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Adaptation to and Biodegradation of Xenobiotic Compounds by Microbial Communities from a Pristine Aquifer

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The ability of subsurface microbial communities to adapt to the biodegradation of xenobiotic compounds was examined in aquifer solids samples from a pristine aquifer. An increase in the rates of mineralization of radiolabeled substrates with exposure was used as an indication of adaptation. For some compounds, such as chlorobenzene and 1,2,4-trichlorobenzene, slight mineralization was observed but no adaptation was apparent during incubations of over 8 months. Other compounds demonstrated three patterns of response. For *m*-cresol, *m*-aminophenol, and aniline intermediate rates of biodegradation and a linear increase in the percent mineralized with time were observed. Phenol, *p*-chlorophenol, and ethylene dibromide were rapidly metabolized initially, with a nonlinear increase in the percent mineralized with time, indicating that the community was already adapted to the biodegradation of these compounds. Only *p*-nitrophenol demonstrated a typical adaptation response. In different samples of soil from the same layer in the aquifer, the adaptation period to *p*-nitrophenol varied from a few days to as long as 6 weeks. In most cases the concentration of xenobiotic added, over the range from a few nanograms to micrograms per gram, made no difference in the response. Most-probable-number counts demonstrated that adaptation is accompanied by an increase in specific degrader numbers. This study has shown that diverse patterns of response occur in the subsurface microbial community.

Ground water pollution has become a major concern in the United States where much of the population relies on ground water as its major source of drinking water (1). Chemical contamination of aquifers has been documented (9, 11) and poses a potential health hazard. The ability of polluted aquifers to recover from chemical contamination is dependent primarily on biological reactions, which may occur more rapidly in the subsurface than chemical and physical reactions. Because the subsurface biological community is composed primarily of bacteria (17, 18), it is essential to investigate the interaction of the subsurface microbial community with xenobiotic compounds, including biodegradation and adaptation processes.

The potential health hazard of a xenobiotic compound is a function of its persistence in the environment as well as the toxicity of the chemical class. Persistence is a function of the biotransformation rate, which influences the compound's form, residence time, and mobility in the subsurface environment. The ability of the subsurface microbial community to adapt to xenobiotic compounds and the time necessary for the adaptation process to occur will be principal factors determining persistence for less readily degraded compounds (19). Adaptation is defined functionally as an increase in the rate of degradation with exposure to a chemical. Adaptation has been found to occur in many different environments including surface soil systems as a result of repeated treatment with insecticides (2, 7, 8), estuarine ecosystems (12), and freshwater environments (14–16, 21). Studies at contaminated field sites have shown that subsurface communities exposed to hydrocarbons degraded them at a faster rate than the community at unexposed sites (19) and that the microbes adapted to degrade several compounds simultaneously (20). Little information currently exists, however, on the relationship between exposure and adaptation in the subsurface microbial community.

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The objectives of this research were to examine the ability of a subsurface microbial community previously exposed to no known pollutants to degrade a wide variety of xenobiotic compounds, determine rates of degradation, and identify patterns of degradation. This was accomplished by measuring mineralization of nine radiolabeled substrates over time. From these studies we were able to identify the xenobiotic compounds to which the subsurface soil microbial community could adapt and the duration of the adaptation period. Finally, the number of specific degraders was calculated before and after the adaptation process to determine whether changes in the degrading population had occurred during the adaptation period.

MATERIALS AND METHODS

Chemicals. The radiolabeled substrates used included *m*-[U-^{14}C]cresol, [U-^{14}C]ethylene dibromide, [U-^{14}C]phenol, and *m*-[U-^{14}C]aminophenol purchased from Amersham Corp. (Arlington Heights, Ill.); [U-^{14}C]chlorobenzene, [U-^{14}C]1,2,4-trichlorobenzene, *p*-[U-^{14}C]chlorophenol, and *p*-[U-^{14}C]nitrophenol purchased from Pathfinder Laboratories, Inc. (St. Louis, Mo.); [U-^{14}C]aniline hydrochloride purchased from Research Products International Corp. (Mount Prospect, Ill.); and [^{14}C]barium carbonate purchased from New England Nuclear Corp. (Boston, Mass.).

Subsurface soil samples. Aquifer solids samples were provided by the U.S. Environmental Protection Agency from an uncontaminated aquifer site, in unconsolidated material from the margin of the flood plain of a small river near Lula, Okla. The soil samples were aseptically removed from the site (5, 18). A uniform fine sand from the saturated zone of the profile was used in all experiments. This area of the profile, at a depth of 4.5 to 5.6 m below the surface, is under artesian head. There was approximately 0.22 ml of pore water per g of soil in this layer of the aquifer.

Respiration measurements. To evaluate the rates of mineralization by the microbial community, $^{14}CO_2$ evolution from

a number of radiolabeled compounds was monitored over time. Because the microbes originated from aquifer solids, concentrations are presented on a solids dry weight basis. It should be recognized however, that all incubation vials contained approximately 1 g of aquifer solids and 24 ml of sterile distilled water; thus the microorganisms were in a more dilute suspension than the concentrations reflect. For the majority of the radiolabeled compounds studied, two concentrations were used, approximately 10 to 50 and 500 to 1,000 ng/g of soil. These represent environmentally relevant concentrations yet were distinct enough to cause differences in microbial responses due to concentration.

Aquifer material was placed in a blender with aerated sterile distilled water, and a slurry was made. Samples were dispensed into 20-ml vials. Sodium azide was added as a metabolic inhibitor to create a series of dead control vials to account for the influence of abiotic processes such as sorption. The radiolabeled isotope was added to all of the vials, and the remaining volume was filled with sterile distilled water. The headspace-free vials were inverted during incubations to minimize loss through the cap and Teflon liner. Incubations were carried out in the dark at 17°C, the ambient temperature at the Lula site.

At specific time intervals three or four live replicate samples and three controls were transferred individually to 40-ml vials by using a connector cap, which created a headspace. The samples were then acidified with phosphoric acid to pH 2, and a center well containing 0.15 ml of 1 N KOH was placed in the vial headspace. The vials were placed on a rotary shaker (Labline Instruments, Inc.) at 80 rpm for approximately 18 h, during which the $^{14}CO_2$ was trapped in the base. The base containing the $^{14}CO_2$ was absorbed onto a filter paper wick, which was placed in a liquid scintillation vial with 10 ml of scintillation cocktail (Scinti Verse II; Fisher Scientific Co., Fairlawn, N.J.). The samples were kept in the dark overnight to reduce chemiluminescence, and the amount of activity recovered (disintegrations per minute) was determined by using a liquid scintillation counter (Packard Tri-Carb 300D) with the appropriate quench curve. A series of $Ba^{14}CO_3$ controls, which had been processed simultaneously, were counted to determine the efficiency of this method for trapping $^{14}CO_2$. The percentage of the radiolabeled substrate respired was calculated as the percentage of the initial label added that was measured as $^{14}CO_2$ after correction for the recovery efficiency. Adaptation was assessed as an increase in the rate of respiration with exposure to the radiolabeled substrate.

Enumeration of specific degraders. Enumeration of specific degraders was based on the most-probable-number (MPN) technique (10) as modified by Somerville et al. (13). Two sets of MPNs were set up simultaneously with the same slurry of Lula 9NN6 soil as the *p*-nitrophenol respiration experiment (see Fig. 3B) to assess changes in the numbers of degraders during adaptation. To determine the MPN before adaptation, radiolabeled *p*-nitrophenol (100 ng per vial) was added to the first set of MPN vials, which were incubated for 22 days. A second set was used to assess the MPN after adaptation and initially incubated with unlabeled *p*-nitrophenol at two concentrations, 40 and 200 ng per vial. After adaptation had been observed in the respiration experiment (day 35), radiolabeled *p*-nitrophenol (100 ng per vial) was added to all of the vials, which were allowed to incubate for an additional 26 days.

For both sets of MPNs, the same amount of the appropriate labeled or unlabeled substrate was added to 40 4-ml tubes which included five sample vials and three control vials for each of five decimal dilutions. The first dilution contained approximately 0.1 g (dry weight) of soil in 1 ml of sterile distilled water. Replicate samples were scored positive if the $^{14}CO_2$ disintegrations per minute produced were greater than or equal to three times the control values for that dilution series. The MPN values were then calculated from these data by using the computer program of Clarke and Owens (3).

RESULTS

Several patterns of mineralization were observed for the different compounds studied (Table 1). No significant mineralization was found for chlorobenzene (27 and 315 ng/g) or 1,2,4-trichlorobenzene (78 and 434 ng/g) after 8 months of incubation. The greatest percent mineralized for either of these compounds was less than 0.3% of the initial label added. Some loss of the compounds did occur, yet after 100 days approximately 50% of the initial label added was still in solution. Thus volatilization did not account for the lack of mineralization of these compounds.

Unlike the chlorobenzenes, the percent mineralized increased slowly and linearly with time for *m*-cresol at 39 ng/g, for *m*-aminophenol 54 ng/g (data not shown) and 559 ng/g, and for aniline at 13 and 224 ng/g (Fig. 1, Table 1). Little mineralization was observed for *m*-cresol at 788 ng/g, perhaps because of toxicity at higher concentrations. No adaptation period was observed during the incubation for any of these compounds, although mineralization accounted for only 5 to 15% of the initial radiolabel added. For *m*-aminophenol and aniline, a slightly greater percentage of the initial label added was mineralized at the lower concentration, but generally concentration did not strongly influence the percent mineralized.

Phenol, *p*-chlorophenol, and ethylene dibromide (EDB) were mineralized much more rapidly and to a greater extent than *m*-cresol, *m*-aminophenol, or aniline, approximately 30% versus 10%, respectively (Fig. 2, Table 1). No adaptation period appears to be required for these compounds, and significant degradation was observed immediately. The first time point taken (day 5) accounted for approximately 25 and 35% of the initial phenol added at 9 and 461 ng/g (data not shown), respectively, 15% of the initial *p*-chlorophenol added (day 3), and 10 to 15% of the initial EDB added (day 7). After the initial time point, identical percents mineralized were seen at the two phenol concentrations tested, and the percent mineralized stayed essentially constant throughout the incubation period. A slightly greater percent of EDB was mineralized at the lower concentration, however.

In contrast to the above results, adaptation to *p*-nitrophenol was evident. In most cases a distinct lag period was observed during which no mineralization was measured, followed by a rapid increase in mineralization. Only the lowest concentration used (14 ng/g) did not show a lag period or significant mineralization (soil 9MM2). Distinct differences in the length of the adaptation period were seen in three different samples of the same layer in Lula soils 9MM2, 9NN6, and 9NN7 (Fig. 3, Table 1). The adaptation period lasted approximately 5 to 6 weeks in the first case, 2 to 3 weeks in the second case, and 1 to 2 weeks in the third case. The maximum percent mineralized however, was approximately the same for the four highest concentrations used (50 to 60%), which was much greater than for any of the other compounds studied.

No degraders were detected by the ^{14}C MPN technique in the Lula 9NN6 soil which was sacrificed after 22 days

TABLE 1. Summary of responses of subsurface soil microbes to xenobiotic compounds

Response	Substrate	Lula soil sample	Substrate concn (ng/g of soil)	% Mineralized day^{-1}	r^2	Maximum % respired
No adaptation observed						
No metabolism	Chlorobenzene	9NN7	27			<0.3
	Chlorobenzene	9NN7	315			<0.3
	1,2,4-Trichlorobenzene	9NN7	78			<0.2
	1,2,4-Trichlorobenzene	9NN7	434			<0.2
Metabolism						
Linear response	*m*-Cresol	9LL7	39	0.07	0.75	10
	m-Aminophenol	9LL7	54	0.07	0.74	15
	m-Aminophenol	9LL7	559	0.04	0.83	10
	Aniline	9HH11	13	0.17	0.83	15
	Aniline	9HH11	224	0.06	0.92	5
Rapid response	Phenol	9LL7	9	2.56	0.83	35
	Phenol	9LL7	461	2.61	0.64	35
	p-Chlorophenol	9LL7	34	0.47	0.65	35
	EDB	9LL7	14	1.11	0.75	25
	EDB	9LL7	166	0.93	0.91	20
Adaptation period observed						
Adaptation after:						
35 to 42 days	*p*-Nitrophenol	9MM2	529	2.11	0.72	50
10 to 21 days	*p*-Nitrophenol	9NN6	31	0.72	0.87	35
10 to 21 days	*p*-Nitrophenol	9NN6	196	2.95	0.87	60
10 to 21 days	*p*-Nitrophenol	9NN6	452	2.79	0.99	60
7 to 14 days	*p*-Nitrophenol	9NN7	485	2.03	0.96	50

incubation, before the observation of adaptation (Fig. 3B). After adaptation (35 days) 1,091 degraders per g (95% confidence interval, 446 to 2662) were detected in the vials preexposed to 40 ng/g, and 6,450 degraders per g (95% confidence interval, 2,897 to 14,362) in the vials preexposed to 200 ng/g per vial. Thus, there was not only a marked increase in the number of *p*-nitrophenol degraders in the adapted versus nonadapted community, but also an effect of preexposure concentration.

DISCUSSION

A summary of the observed patterns of biodegradation exhibited and the degradation rates measured is presented in Table 1. The Lula aquifer solids microbial community was capable of degrading a wide variety of xenobiotic compounds. The time frame of degradation varied from days for phenol, *p*-chlorophenol, and EDB, to weeks or months for *m*-cresol, *m*-aminophenol, aniline, and *p*-nitrophenol, and to perhaps years for the chlorinated benzenes.

The first category of response observed included the chlorinated benzenes chlorobenzene and 1,2,4-trichlorobenzene, neither of which was significantly degraded at either concentration tested over many months of incubation. It seems clear that if adaptation to these compounds occurs in nature it may require long time periods. It was not possible to calculate degradation rates for these two compounds. Incubation times longer than 6 months may be needed for adaptation and biodegradation; alternatively, the microbial community of the fine sand portion of the aquifer may simply not contain members able to metabolize these compounds. Also, any biotransformation products or cellular incorporation which may have occurred were not detected by our analytical technique, and therefore the measured mineralization underestimated the amount metabolized.

The second category demonstrated two distinct responses and included compounds which were degraded but for which no adaptation period was observed (Table 1). The first response observed with *m*-cresol, *m*-aminophenol, and aniline was an initially slow rate of degradation which was maintained throughout the incubation period, giving a linear increase in percent mineralized with time. Mineralization rates calculated from linear regression of all replicates for all data points were low, ranging from 0.04 to 0.18% per day with r^2 values ranging from 0.743 to 0.924. The percent degraded per day for the lower concentrations (<100 ng/g) of these compounds was generally twice that of the higher concentrations (>200 ng/g).

The second type response in this group, observed with phenol, *p*-chlorophenol, and EDB was an initially rapid rate of mineralization, which leveled off such that a maximum percent respired was reached, normally within weeks. Initial degradation rates were calculated from linear regression of all replicates of data points from only the initial linear portion of percent respired-versus-time figures. All rates were greater than the previous group, 0.47% per day for *p*-chlorophenol, 2.6% per day for phenol, and approximately 1.0% per day for EDB. It appears that the community is already adapted to the utilization of these materials.

With the exception of the high concentration of *m*-cresol, for which little mineralization was measured, and for some substrates that had a slightly higher proportion of the compound metabolized at the lower concentration, biodegradation was not influenced by the amount of initial substrate added. The community appears to respond to low part-per-billion concentrations, which is usually considered to be below the level that supports an active community of degraders. It has been shown previously, in other environments, that very low concentrations can lead to an adapted community (12).

The third category included compounds which were degraded and for which an adaptation period was required. Of the compounds studied, only *p*-nitrophenol fell within this group. A greater percent of the *p*-nitrophenol was mineralized at the higher concentrations and thus a significantly greater amount. Degradation rates were calculated from

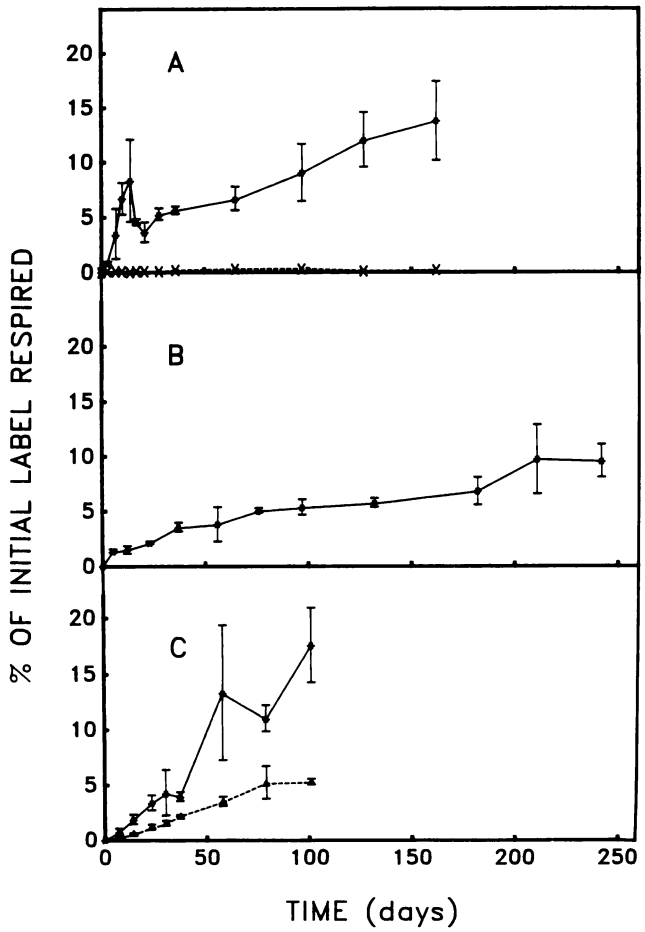

FIG. 1. Percent respired over time for (A) *m*-cresol at 39 ng/g (—*—) and 788 ng/g (--×--), ± standard deviation (Lula soil 9LL7); (B) *m*-aminophenol at 559 ng/g, ± standard deviation (Lula soil 9LL7); and (C) aniline at 13 ng/g (—*—) and 224 ng/g (--▲--), ± standard deviation (Lula soil 9HH11).

replicates of data points after the lag phase, at the onset of degradation. Rates were higher than for other compounds studied, 2 to 3% per day for the higher concentrations (200 to 500 ng/g) and 0.7% per day at 31 ng/g.

A threefold difference in the length of the adaptation period was seen for *p*-nitrophenol with three Lula aquifer samples which came from the same soil core and the same portion of the soil profile. The maximum percent of *p*-nitrophenol ultimately degraded, however, was not affected by the soil used. Other workers also have shown variability within subsurface soil profiles (4, 6, 18, 20). This suggests that there is a great deal of intrasite variability, including spatial heterogeneity and bacterial patchiness associated with soil particles in this subsurface environment.

Currently it is believed that the adaptation process may involve one or a combination of (i) induction or derepression of enzymes specific for degradation pathways of a particular compound, (ii) a random mutation in which new metabolic capabilities are produced which allow degradation that was not previously possible, or (iii) an increase in the number of organisms in the degrading population (14). At sites where the community has been exposed to known pollutants for long periods, it is clear that adaptation can occur (19). Defined functionally as an increase in rate of degradation with exposure to a compound, adaptation was evident for only *p*-nitrophenol among the nine compounds tested, although adaptation may occur under conditions different than those used in this study. The other compounds were mineralized, and adaptation periods may have occurred before the initial sampling time and gone undetected for the compounds initially degraded rapidly, such as phenol, *p*-chlorophenol, and EDB. Short time frames, however (less than 1 week), may be considered essentially instantaneous in this pristine aquifer environment.

Wyndham (21) attributed the adaptation to aniline by *Acinetobacter calcoaceticus* to a mutation in the natural population with the involvement of a plasmid carried gene. He found adaptation to aniline in periods of 24 h for microbes in continuous culture taken from the disturbed sediment-water interface of a river bed. He suggested that parent and mutant populations with different activities for assimilation of aniline were both selected, and that the population dynamics of the parent and mutant may control the length of the adaptation period and ultimate rates of degradation.

Spain et al. (14) and Spain and Van Veld (15) found adaptation to *p*-nitrophenol in river water-sediment ecocores. The length of the adaptation period observed in our study was generally 5 to 10 times longer than those deter-

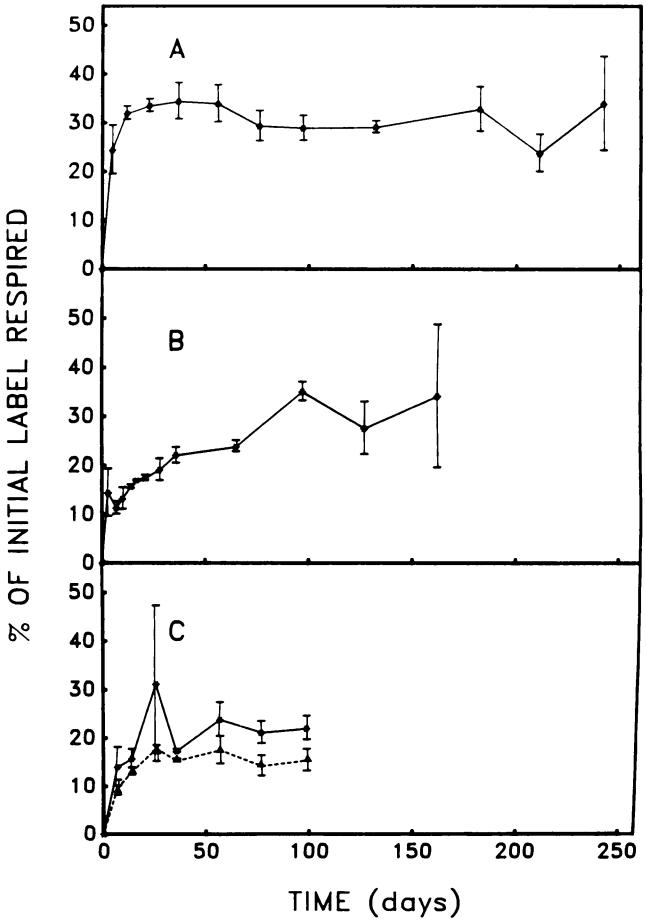

FIG. 2. Percent respired over time for (A) phenol at 9 ng/g, ± standard deviation (Lula soil 9LL7); (B) *p*-chlorophenol at 34 ng/g, ± standard deviation (Lula soil 9LL7); and (C) EDB at 14 ng/g (—*—) and 150 ng/g (--▲--), ± standard deviation (Lula soil 9LL7).

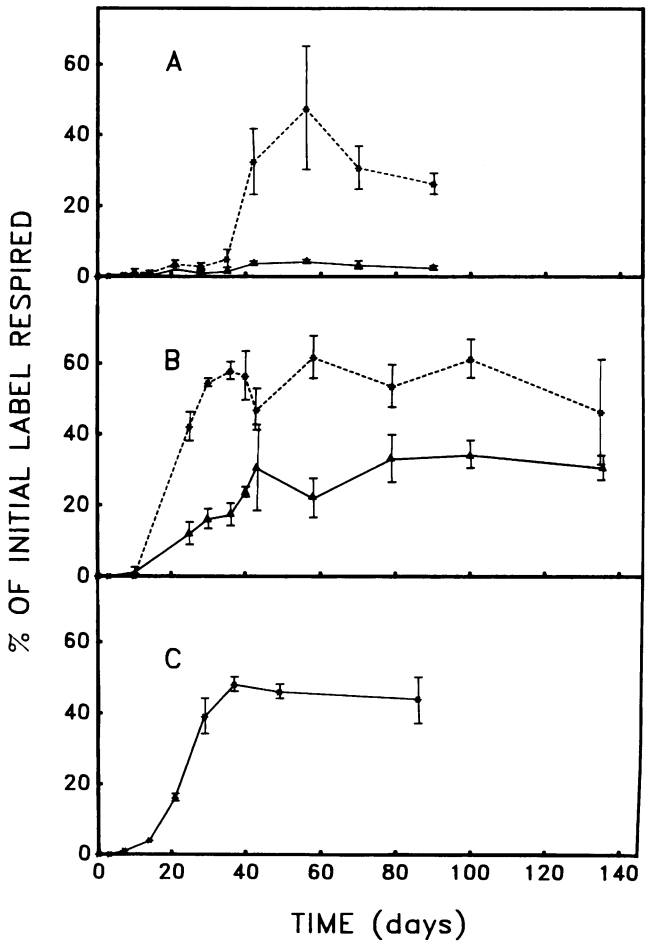

FIG. 3. Percent *p*-nitrophenol respired for (A) Lula soil 9MM2 at 14 ng/g (—▲—) and 529 ng/g (---*---), ± standard deviation; (B) Lula soil 9NN6 at 31 ng/g (—▲—) and 452 ng/g (--*--), ± standard deviation; and (C) Lula soil 9NN7 at 485 ng/g, ± standard deviation.

mined for this compound by Spain et al. (14) and Spain and Van Veld (15) in their ecocores, 4 and 3 days, respectively. They also found that the concentration of *p*-nitrophenol used influenced the maximum percent respired. Spain et al. (16) found that an increase in the *p*-nitrophenol-degrading population correlated well with increases in biodegradation rates and that the total bacterial population did not change during the adaptation process. They suggested that adaptation was not due to a random mutation or recruitment of a plasmid gene but instead was the result of selection of organisms able to grow at the expense of the substrate (16).

Our data showed that the number of degraders increased during the adaptation period. However, it is unlikely that the adaptation period was due solely to a continuous growth of a small population of degraders, because the adaptation period was very long and the onset of degradation was, in some instances, very rapid after this period. In addition, in some cases the concentrations that elicited adaptation were quite low relative to what is commonly thought to be necessary to support growth. During a long adaptation period it seems possible that a change in the population may occur which selects for a certain portion of the community and allows the number of degraders to increase over a relatively short time with a subsequent rapid increase in the percent mineralized.

It is difficult to extrapolate results from a laboratory study, such as that reported here, to field sites subject to spills of xenobiotic chemicals. Our results indicate that the pristine microbial community is capable of biodegrading xenobiotic compounds, sometimes quite rapidly, and that the community is preadapted to some compounds. In field situations, however, the community has to deal with physical, chemical, and hydrogeological constraints that do not exist in laboratory incubations. Further research is clearly needed to relate laboratory results to field situations.

The steps involved in the adaptation process were not identified in this study. A great deal more research is needed to understand microbial population interactions with both the compound and each other during exposure to organic chemicals. Although it is encouraging that the aquifer solids microbes in this study were able to degrade a variety of compounds, results with a greater variety of chemicals and soil types must be generated before generalizations can be made concerning biodegradation capabilities. Finally, to understand the adaptation process in the subsurface system it is essential to identify the mechanism of the process and assess the microbial population response during the adaptation period.

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