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Role of Chemical Concentration and Second Carbon Sources in Acclimation of Microbial Communities for Biodegradation

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A study was conducted to determine the role of concentration of the test chemical, of a second organic compound, and of mutation in the acclimation period before the mineralization of organic compounds in sewage. The acclimation period for the mineralization in sewage of 2 μg of 4-nitrophenol (PNP) per liter increased from 6 to 12 days in the presence of 10 mg of 2,4-dinitrophenol per liter. The extension of the acclimation period was equivalent to the time required for mineralization of 2,4-dinitrophenol. In contrast, the time for acclimation for the degradation of 2 μg of PNP per liter was reduced when 10 or 100 mg of phenol per liter was added. Lower phenol levels increased the acclimation period to 8 days. The length of the acclimation period for PNP mineralization decreased as the initial concentration of PNP increased from 2 μg to 100 mg/liter. The acclimation period for phenol mineralization was lengthened as the phenol concentration increased from 100 to 1,400 mg/liter. The length of the acclimation period for PNP and phenol biodegradation was reproducible, but it varied among replicates for the biodegradation of other nitro-substituted compounds added to sewage or lake water, suggesting that a mutation was responsible for acclimation to these other compounds. The acclimation period may thus reflect the time required for the destruction of toxins, and it also may be affected by the concentration of the test compound or the presence of other substrates.

The mineralization of many organic compounds that are introduced into treatment systems or into natural environments is often preceded by an acclimation period. The acclimation period is taken to mean the time interval during which biodegradation is not detected, and the term does not imply an explanation for the phenomenon or the way in which the biodegrading populations are growing or metabolizing during that period. Because no detectable mineralization occurs during the acclimation period, a compound may pass through treatment systems and into natural environments during this time. To minimize the environmental impact of these chemicals, it is important to understand the mechanisms involved in the acclimation of microbial communities.

In our previous study, data were presented to show the existence of several mechanisms for acclimation before the onset of rapid biodegradation (13). The results of that investigation indicated that acclimation for the mineralization of 4-nitrophenol (PNP) in sewage and lake water resulted from the time needed for small populations to become sufficiently large to give detectable loss of the chemical. The growth of the mineralizing organisms was affected by predation by protozoa and competition for inorganic nutrients. An acclimation period may also occur because of environmental conditions at the site where the chemical is discharged or because of the rarity in nature of microorganisms able to mineralize certain chemicals. Of particular importance in many municipal waste streams and in groundwaters adjacent to disposal sites for toxic wastes is the presence of compounds inhibiting microorganisms. Inhibitory compounds may influence the length of time before commencement of microbial decomposition, as indicated by the finding that biodegradation of oil in seawater is initiated only after toxic constituents of the oil disappear because of volatilization (1).

The inhibitor of biodegradation need not be another compound, because the concentrations of many chemicals in industrial waste streams and waste disposal sites are probably sufficiently high to suppress the microorganisms having the capacity to metabolize those compounds. Indeed, evidence exists that the acclimation period is increased at high chemical concentrations. Grover (4) observed that the acclimation period for degradation in soil of the herbicide picloram increased as its concentration increased, and Lappin et al. (6) reported that the acclimation period for decomposition of mecoprop by a five-member microbial consortium became longer with increasing concentrations of this herbicide. Similarly, Rossin et al. (8) observed that the acclimation period for degradation of nitrilotriacetate in sewage treatment plants was longer at high concentrations of this chelating agent.

Other evidence exists that one compound may shorten the acclimation period needed before another is degraded. Thus, Papanastasiou and Maier (7) showed that the mineralization of 2,4-dichlorophenoxyacetate in sewage that had been previously exposed to this pesticide was stimulated by glucose, and Haller (5) observed that acclimation of the sewage microflora to 3-chlorobenzoate or 4-chlorophenol reduced the time before the first detectable mineralization of other mono-substituted aromatic hydrocarbons.

The creation of a new genotype may also be an explanation for acclimation. Wyndham (14) noted the appearance of a mutant during the acclimation of river water microbial communities for aniline mineralization. However, because of the consistency of the length of the acclimation period for PNP mineralization, Spain et al. (11) suggested that acclimation was not a result of a mutation or of plasmid gene recruitment.

A study was undertaken to determine whether the presence of toxins, the presence of other organic compounds, and substrate concentration explain the frequently lengthy time interval before biodegradation becomes evident. Thus, although acclimation is defined in terms of the substrate of

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interest, the explanations being evaluated are microbiological.

MATERIALS AND METHODS

Fresh, untreated sewage was obtained from the primary settling tank of the sewage treatment system of Ithaca, N.Y. The sewage was passed through a Whatman no. 41 filter, and it was used within 2 h of collection. Sterile sewage was prepared by autoclaving for 20 min. Surface water from Cayuga Lake was used within 6 h after collection.

[2,6-^{14}C]PNP (specific activity, 30 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. [U-^{14}C]phenol (specific activity, 98 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. In experiments in which the PNP concentration was 2 μg/liter, only the labeled compound was added. In experiments in which the concentrations of PNP or phenol ranged from 10 μg to 1.6 g/liter, the radioactive chemical was added to give 500 to 1,500 dpm/ml, and the unlabeled compound was added to give the desired concentration. The two chemicals were sterilized by filtration and added to triplicate 50-ml samples of sewage contained in 250-ml Erlenmeyer flasks. The flasks were incubated at 28°C without shaking. At various intervals, subsamples from each flask were acidified and bubbled with air to drive off radioactive CO_2. The subsamples were mixed with Liquiscint scintillation fluid (National Diagnostics, Highland Park, N.J.), and the amount of radioactivity remaining in the solution was determined with a Beckman liquid scintillation counter (model LS7500; Beckman Instruments, Inc., Fullerton, Calif.). Full details of this method have been described previously (12). The disappearance of 2,4-dinitrophenol (DNP) was determined spectrophotometrically at 400 nm with a Spectronic 88 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

The length of the acclimation period was determined from the point of intersection of the horizontal line and the linearized curve of the data showing active mineralization (11).

For determination of the most probable number (MPN) of organisms able to use PNP or phenol, samples of fresh sewage were diluted in 10-fold steps in autoclaved sewage. The test substrate was then added to each dilution at the appropriate concentration, and 3-ml portions of each dilution were placed into each of five test tubes. The tubes were incubated at 28°C for 3, 2, or 4 weeks when the substrate was 2 μg or 10 mg of PNP per liter, 100 mg of phenol per liter, or 1.0 g of phenol per liter, respectively. When the substrate was 10 mg of PNP per liter, each tube was observed for loss of color, and a colorless tube was recorded as positive. When the substrate was 2 μg of PNP per liter, radioactive PNP was used. At the end of the incubation period, the contents of each tube were acidified with 1 drop of 20% concentrated H_2SO_4 and then bubbled with air for 5 min to drive off radioactive CO_2 before determination of the radioactivity remaining in solution. If more than 10% of the PNP was mineralized as compared with sterile controls, the tube was recorded as positive.

For MPN determinations with 100 and 1.0 g of phenol per liter as the substrate, the tubes were treated by using a modification of the test for phenols given by Feigl (3). Concentrated H_2SO_4 (0.5 ml) containing 1% $NaNO_2$ was added to each test tube containing 3 ml of sample. The contents were mixed well, and 6 ml of 3.57 N KOH was then added to each tube. Any phenol present was converted to nitrophenol in the presence of the acid and $NaNO_2$, and the yellow color of the nitrophenol became evident upon the addition of base. Thus, tubes which were colorless were recorded as positive. MPN estimates were determined with a computer program we wrote that approximates the solution to the MPN equation given by Cochran (2).

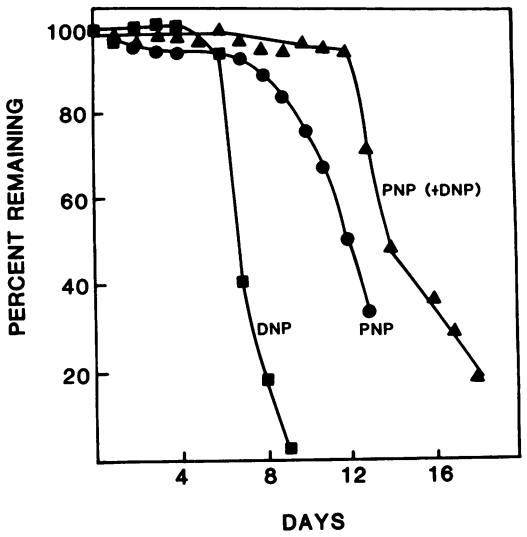

FIG. 1. Mineralization of PNP (2 μg/liter), DNP (10 mg/liter), and PNP (2 μg/liter) in the presence of DNP (10 mg/liter) [PNP (+DNP)].

To test for the possible occurrence of a mutation that results in the capacity for biodegradation of organic compounds, various nitro compounds were added to 100 ml of fresh sewage or Cayuga Lake water, respectively, at the following concentrations (expressed as milligrams per liter): 2-nitrophenol, 17 and 25; 1-nitroaniline, 14 and 16; 3-nitroaniline, 45 and 44; 4-nitroaniline, 32 and 43; 2-methyl-6-nitroaniline, 16 and 15; and 4-nitrophenylhydrazine, 37 and 35. Each 100-ml sample was then divided into 10 10-ml portions, each portion was put into a 50-ml Erlenmeyer flask, and all flasks were incubated at 28°C. The flasks were inspected daily for loss of the yellow color, which indicated that the parent compound had disappeared.

RESULTS

A study was undertaken to determine the effect of toxic compounds on acclimation. An experiment was first performed to determine the highest concentration of DNP that is mineralized in sewage. DNP was added to flasks of sewage at concentrations of 1, 5, 10, 25, and 50 mg/liter, and biodegradation was determined by loss of the yellow color. The highest concentration that disappeared was 10 mg/liter, and DNP at this concentration disappeared in 10 days.

To test the effect of DNP on the acclimation for PNP mineralization, DNP and radioactive PNP were added to samples of sewage at concentrations of 10 mg and 2 μg/liter, respectively. The acclimation period for PNP mineralization markedly increased in the presence of DNP (Fig. 1). In solutions containing DNP as the sole added carbon source, the compound disappeared in 6 days. In some samples of sewage, however, DNP had no effect on PNP acclimation.

Nontoxic concentrations of phenol also affected the acclimation period for PNP mineralization. The addition to sewage of 10 and 100 mg of phenol per liter reduced the acclimation period for the mineralization of 2 μg of PNP per

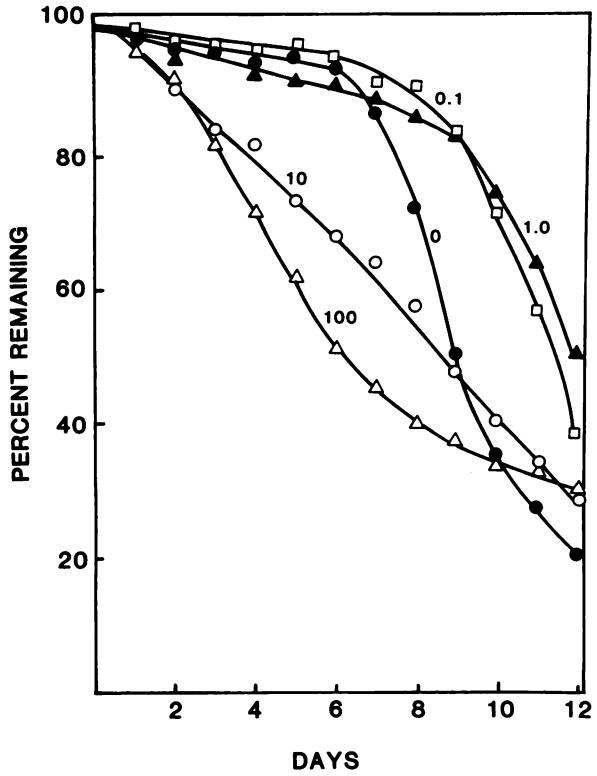

FIG. 2. Effect of phenol at 100, 10, 1.0, and 0.1 mg/liter on the mineralization of 2 μg of PNP per liter of sewage.

liter from 6 days to 1 day (Fig. 2). On the other hand, 1 and 0.1 mg of phenol per liter extended the length of the acclimation period to 8 days. Phenol at 100 mg/liter completely inhibited the mineralization of 100 mg of PNP per liter (data not shown).

The toxicity of the compound whose biodegradation is being determined may also affect the acclimation period. This is evident in a study in which phenol at levels of 100 to 2,000 mg/liter was added to sewage. At a concentration of 1,400 mg/liter, the acclimation period was 10 days, but it was 4 days at 1,000 mg/liter and less than 1 day at 100 mg/liter (Fig. 3). Although the rates presented in Fig. 3 suggest that, on a percentage basis, higher concentrations of phenol slowed the biodegradation, the actual rates were somewhat greater; thus, the rates of phenol mineralization were 2.2, 2.8, and 3.4 mg/liter per h at 100, 1,000, and 1,400 mg/liter, respectively. At concentrations of 1,800 and 2,000 mg/liter, phenol was not mineralized. MPN estimates showed that the sewage initially contained 4.3×10^4 organisms per ml that were able to mineralize 100 mg of phenol per liter but only 39 cells per ml that could mineralize 1,000 mg of phenol per liter. These data show that the acclimation period is increased by high concentrations of a toxic compound and suggest that the extension results from the presence initially of fewer organisms capable of mineralizing phenol at the high concentrations.

A study was conducted to determine the effect of the concentration of PNP on the length of the acclimation period for PNP biodegradation in sewage. The acclimation period for 2 μg of PNP per liter was 11 days, and the interval decreased as the concentration of PNP increased (Fig. 4). At PNP concentrations of 10 and 50 mg/liter, the acclimation required only 2 days. In sewage collected at another time,

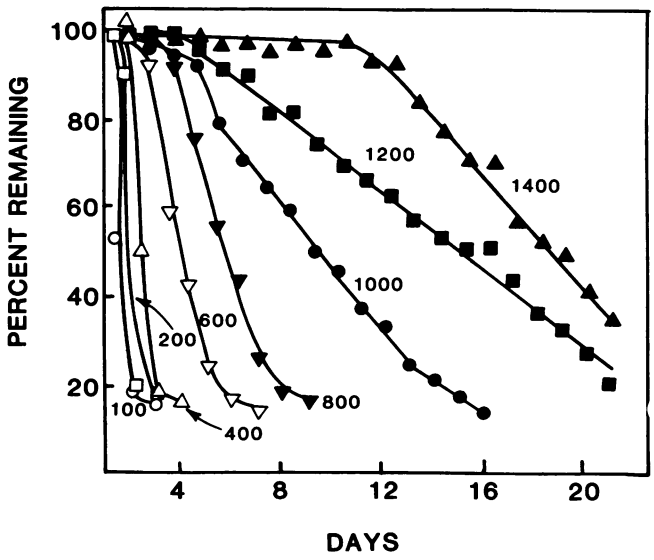

FIG. 3. Mineralization of phenol at levels of 100 to 1,400 mg/liter.

the acclimation period was 6 days for 2 μg/liter, 4 days for 10, 100, and 1,000 μg/liter, and less than 4 days for 100 mg/liter. In a sewage sample collected at still another time, the acclimation required 5 days for 2 μg/liter and 4 days for 10 μg, 1 mg, and 100 mg/liter.

Estimates were made of the numbers of organisms capable of mineralizing high and low concentrations of PNP. In one sample of fresh sewage, 2 cells per ml were found to be capable of mineralizing 2 μg of PNP per liter, and 35 cells per

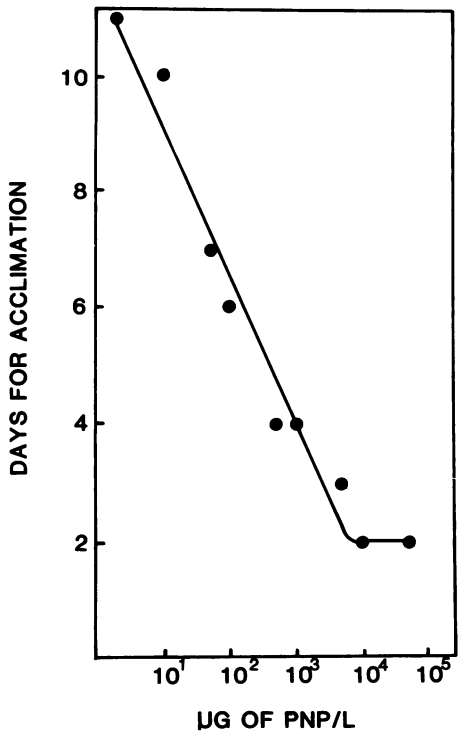

FIG. 4. Effect of PNP concentration on the length of the acclimation period for PNP mineralization.

TABLE 1. Length of acclimation period for mineralization of four compounds in replicate samples of lake water

Compound	Days for mineralization in individual samples of:	
	Sewage	Lake water
2-Nitrophenol	9, 10,[a] 12	11, 12, 12, 14, 15, 18, 20 (NB: 3)[b]
4-Nitroaniline	14, 17, 22, 22, 23, 23, 33, 33, 62 (NB: 1)	NB: 10
5-Nitrosalicylic acid	16, 19, 22, 24, 33, 53, 60 (NB: 3)	24, 25 (NB: 8)
3-Nitrosalicylic acid	28, 32, 61 (NB: 7)	12, 12, 16, 25, 25, 29 (NB: 4)

[a] Eight replicates.
[b] NB, No biodegradation. The number after the colon is the number of replicates in which biodegradation was not detected.

ml were able to mineralize 10 mg/liter. In a sample of sewage collected at another time, 80 cells per ml were able to use 2 μg and 10 mg of PNP per liter.

The possible role of mutation in determining the length of the acclimation period was investigated by using eight nitro-substituted aromatic compounds. Each of the 10 replicates from sewage containing 2-nitrophenol was mineralized after an acclimation period of approximately 10 days (Table 1). In lake water, mineralization of 2-nitrophenol occurred in seven replicates within 10 to 20 days, and no mineralization was evident in the remaining three replicates. In contrast, acclimation for biodegradation of 3-nitrosalicylic acid required 28, 32, and 61 days for three of the replicates, but mineralization did not occur in the remaining seven flasks containing sewage samples. The lengths of the acclimation periods for destruction of 5-nitrosalicylic acid in sewage samples differed greatly, and three flasks showed no activity. In lake water, 5-nitrosalicylic acid was mineralized in only two flasks, but those two started mineralization at approximately 24 days. The lengths of the acclimation periods for 4-nitroaniline mineralization varied among replicates of sewage samples, but 9 of 10 flasks exhibited biodegradation. 4-Nitroaniline was not destroyed in any replicate from lake water during the 64-day incubation period. 2-Nitroaniline, 3-nitroaniline, 2-methyl-6-nitroaniline, and 4-nitrophenylhydrazine were not mineralized in samples from sewage and lake water during the 64-day incubation period.

DISCUSSION

The data show that mechanisms in addition to those presented earlier (13) may explain the acclimation period before mineralization. Thus, the presence of chemicals at inhibitory levels may be responsible for prolonged acclimation. The inhibitor may be the substrate itself or another compound that has antimicrobial activity. In the present study, DNP was found to extend the acclimation period for PNP mineralization. Because the time required for DNP to be mineralized was the same as the DNP-induced extension of the acclimation period for PNP mineralization, DNP probably slowed or prevented growth of the PNP-mineralizing populations. Once DNP was destroyed, however, the PNP-mineralizing organisms could grow, and the length of the subsequent acclimation period was similar to that when DNP was not present initially. The acclimation period after DNP was decomposed presumably was a reflection of the time for the initially small numbers of PNP utilizers to reach densities high enough to mineralize a detectable amount of PNP. If the delay in mineralization caused by DNP was a result of its being used in preference to PNP, then PNP should have been mineralized by the large population of cells (produced from DNP) immediately after DNP had been destroyed. The finding that DNP did not affect the acclimation for PNP in all samples of sewage may have resulted from variations in composition of the microbial community.

High concentrations of phenol appeared to be toxic to the PNP mineralizers, as no mineralization was observed. At lower concentrations of phenol, however, the PNP mineralizers appeared to be stimulated by phenol. The shortened acclimation period for PNP mineralization in the presence of 10 and 100 mg of phenol per liter may result from the ability of the PNP utilizers to grow and reach high cell densities by using phenol and then mineralizing the PNP. The reason that 0.1 and 1 mg of phenol per liter affected the acclimation period for PNP mineralization is uncertain.

The acclimation period also may be affected by the concentration of the compound (4, 6, 8). At very low concentrations, the long acclimation may be the result of slow growth of the mineralizing organisms on very low concentrations of substrate. As the substrate concentration increases, so does the growth rate, and the more rapid proliferation at these higher concentrations would be reflected in the shorter acclimation periods that were observed. The shortening of the acclimation period is not the result of more cells capable of using the higher concentrations, because sewage contained similar numbers of cells able to use 2 μg and 10 mg of PNP per liter. At very high substrate levels, however, the toxicity of the compound may reduce the number of active organisms and thus increase the acclimation period, as noted with the highest phenol concentration tested. An increasing acclimation period with increasing substrate concentration may reflect the greater time required to detect chemical loss rather than toxicity as the substrate level increases. However, the longer acclimation periods for phenol mineralization at high concentrations of this compound are more likely a consequence of the presence of fewer organisms capable of growing at the high concentrations. Even though the mineralization rates are slightly greater at the higher concentrations, it takes longer for the fewer cells to give a detectable loss of phenol.

An acclimation period may result from the time required for the appearance of a new genotype after a mutation or genetic exchange, which presumably occurs during the time the microorganisms are exposed to the compound. The new organism then grows and mineralizes the compound. Because mutations and gene transfer are rare events, they should appear in only a small and random percentage of samples, and the acclimation period that results from the time required for a mutation to occur should vary in length among replicates. The length of the acclimation periods for some of the compounds that were tested in sewage in this study (2-nitrophenol and phenol) and previously for PNP (13) were consistent and reproducible. The lengths did not vary by more than 1 day (or rarely 2 days) among replicate samples from the same batch of sewage and did not vary by more than 2 days (or rarely 3 days) among batches, arguing against the occurrence of a mutation for the mineralization of these compounds. Conversely, the large variation in acclimation period for 3- or 5-nitrosalicylic acid or 4-nitroaniline in sewage suggests that mutation was required to produce an organism capable of mineralizing these compounds. In contrast, the small variation in the lengths of the acclimation periods for 5-nitrosalicylic acid in lake water suggests that mutation did not occur, but the small number of flasks exhibiting mineralization indicates that the mineralization

was the result of the presence of a rare organism. However, because of the necessity of eliminating the possibility that a rare organism initially present in the sample can mineralize the compound, the experiments described above do not prove that a mutation occurred.

For compounds that exhibit acclimation periods longer than 1 month, it is unlikely that the growth of cells initially present in the sample can account for acclimation. Thus, if one cell able to mineralize the compound was present initially in 50 ml of sewage and if it grew with a generation time of 2 days, only 26 days would be required for that cell to multiply to a high enough density for the mineralization of 2 μg of the compound per liter to be detected. Hence, the random occurrence of a mutation or genetic exchange followed by subsequent growth of that species probably accounts for very long acclimation periods. However, as yet unrecognized causes may exist for the long or variable acclimation periods.

The apparently linear kinetics observed for the mineralization of the higher concentrations of phenol may seem to be anomalous in view of the likelihood that the bacteria are growing at these substrate concentrations. However, most of the active growth period of the responsible organisms would have been before phenol mineralization was detected, and the interval during which mineralization was detected would correspond to only about the last three doublings of the growth period. Bacterial growth at limiting substrate concentrations is often logistic, and the logistic curve is approximated by a straight line during the period of time during which the number of organisms increases from 10 to 90% of the maximum cell density (9, 10).

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