APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1982, p. 1133–1138
0099-2240/82/051133-06$02.00/0
Vol. 43, No. 5

Rates of Mineralization of Trace Concentrations of Aromatic Compounds in Lake Water and Sewage Samples

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Received 27 April 1981/Accepted 1 February 1982

The rates of mineralization of phenol, benzoate, benzylamine, *p*-nitrophenol, and di(2-ethylhexyl) phthalate added to lake water at concentrations ranging from a few picograms to nanograms per milliliter were directly proportional to chemical concentration. The rates were still linear at levels of <1 pg of phenol or *p*-nitrophenol per ml, but it was less than the predicted value at 1.53 pg of 2,4-dichlorophenoxyacetate per ml. Mineralization of 2,4-dichlorophenoxyacetate was not detected in samples of lake water containing 200 ng of the chemical per ml. The slope of a plot of the rate of phenol mineralization in samples of three lakes as a function of its initial concentration was lower at levels of 1 to 100 μg/ml than at higher concentrations. In lake water and sewage supplemented with <60 ng of ^{14}C-labeled benzoate or phenylacetate per ml, 95 to 99% of the radioactivity disappeared from solution, indicating that the microflora assimilated little or none of the carbon. The extent of mineralization of some compounds in samples of two lakes and sewage was least in the water with the lowest nutrient levels. No mineralization of 2,4-dichlorophenoxyacetate and the phthalate ester was observed in samples of an oligotrophic lake. These data suggest that mineralization of some chemicals at concentrations of <1 μg/ml is the result of activities of organisms different from those functioning at higher concentrations or of organisms that metabolize the chemicals at low concentrations but assimilate little or none of the substrate carbon.

Predicting the rate of decomposition of organic compounds in natural waters is of considerable importance, especially for toxic chemicals. However, many of the routine tests used to assess rates of biodegradation involve chemical concentrations far in excess of those likely to be found in natural waters. Insufficient data exist to allow for extrapolation from the high levels commonly used in laboratory studies to the far lower levels characteristic of freshwaters or marine waters.

The rate of mineralization may be directly related to substrate concentration at all concentrations below those supporting the maximum rate (V_{max}) for a particular chemical. Conversely, a threshold level may exist below which the rate of decomposition is either less than that predicted from a linear extrapolation of the rates at higher substrate levels or is zero. Jannasch (5) demonstrated the existence of a threshold concentration below which glucose decomposition did not take place in culture. Law and Button (6) later found that the threshold concentration for glucose could be lowered by the addition of amino acids. A subsequent study suggested that a threshold level for certain compounds existed in natural waters (3).

This investigation was designed to determine the kinetics of mineralization of several compounds in samples of freshwater and sewage. For this purpose, a method was developed to test the rates at chemical concentrations lower than those that have been studied heretofore.

MATERIALS AND METHODS

[*U*-^{14}C]phenol (specific activity, 87 mCi/mmol) and 2,4-dichlorophenoxy[2-^{14}C]acetic acid (28 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. [Carboxyl-^{14}C]benzoic acid (25.6 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. [Methylene-^{14}C]benzylamine·HCl (6.9 mCi/mmol), phenyl[1-^{14}C]acetic acid (1.0 mCi/mmol), and [carboxyl-^{14}C]di(2-ethylhexyl) phthalate (2.7 mCi/mmol) were obtained from California Bionuclear Corp., Sun Valley, Calif. *p*-Nitro[2,6-^{14}C]phenol (26.6 mCi/mmol) was obtained from Tracerlab, Waltham, Mass.

Samples of freshwater were taken from Beebe Lake and Cayuga Lake in Ithaca, N.Y., and White Lake near Old Forge, N.Y. These lakes are eutrophic, mesotrophic, and oligotrophic, respectively. Fresh sewage was from the waste treatment facility of Ithaca, N.Y.

To study mineralization rates, which are taken to be the loss of ^{14}C from the test system, freshly sampled lake water was passed through a glass fiber filter to

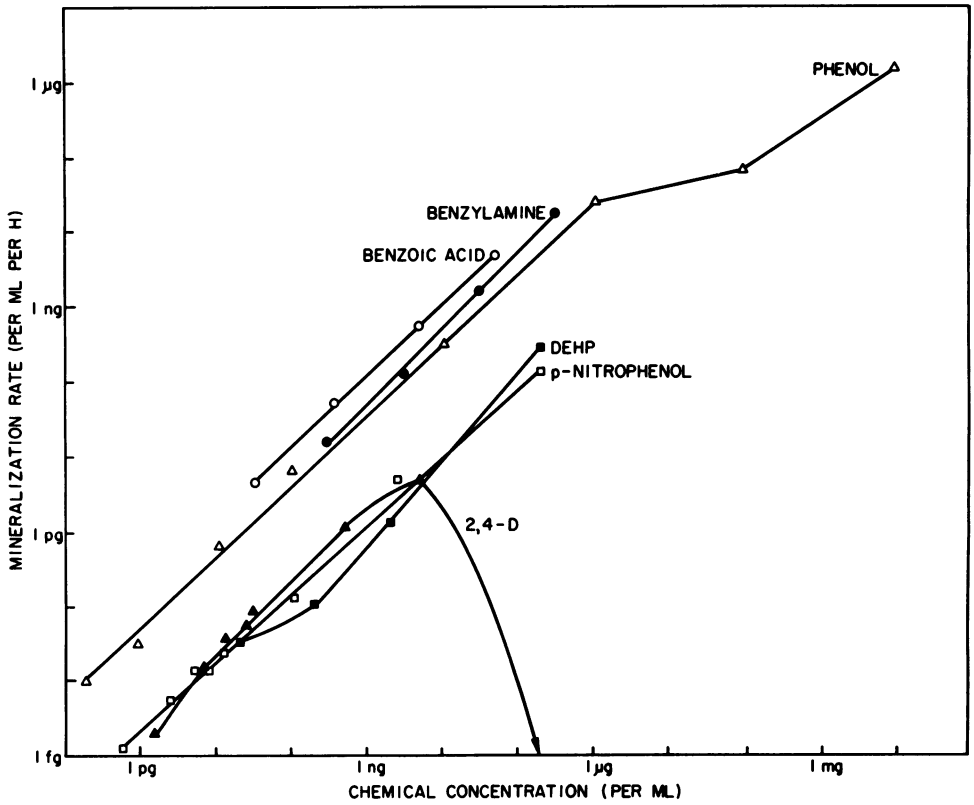

FIG. 1. Rates of mineralization of several compounds added at different concentrations to Beebe Lake Water. DEHP, di(2-ethylhexyl) phthalate.

remove the large particulates. The liquid was then amended with the ^{14}C-labeled compound to be tested and incubated without shaking in the dark at 29°C. All tests were conducted in triplicate with a single set of analyses performed on the contents of each flask. The volume of lake water used varied from 100 ml to 8 liters depending on the concentration and specific activity of the chemical. The ^{14}C-amended waters were contained in 250-ml to 12-liter Erlenmeyer or flat-bottomed Florence flasks. At regular intervals, 5.0- to 500-ml samples were removed and acidified with concentrated H_2SO_4 to pH 2. The amount of radioactivity remaining was measured by liquid scintillation counting, either after bubbling the sample for 60 min with air to remove the $^{14}CO_2$ or after solvent extraction of the substrate from the water sample. Details of the method and some of the data from which the rates given here are calculated are given elsewhere (9). Because of its low water solubility, di(2-ethylhexyl) phthalate was dissolved in acetone before it was added to the reaction vessel. The acetone was allowed to evaporate before the lake water was added. Abiotic and microbial mechanisms leading to the loss of each chemical were distinguished by adding the compound to samples of lake water supplemented with 0.2% NaCN; no losses were detected under these circumstances. A linear relationship was observed for the rate of disappearance of all chemicals as a function of time, except for *p*-nitrophenol, for which the relationship was linear at 605 fg/ml but not at higher concentrations (9); hence, the rates of disappearance of each chemical at each concentration were determined from the linear portion of a plot of the amount of chemical that disappeared with time.

RESULTS

The effect of chemical concentration on the rates of mineralization of several compounds added to Beebe Lake water is shown in Fig. 1. To present all of the data in a single figure, the rates and concentrations are expressed on a logarithmic scale. Benzoate was the most rapidly mineralized compound, and benzylamine and phenol were mineralized somewhat more slowly. The rates of mineralization were slowest for 2,4-dichlorophenoxyacetate (2,4-D), *p*-nitrophenol, and di(2-ethylhexyl) phthalate.

These data show that a linear relationship existed between mineralization rate and chemical concentration for all compounds over part of or the entire range of concentrations studied. The correlation coefficient for the plot of the logarithm of rate of carbon loss versus the

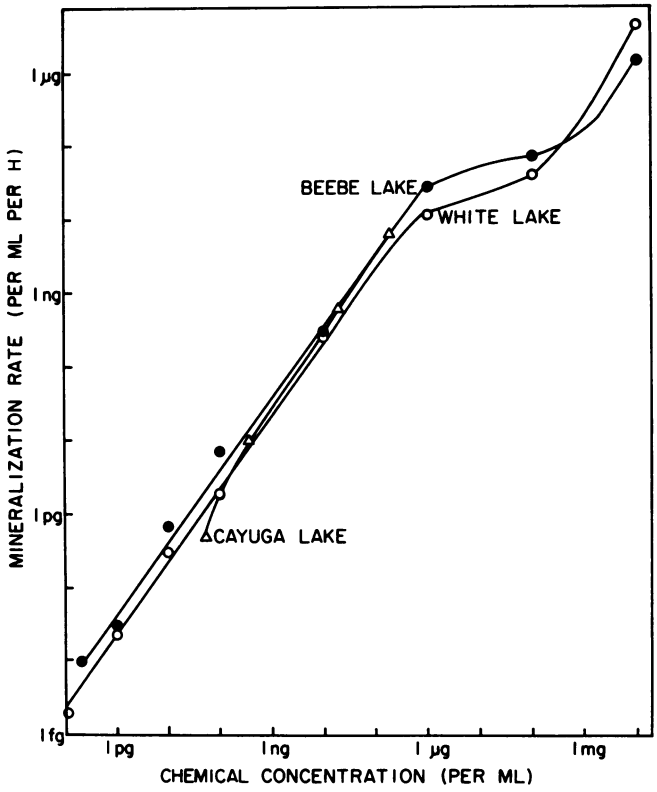

FIG. 2. Rate of mineralization of several concentrations of phenol in waters from three lakes.

logarithm of 2,4-D concentration between 7.2 and 500 pg/ml was 0.998. The observed rates for 1.53 pg and 4.9 ng of 2,4-D per ml were 43 and 58% of the predicted rates and were outside the 95% confidence limits for the regression line. Because no mineralization of 2,4-D occurred at 200 ng of 2,4-D per ml (shown by the arrow pointing downward in Fig. 1), the finding of a slower than predicted rate at 4.9 ng of 2,4-D per ml is consistent with the apparent toxicity. However, the less than predicted rate at 1.5 pg/ml is noteworthy. A similar statistical analysis indicated that the rate of mineralization of di(2-ethylhexyl) phthalate at an initial concentration of 20.7 pg/ml was four times greater than the predicted value and was outside the 95% confidence limits for the regression line, which had a correlation coefficient of 0.996.

The data in Fig. 1 show that the rates of phenol and *p*-nitrophenol mineralization were directly related to concentration from levels of <1.0 pg/ml to >100 ng/ml, i.e., by 5 or more orders of magnitude. The rate at 100 µg of phenol per ml was much lower than the rate predicted from the linear plot derived from the lower concentrations; moreover, the plot at the still higher concentration had a greater slope.

Phenol mineralization was studied further with samples of three different natural waters (Fig. 2). Statistical analysis indicated that the three plots were different. The rates at every concentration were less in White Lake water than in Beebe Lake water, except at 10 mg/ml, the highest level tested. The decline in the slopes of the lines because of the rate at 100 µg/ml and the increase in slope at a concentration of >100 µg/ml are noteworthy. The differences in rates between the two waters seem small on a logarithmic plot, but the rates in White Lake water were 60 and 56% of those in Beebe Lake water at 2 µg/ml and 200 fg/ml, respectively. A threshold was not observed for phenol mineralization, even at 102 fg/ml in White Lake and at 191 fg/ml in Beebe Lake water.

Mineralization was tested in samples of aquatic microbial habitats containing dissimilar nutrient levels and communities. The samples were taken in January. The data are presented as percentage of the benzoate and phenylacetate mineralized in 7 days and *p*-nitrophenol and 2,4-D mineralized in 28 days (Table 1). The extent of mineralization of one or both levels of phenylacetate and *p*-nitrophenol was greater in Beebe Lake water and sewage, and 2,4-D was mineral-

TABLE 1. Mineralization of aromatic compounds in lake waters and sewage

Test chemical	Initial concn (pg/ml)	% Mineralized		
		Cayuga Lake	Beebe Lake	Sewage
Benzoate	59	94.5	96.3	99.4
	59,000	98.8	98.6	99.5
Phenylacetate	19	77.8	98.3	98.3
	657	70.9	96.3	95.5
p-Nitrophenol	14.4	9.5	27.9	62.3
	934	58.4	56.3	57.6
2,4-D	36.6	0	0	93.7
	48,900	0	0	91.2

ized in this trial only in sewage. Thus, the activity seems to be related to the nutrient level, or possibly the community composition, of these environments. In two of the three environmental samples, the percentage of *p*-nitrophenol mineralized was greater at the higher substrate concentration. The almost complete disappearance of radioactivity from solutions that initially were amended with benzoate and phenylacetate indicates that little or none of the substrate-carbon was assimilated by the microflora. The previous finding of 2,4-D breakdown in Beebe Lake water was made with samples taken in the spring.

The mineralization of *p*-nitrophenol in samples of sewage and lake water is shown in Fig. 3. In several instances, an apparent lag phase was evident before loss of the chemical was detected. This apparent lag phase was shorter for comparable chemical concentrations in samples from sewage than from Cayuga Lake water. The reason for the decline in rate followed by a reinitiation of mineralization in Beebe Lake water is unknown. At the lower concentration, mineralization was slowest in Cayuga Lake water. The amount of substrate mineralized at any time period was different among the three environmental samples, the percent mineralization of the lower concentration increasing as the nutrient status increased, i.e., in the order of Cayuga Lake, Beebe Lake, and sewage.

When White Lake water was amended with 2,4-D and di(2-ethylhexyl) phthalate at various

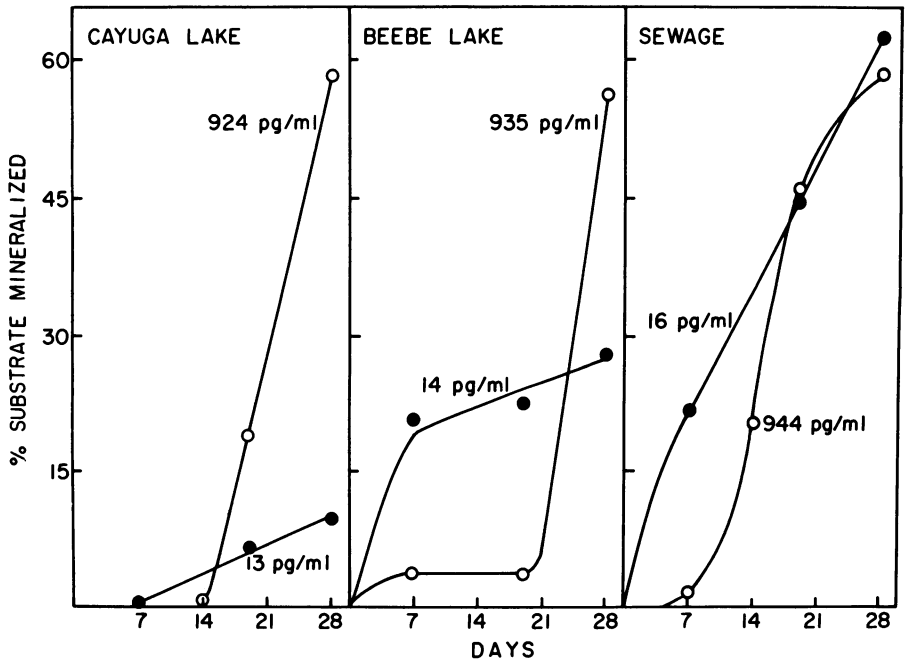

FIG. 3. Mineralization of *p*-nitrophenol in samples of sewage and lake water.

concentrations, no mineralization was detected in 60 days. In Beebe Lake water, in contrast, mineralization occurred immediately with the phthalate ester and within 8 days for 2,4-D.

DISCUSSION

The rate of phenol mineralization is a linear function of concentration at levels below 1 μg/ml, falls off between 1 and 100 μg/ml, and is again high at levels above 100 μg/ml. These findings may reflect the activity of two different kinds of organisms, oligotrophs (or oligocarbophiles) active at the lower concentrations and eutrophs active at the higher concentrations. The hypothesis that two such types of microorganisms exist is consistent with studies of axenic cultures. The occurrence of eutrophs is shown by the studies of Jannasch (5) and Shehata and Marr (7), who found that stable microbial populations could not be maintained in axenic continuous culture at low levels of organic substrates, and of Boethling and Alexander (4), who noted that bacteria in axenic culture degraded 18 pg of glucose per ml at rates far below those predicted by Michaelis-Menten kinetics. The existence of oligotrophs has been the subject of considerable recent concern (8), and such organisms may grow well on carbon sources added to media at concentrations of <10 μg of C per ml (1). The presence of sensitive oligotrophs in the water samples may explain the total inhibition of 2,4-D mineralization at 200 ng/ml, a sensitivity previously undescribed for heterotrophs.

Threshold concentrations below which there is little or no decomposition of organic compounds have been reported for natural waters (3). A threshold for a given compound may be evident in ecosystems containing eutrophs but not oligotrophs capable of metabolizing a test chemical. Alternatively, in waters containing organic compounds in addition to the substrate of interest, a eutroph may have a lower threshold for a given compound than might be predicted from studies involving only a single substrate. Thus, Law and Button (6) reported that the 210-ng/ml threshold for glucose of a marine bacterium was reduced to <10 ng/ml by a mixture of amino acids. Effects of nutrient levels in aquatic habitats on mineralization are suggested by the present investigation. Nevertheless, it is not clear whether the absence of a detectable threshold noted here for most chemicals is a reflection of the existence of an oligotrophic population or of diminished thresholds associated with other nutrients. The lower than predicted rate at 1.53 pg of 2,4-D per ml suggests that a threshold exists for this compound at still lower concentrations.

Another possibility is raised by our observation that more than 95% of the radioactivity in samples of lake water or sewage or both receiving benzoate and phenylacetate and 93.7% of the radioactivity in sewage amended with 36.6 pg of 2,4-D per ml disappeared. Thus, little or none of the carbon had been assimilated. It is thus possible that microorganisms carrying out such transformations at these low concentrations have the previously unreported capacity of mineralizing organic compounds without obtaining carbon, and presumably energy, from the substrate. This phenomenon is similar to cometabolism, the cometabolizing populations deriving neither a nutrient nor energy from the substrate they metabolize, but it is generally believed that mineralization is not a consequence of cometabolism (2).

The finding of mineralization of 2,4-D and di(2-ethylhexyl) phthalate in samples from Beebe Lake but not from White Lake points to a feature of the environmental metabolism of synthetic chemicals that is often overlooked: a mineralizable molecule may not be destroyed in a particular ecosystem, particularly in oligotrophic waters. The lack of decomposition may be attributable either to the absence of populations having the necessary catabolic enzymes or to the existence of species that are potentially active but which require growth factors that are not present in the nutrient-poor water. Moreover, because 2,4-D decomposition was found in Beebe Lake water sampled at one season but not another, time of year may determine the occurrence of mineralization by means other than those linked with suppressed activity at low temperature. The effect of season during which the sample was taken from freshwaters also has been reported for the metabolism of *N*-nitrosodiethanolamine (10).

ACKNOWLEDGMENT

This project was supported by the U.S. Environmental Protection Agency under cooperative agreement CR806887.

LITERATURE CITED

1. **Akagi, Y., U. Simidu, and N. Taga.** 1980. Growth responses of oligotrophic and heterotrophic marine bacteria in various substrate concentrations, and taxonomic studies on them. Can. J. Microbiol. **26**:800–806.
2. **Alexander, M.** 1981. Biodegradation of chemicals of environmental concern. Science **211**:132–138.
3. **Boethling, R. S., and M. Alexander.** 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. **37**:1211–1216.
4. **Boethling, R. S., and M. Alexander.** 1979. Microbial degradation of organic compounds at trace levels. Environ. Sci. Technol. **13**:989–991.
5. **Jannasch, H. W.** 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. **12**:264–271.
6. **Law, A. T., and D. K. Button.** 1977. Multiple-carbon-source-limited growth kinetics of a marine coryneform bacterium. J. Bacteriol. **128**:115–123.
7. **Shehata, T. E., and A. G. Marr.** 1971. Effect of nutrient

concentration on the growth of *Escherichia coli*. J. Bacteriol. **107**:210–216.
8. **Shilo, M. (ed.).** 1979. Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, West Germany.
9. **Subba-Rao, R. V., H. E. Rubin, and M. Alexander.** 1982. Kinetics and extent of mineralization of organic chemicals at trace levels in freshwater and sewage. Appl. Environ. Microbiol. **43**:1139–1150.
10. **Yordy, J. R., and M. Alexander.** 1980. Microbial metabolism of *N*-nitrosodiethanolamine in lake water and sewage. Appl. Environ. Microbiol. **39**:559–565.