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Effect of Substrate Concentration and Organic and Inorganic Compounds on the Occurrence and Rate of Mineralization and Cometabolism

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Isopropyl *N*-phenylcarbamate (IPC) at 400 pg and 1 μg/ml was mineralized in samples of sewage, but only the lower concentration was mineralized in lake water samples in a 50-day period. IPC at 1 μg/ml disappeared from lake water, but it was converted to organic products. Mineralization of IPC at 400 pg/ml in lake water was enhanced by additions of inorganic nutrients or a mixture of nonchlorinated water pollutants but not by yeast extract or mixtures containing aromatic compounds or excretions of primary producers. The mineralization of 200 pg of 2,4-dichlorophenoxyacetate per ml of lake water was not affected by additions of low levels of yeast extract or compounds excreted by primary producers but was enhanced by low concentrations of mixtures of water pollutants. It is suggested that some chemicals that are found to be converted only to organic products, presumably by cometabolism, in tests using the concentrations commonly employed in laboratory evaluations may be mineralized at the lower concentrations prevailing in natural waters.

Assessments of biodegradation are commonly performed using concentrations of organic chemicals far higher than those found in most natural waters, and these laboratory tests serve as the basis for predicting the fate of the compounds in natural ecosystems. However, recent evidence indicates that extrapolations using data from tests at high concentrations, for certain chemicals at least, may not reflect what takes place at low concentrations. For example, the microflora of stream water mineralized little 2,4-dichlorophenoxyacetate (2,4-D) and 1-naphthyl-*N*-methylcarbamate (Sevin), or the naphthol formed from Sevin, at concentrations of 2 to 3 ng/ml or less, but it mineralized 60% or more of the compounds in 6 days when they were provided at higher levels (4). Conversely, 2,4-D was not mineralized at a concentration of 200 ng/ml by the microflora of a lake, but it was mineralized at levels below 5 ng/ml (20). Moreover, in contrast to the kinetics of microbial processes at high concentrations, mineralization of a number of organic compounds in lake water is linear with time (21).

The present study was designed to determine whether compounds that have been reported to be mineralized in natural waters at only one of two concentrations were metabolized at both test levels. The reason for the study is the need to establish whether a compound giving a negative mineralization test is transformed in nature to organic products. In addition, an investigation was conducted to determine whether nutrients and organic chemicals present at low levels in natural waters affect the rate and extent of mineralization of low concentrations of organic substrates.

MATERIALS AND METHODS

2,4-Dichlorophenoxy[2-^{14}C]acetic acid (specific activity, 28 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill., and unlabeled 2,4-D was obtained from Applied Science Laboratories, State College, Pa. Isopropyl *N*-[ring-*U*-^{14}C]phenylcarbamate (specific activity, 4.28 mCi/mmol) (IPC) was purchased from New England Nuclear Corp., Boston, Mass., and unlabeled IPC was obtained from PPG Industries, Pittsburgh, Pa. The purity of these compounds as determined by thin-layer chromatography exceeded 99%. Solvents of the highest purity available commercially were used without further purification. The glassware was soaked in sulfuric acid containing a metal-free inorganic oxidizer (Nochromix, Godak Laboratories, New York, N.Y.) for 2 h and rinsed three or four times with reagent-grade distilled water.

Samples of fresh water were taken in July 1982 from Beebe Lake, Ithaca, N.Y. The pH value was 7.6, and the dissolved organic matter content determined by combustion ranged from 4.8 to 5.2 μg/ml. Sewage was obtained in September 1982 from the settling tanks of the Ithaca waste treatment plant. Before use, lake water and sewage samples were passed through a glass fiber filter (no. 66085; Gelman Sciences, Ann Arbor, Mich.) to remove particulate matter. Distilled water was used after purification by passage through the Milli-Q Reagent-Grade Water System (Millipore Corp., Bedford, Mass).

Thin-layer chromatography was carried out with precoated silica gel plates containing a fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). Before placing samples on the plates, 3 drops of a solution of 250 μg of unlabeled IPC per ml of acetone and 1 drop of acetone containing aniline were added to the samples. The separated compounds were detected under a short-wave UV lamp (254 nm). Gas chromatography was performed with a Perkin-Elmer gas chromatograph, model 3920B, equipped with a flame ionization detector. The stainless steel column (183 by 0.3 cm) was packed with 3% Silar 10C on 100/120 Gas-Chrom Q (Applied Science Laboratories). The flow rate of the carrier gas, N_2, was maintained at 45 ml/min. The temperatures of the interface and injection port were 260 and 240°C, respectively. The temperature of the column was

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maintained at 100°C for 1 min, and it was then increased to 240°C at a rate 16°C/min.

IPC was added to freshly sampled and filtered lake water or sewage (either 100 ml in 250-ml Erlenmeyer flasks for the higher IPC level or 250 ml in 500-ml flasks for the lower level). The tests were conducted in triplicate. For the higher concentration, 1.0 μg of unlabeled IPC was added per ml, and the labeled chemical was added to give 250 dpm/ml. At an IPC concentration of 400 pg/ml, only the labeled compound was added. The flasks were stoppered with foam plugs and incubated without shaking in the dark at 29°C for lake water and at 20 to 22°C for sewage samples.

To determine mineralization rates, 1.0-ml portions of samples initially containing 1 μg of IPC per ml and 10-ml portions of samples initially containing 400 pg of IPC per ml were removed at regular intervals, and the liquid was acidified with concentrated H_2SO_4 to pH 2 and bubbled with air to remove $^{14}CO_2$. The treated sample was placed in 20-ml glass scintillation vials, and 7.0 ml of Aqueous Counting Scintillant (Amersham) was added to the 1.0-ml samples and 10 ml of Redi-solve MP (Beckman Instruments, Fullerton, Calif.) was added to the 10-ml samples. The radioactivity of the samples was measured for 10 min using a Beckman LS7500 liquid scintillation system. Details of the method are given elsewhere (20, 21).

For samples that were to be analyzed by gas and thin-layer chromatography, 1,000 ml of a solution initially containing 1 μg of IPC per ml and 2,500 ml of a solution initially containing 400 pg of IPC per ml were incubated at 29°C in the dark without shaking in 2- and 4-liter flasks, respectively. Only unlabeled IPC was added to flasks to be analyzed by gas chromatography. Portions of the liquid were removed from the flasks, either 100 and 250 ml from lake water samples or 10 and 100 ml from sewage samples receiving the higher and lower IPC levels, respectively. The liquid was extracted three times with ethyl ether, and the extracts were then pooled, dried over anhydrous Na_2SO_4, and concentrated in a rotary flash evaporator to dryness. The residue was dissolved in acetone before thin-layer and gas chromatographic analysis. Thin-layer chromatograms were developed with chloroform-acetone (9:1) as the solvent system. Portions of the material on the plates were scraped off, and the silica gel was placed in scintillation vials containing 7.0 ml of Aqueous Counting Scintillant for radioactivity measurements.

In studies of the effects of nutrients and organic compounds on the mineralization of 2,4-D and IPC, eight groups of substances were used: (i) major inorganic nutrients, (ii) minor inorganic nutrients, (iii) yeast extract, (iv) a mixture of aromatic monomers of lignin and humic substances, (v) compounds excreted by primary producers in natural waters, (vi) nonchlorinated pollutants, (vii) chlorinated pollutants, and (viii) a combination of these chemicals. Yeast extract was obtained from BBL Microbiology Systems, Cockeysville, Md. The aromatic compounds considered as monomers, or related to monomers, of lignin and humic substances were catechol, resorcinol, *o*-methoxyphenol, protocatechuic acid, 3,5-dihydroxybenzoic acid, vanillin, vanillic acid, syringic acid, *p*-hydroxybenzoic acid, *trans*-ferulic acid, *p*-hydroxybenzaldehyde, and *p*-cresol (1). Compounds that represented excretions of primary producers in natural waters were lactic acid, succinic acid, pyruvic acid, acetic acid, oxalic acid, tartaric acid, glycolic acid, glucose, xylose, glucuronic acid, galactose, arabinose, *p*-cresol, glycerol, glycine, mannitol, α-methylglucoside, sucrose, urea, and inositol (17). The mixture of nonchlorinated pollutants contained hexadecane, eicosane, dotriacontane, dioctyl phthalate, naphthalene, anthracene, phenanthrene, aniline, diethylamine, toluene, benzene, octane, *p*-phenylphenol, *n*-docosane, linoleic acid, oleic acid, palmitic acid, and stearic acid (6, 9, 17). The mixture of chlorinated pollutants contained α-BHC, aldrin, dieldrin, heptachlor, methoxychlor, *p,p'*-DDT, *p,p'*-DDD, endrin, *p*-dichlorobenzene, 2,4,5-trichlorophenoxyacetic acid, 4,4-dichlorobiphenyl, chloroform, carbon tetrachloride, and 1,2-dichloroethane (8).

Each group of these chemicals was added at two concentrations to filtered lake water. The major inorganic nutrients were added to final concentrations of 1.5 μg of $CaCl_2$, 0.1 μg of $FeSO_4$, 20 μg of $MgSO_4 \cdot 7H_20$, 4 μg of KCl, 25 μg of NH_4Cl, 100 μg of $NaNO_3$, 10.5 μg of Na_2HPO_4, and 4.5 μg of NaH_2PO_4 (10) or at 100-fold lower levels to 1 liter of lake water. The minor inorganic nutrients were added to final concentrations of 0.5 μg of $CuSO_4$, 1.0 μg of H_3BO_3, 1.0 μg of $MnSO_4 \cdot 5H_2O$, 7.0 μg of $ZnSO_4 \cdot 7H_2O$, and 1.0 μg of MoO3 (10) or at 100-fold lower levels to 1 liter of lake water. The compounds in the remaining six groups of chemicals were added at final concentrations (for the total of all compounds in the group) of 10 ng or 1.0 μg/ml. The chemicals in each group were in equal amounts to prepare the mixture of all chemicals. To prepare a mixture of nutrients and other chemicals (group viii), inorganic nutrients and yeast extract at the concentrations given above and one-fourth the amounts of chemicals in groups iv to vii were added to lake water to give a final concentration of 1.0 μg or 10 ng/ml. Because of their low solubility in water, compounds in groups iv, vi, and vii were added as an acetone solution to the empty flasks, and the solvent was allowed to evaporate before the lake water was added.

To the flasks containing these solutions was then added 1.0 μg or 200 pg of 2,4-D or 1.0 μg or 400 pg of IPC per ml. At the higher concentration, 1.0 μg of unlabeled chemical per ml was supplemented with approximately 500 and 250 dpm/ml for 2,4-D and IPC, respectively. At the lower concentration, only the labeled compounds were added. At the higher concentration, 100 ml of solution was incubated in 250-ml Erlenmeyer flasks. At the lower concentration, 250 ml of solution was incubated in 500-ml flasks. The flasks were stoppered with foam plugs and incubated without shaking in the dark at 29°C. All tests were conducted in triplicate. The sample sizes taken, the means of preparing samples for determining radioactivity, and methods of measuring mineralization of the labeled compounds were the same as those described above.

RESULTS

The mineralization of IPC in samples of Beebe Lake water and sewage is shown in Fig. 1. Mineralization of the lower concentration was observed in the lake water after 10 days, and the percentage of IPC-carbon that was mineralized reached 70% in 46 days. No mineralization of IPC at the higher concentration was detected in the lake water in 50 days. In the sewage samples, IPC was mineralized at both concentrations. In the test period, 60 and ca. 80% of the carbon was mineralized in sewage samples receiving 1.0 μg and 400 pg of IPC per ml, respectively. Algae developed in the sewage samples because they were incubated on the laboratory bench, but the algal contribution to mineralization was not evaluated.

Because the sewage samples had been incubated in the light, measurements were made of the possible photodecomposition of IPC. Flasks containing either distilled water or sewage samples were amended with 1.0 μg and 400 pg of

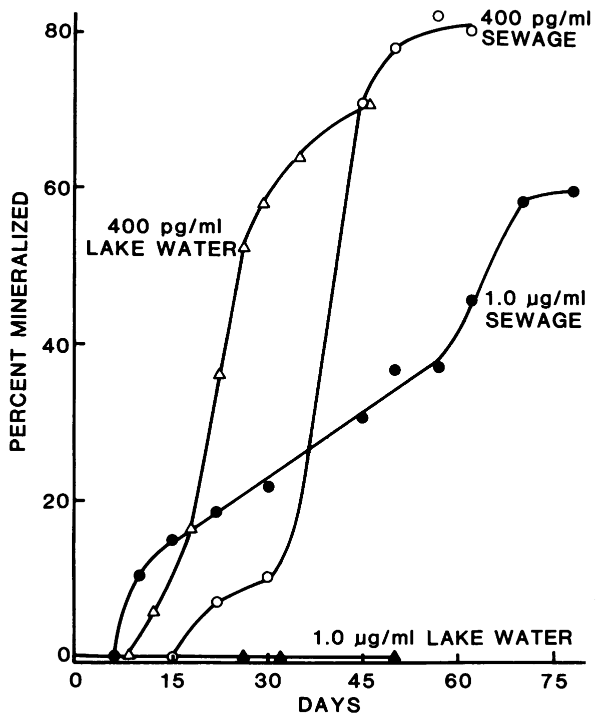

FIG. 1. Mineralization of IPC added at two concentrations to samples of lake water and sewage.

labeled IPC per ml. In the former instance, unlabeled chemical was also added. The solutions were amended with 100 μg of NaCN per ml to prevent microbial growth, and they were incubated under ambient light in the laboratory for 8 days for distilled water samples or 32 days for sewage samples. Identical tests were run with the flasks incubated in the dark at 29°C. The solutions were examined at the start and the end of the incubation period by thin-layer chromatography and by assessing the possible formation of $^{14}\text{CO}_2$. Photochemical decomposition or nonbiological degradation of IPC was not detected in these cyanide-treated samples. These data indicate that the changes noted in the nonsterile samples incubated in the light for part of the test period did not result from photochemical breakdown of IPC.

The finding that IPC was extensively mineralized in nonsterile lake water samples receiving 400 pg/ml but not in samples amended with 1.0 μg/ml was surprising. A study was conducted to determine whether the chemical at the higher level might be converted to organic products even it if was not transformed to CO_2. IPC-amended lake water samples were incubated for 46 days, samples were regularly taken and extracted, and the products were separated by thin-layer chromatography. Because of the small amount of chemical added to the lake water, the products could not be detected with a UV lamp. Therefore, the silica gel from the thin-layer plates was scraped off as several bands, and the radioactivity in each band was determined by scintillation counting. No decline in total counts was evident in the lake water receiving 1.0 μg of IPC per ml, confirming the absence of mineralization (Table 1). However, the amount of radioactivity that was recovered at the spot that chromatographed with IPC declined from 98.9% at day zero to 80.2% at day 46. This spot at R_f 1.00 to 0.80 contains IPC but could contain other compounds as well. As the IPC disappeared, products with lower R_f values appeared. In contrast, radioactivity disappeared with time in lake water samples receiving 400 pg of IPC per ml, reflecting the mineralization that was taking place. At the same time, organic products were detected on the chromatograms.

The formation of products during the decomposition of IPC in sewage samples is shown in Table 2. The extent of mineralization is evident by the loss of radioactive carbon from solution. The percentage of the radioactivity at the spot cochromatographing with IPC (R_f 1.00 to 0.80) declined to 47.6 and 1.02% of its initial value in sewage initially receiving 1.0 μg and 400 pg of IPC, respectively, indicating that at least 52 and 99% of the pesticide was metabolized. During the incubation, polar products were formed, and some of these were then further metabolized. Thus, although mineralization occurred at both concentrations, a variety of organic products were generated and some persisted.

The extracts from this experiment (samples receiving 1.0 μg of IPC per ml) were also analyzed by gas chromatography. The extraction efficiency for IPC was 55 ± 5%. IPC and aniline showed distinct, sharp peaks on a 3% Silar 10C column with retention times of 7.0 and 3.8 min, respectively. Based on peak heights, the concentration of IPC decreased in lake water and sewage samples. A trace of aniline was present even at day zero, probably as a contaminant in the IPC preparation, and its concentration in the treated lake water and sewage never fell below 6.0 ng/ml. The gas chromatograms also contained a series of products that were not present in IPC-amended lakewater and sewage samples at day zero. Thus, at day 8, three new compounds were

TABLE 1. Distribution of radioactivity in products of IPC degradation in Beebe Lake water

IPC concn (per ml)	Days of incubation	Distribution of initial radioactivity (%) at the following R_f values:					Total label remaining in solution (%)[a]
		1.00–0.80	0.80–0.77	0.77–0.60	0.60–0.10	0.10–0.0	
1.0 μg	0	99	1.1	0.0	0.0	0.0	100
	8	93	4.5	1.5	0.49	0.59	100
	18	89	7.7	1.2	0.84	0.92	100
	46	80	17	0.94	1.1	0.79	100
400 pg	0	98	1.7	0.16	0.0	0.0	100
	8	65	15	8.0	4.9	4.6	97
	18	56	6.8	9.3	3.9	8.6	82
	46	25	2.6	2.7	0.32	0.31	29

[a] Radioactivity in 1.0- and 10-ml portions of the unextracted samples receiving 1.0 μg and 400 pg of IPC per ml, respectively. The standard deviation for the values recorded as 100% was 2.1%.

TABLE 2. Distribution of radioactivity in products of IPC degradation in sewage samples

IPC concn (per ml)	Days of incubation	Radioactivity in solution (%)[a]	Distribution of initial radioactivity (%) at the following R_f values:				
			1.00–0.80	0.80–0.77	0.77–0.60	0.60–0.10	0.10–0.00
1.0 µg	0	100	96	0	0.60	1.4	1.6
	2	100	92	0	1.9	2.0	4.1
	10	89	32	6.5	37	14	14
	22	81	60	0	5.4	9.2	6.1
	30	78	58	0	1.6	15	2.9
	45	67	38	6.8	4.6	7.1	11
	60	62	48	0	1.8	2.6	9.6
400 pg	0	100	84	0.9	3.1	1.9	10
	2	100	85	5.0	2.1	3.4	4.1
	6	100	79	4.2	3.3	1.9	11
	10	100	71	9.8	1.6	1.5	16
	15	100	79	2.3	1.8	1.8	15
	22	92	63	1.0	9.1	7.4	11
	30	90	72	2.9	2.2	7.3	5.2
	45	28	8.7	0.23	1.9	6.9	11
	60	18	1.0	0	2.8	6.2	7.5

[a] Radioactivity in 1.0- and 10-ml portions of the unextracted samples receiving 1.0 µg and 400 pg of IPC per ml, respectively.

found in treated lake water, and eight different products were observed at day 28. The identities of these products were not ascertained.

The effect of added inorganic nutrients and organic compounds on the extent of mineralization of 2,4-D (initial concentration of 200 pg/ml) in 50 days and of IPC (initial concentration of 400 pg/ml) in 60 days in Beebe Lake water was evaluated. The minor inorganic nutrients, yeast extract, and the products of primary producers at concentrations of 1.0 µg or 10 ng/ml and the major inorganic nutrients, nonchlorinated or chlorinated pollutants, and the mixture at 10 ng/ml had no effect. As shown in Table 3, however, the major inorganic nutrients, the nonchlorinated and chlorinated pollutants, and the mixture at 1.0 µg/ml had a significant stimulatory effect on the extent of mineralization of 2,4-D and IPC. Surprisingly, the aromatic compounds at 10 ng but not at 1.0 µg/ml enhanced the extent of IPC mineralization.

To assess the rates of mineralization of 2,4-D added to Beebe Lake water at final concentrations of 1.0 µg and 200 pg/ml, measurements were made at regular time intervals. Mineralization was rapid at the higher concentration, and more than 90% of the carbon was converted to CO_2 (Fig. 2). The process was far slower at the lower concentration, slower even than the rate that would be predicted if the rate were directly proportional to substrate concentration. The

TABLE 3. Effect of inorganic nutrients and organic compounds on the extent of mineralization of 2,4-D in 50 days and IPC in 60 days in samples of Beebe Lake water

Amendment	Concn of amendment (µg/ml)	% Mineralized[a]	
		2,4-D	IPC
None		72.0 (C)	69.5 (C)
Major inorganic nutrients	1.0	84.2 (B)	85.8 (A)
Aromatic compounds of humic origin	1.0 0.01	62.7 (C) 67.7 (C)	63.7 (C) 76.9 (B)
Nonchlorinated pollutants	1.0	93.5 (A)	89.3 (A)
Chlorinated pollutants	1.0	94.9 (A)	79.3 (B)
Mixture	1.0	95.8 (A)	87.5 (A)

[a] Values followed by different letters within each column are significant different at the 1% level of probability.

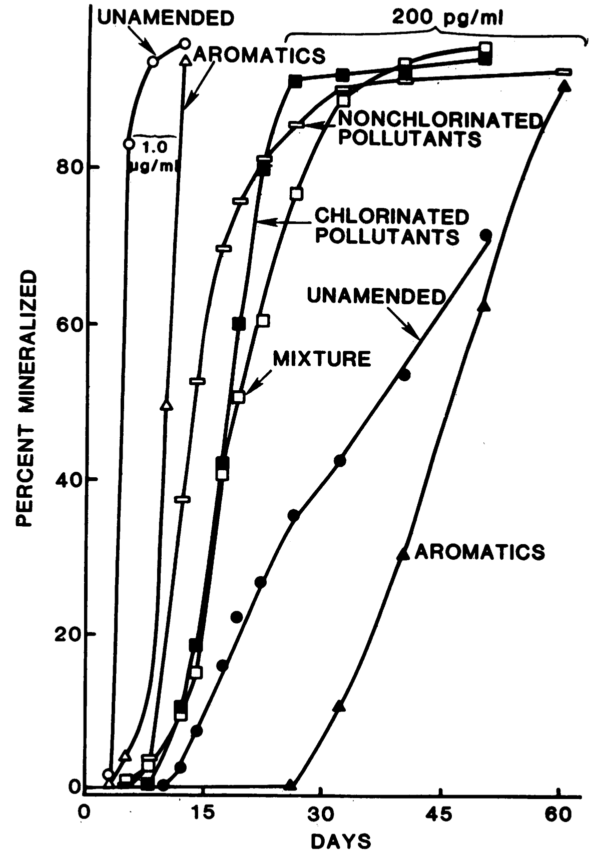

FIG. 2. Mineralization of 1.0 µg or 200 pg of 2,4-D per ml of unamended lake water or lake water amended with a total of 1.0 µg of various compounds per ml.

various amendments at concentrations of 10 ng/ml and the inorganic nutrients, yeast extract, and products of primary production at 1.0 μg/ml had little or no effect on the rates. In contrast, the aromatic compounds of humic origin at a concentration of 1.0 μg/ml markedly increased the time before mineralization of 1.0 μg or 200 pg of 2,4-D per ml was detected (Fig. 2). In addition, the nonchlorinated and chlorinated pollutants and a combination of all chemicals each at final concentrations of 1.0 μg/ml enhanced the rate of mineralization of 200 pg of 2,4-D per ml but not of the higher herbicide level.

No matter which group of chemicals was added to lake water, mineralization of IPC added at a concentration of 1.0 μg/ml did not occur. The incubation was conducted for 80 days.

There was little or no effect of the following on the rate of mineralization of 400 pg of IPC per ml: minor inorganic nutrients, yeast extract, the aromatic mixture, products of primary producers, and the chlorinated pollutants at 10 ng and 1.0 μg/ml and the major inorganic nutrients, the nonchlorinated pollutants, and the mixture at 10 ng/ml. On the other hand, the major inorganic nutrients, the nonchlorinated pollutants, and the mixture at 1.0 μg/ml were stimulatory by shortening the apparent lag phase, increasing the extent of mineralization, or both (Fig. 3).

DISCUSSION

These two test compounds were chosen because they are known to be readily degraded at the usual test concentrations and are used as substrates to support microbial growth.

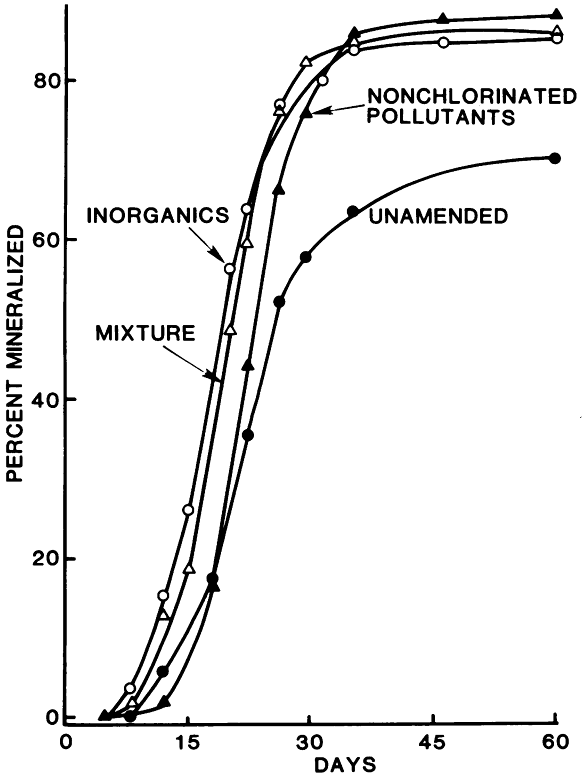

FIG. 3. Mineralization of 400 pg of IPC per ml of unamended lake water or lake water amended with a total of 1.0 μg of various compounds per ml.

Thus, IPC disappears readily from natural soil but persists if the soil is sterilized (15), and it is a carbon source for *Pseudomonas striata* (13). Similarly, 2,4-D is readily destroyed in natural waters (20) and supports the growth of bacteria in culture (11).

The finding that IPC is mineralized at one concentration but is converted only to organic products at another concentration has considerable importance. Comparable results have not been reported for any other compound. The practical significance lies in the fact that measuring the results of tests at only one concentration might not be applicable to circumstances in which the chemical is present at lower concentrations in nature. In the case of IPC at 1.0 μg/ml, metabolism occurred but no CO_2 was produced; this presumably was the result of cometabolism. However, the compound was mineralized at lower concentrations. A chemical that is extensively mineralized presumably gives lower yields of organic products than one that is cometabolized, and hence there may be less concern with the toxicity of these products (2). Moreover, these observations may have relevance to the previous finding that a chemical that is mineralized at concentrations of 1.0 ng/ml is not mineralized at 200 ng/ml levels (20); the compound may not be resistant at the higher level, but rather it may be subject to cometabolism.

It is possible that IPC was mineralized in lake water at the higher concentration to a slight extent and that this mineralization would not have been detected by the methods used. For example, if the extent of mineralization was the same at both the 400 pg/ml and 1.0 μg/ml levels, the percent mineralized at the higher concentration would have been only 0.04% of the percent converted to CO_2 at the lower level. Even if this is true, however, the data still show that measurements made at one concentration may not provide useful information for predictive purposes for the chemical levels in natural waters because the percentages of the compound transformed to inorganic products would be vastly different.

Mineralization of substrates at concentrations of 200 or 400 pg/ml may result from the activities of oligotrophic populations. Such organisms, which have been the subject of recent interest (18), may be markedly inhibited by organic compounds at concentrations of 1.0 μg/ml and hence are inactive. The apparent cometabolism of 1.0 μg of IPC per ml may be the result of activities of eutrophic populations that cannot function at very low substrate levels. A eutrophic *Pseudomonas* sp. strain has been described that rapidly mineralized 18 ng of glucose per ml but had little activity on 18 pg/ml (5), but the possibility that this bacterium could cometabolize a substrate at one concentration but mineralize it at another was not tested. Indeed, a single population, and not two, may mineralize at one concentration in nature and only cometabolize at another.

Ample evidence exists that nutrients or other compounds alter the rates of transformation by heterotrophic microorganisms. For example, the decomposition of 2,6-dichlorobenzamide in soil is enhanced by ethanol and glucose (7), and the metabolism of BHC is stimulated by peptone (16). However, nearly all of these studies were conducted with substrates added at high concentrations. The results of such studies may not be applicable to the transformations of substrates at low concentrations, especially if different physiological groups of microorganisms bring about the conversions at high and at low levels. However, it has been reported that nitrogen and phosphorus compounds enhance oil biodegradation in seawater (3) and that inorganic nutrients enhance 2,4-D degradation in river water (14). It has also been noted that the rates of mineralization of low concentrations of phenol in lake waters were directly related to the trophic levels of the waters, were often increased by inorganic nutrients, but were decreased by glucose (19).

Studies have also been conducted to assess the effect of concentration on rates of mineralization. Thus, the rates of mineralization of diisopropylnaphthalene in activated sludge (22), glucose and aromatic compounds in soil (12), and aromatic compounds in lake water (20, 21) are affected by substrate concentration, but the possibility that mineralization occurs at one concentration and cometabolism at another was not evaluated heretofore.

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