bacteria in minimal media (25). The addition of various organic amendments to sewage and lake water had little or no effect, although the addition of yeast extract to lake and stream water has been reported to reduce the acclimation period for ε-caprolactam disappearance (5).

Little attention has been given to the effect of protozoan predation on acclimation. Predation may reduce the number of bacteria that can mineralize a chemical of interest and thus delay the initiation of rapid mineralization until the number of all bacteria becomes low and the intensity of grazing diminishes. The marked shortening of the acclimation period when eucaryotic organisms were inhibited suggests that grazing by protozoa is important in determining the time for initiation of rapid mineralization in sewage. Such predation is quite likely because protozoa are abundant in sewage (13), and although there exists a prey density below which protozoa do not graze actively (1), bacterial densities in sewage are initially above the threshold for grazing. In contrast, grazing by protozoa is less likely to be a major factor affecting acclimation in lake water because the bacterial densities usually are lower in fresh waters. Because of the role of protozoa in cycling nutrients (7), the absence of mineralization of 2 μg of PNP per ml of lake water and the lesser extent of mineralization in sewage observed when protozoa are inhibited may be related to an inadequate supply of nutrients for the mineralizing species.

The data suggest that the acclimation period in aquatic environments is often a reflection of the time for small populations of organisms capable of mineralizing chemicals of interest to reach the high cell densities necessary to give detectable mineralization. However, some constraint may be present that prevents the low densities of these active organisms from proliferating readily. The identity of the constraint may vary from one environment to another. In lake waters, the low levels of inorganic nutrients may limit the rate of growth of the mineralizing organisms and thus

cause an extended acclimation period. However, in sewage, the total numbers of bacteria are large enough to support active predation by protozoa, and such grazing keeps the densities of the mineralizing organisms low.

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