Effect of Varying the Rate of Partitioning of Phenanthrene in Nonaqueous-Phase Liquids on Biodegradation in Soil Slurries

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A study was conducted to determine the influence of varying the rates of partitioning of phenanthrene from nonaqueous-phase liquids to water on its biodegradation. Partitioning rates from dibutyl phthalate and 2,2,4,6,8,8-heptamethylnonane were rapid in slurries of soil or aquifer solids that were shaken and were affected by the identity and volume of the nonaqueous-phase liquid. Concentrations of the surfactant Alfonic 810-60 that increased partitioning inhibited biodegradation. The rates of mass transfer from the phthalate to water were not influenced by the identity of the environmental sample. Although the rate of mass transfer of phenanthrene did not limit its mineralization by microorganisms in the soil or aquifer solids, treatments that increased the rate of partitioning enhanced biodegradation, presumably because the treatment overcame some other factor that limited degradation of the hydrocarbon.

Introduction

Hydrophobic pollutants at many hazardous waste sites or in subsoils contaminated by leaking underground storage tanks are chiefly present in nonaqueous-phase liquids (NAPLs) rather than in the water phase. For these pollutants to be subject to biodegradation or bioremediation, the compounds presumably must partition from the NAPL to the aqueous phase, in which are found the potentially active microorganisms. When those populations become large, the rate of mass transfer from the NAPL to the water might limit the rate of biodegradation or bioremediation. Because this limitation by the kinetics of partitioning would thus constrain the rate of destruction of hydrophobic pollutants, it is important to establish whether factors that affect partitioning also influence biodegradation by natural microfloras.

A number of studies have shown the slow biodegradation by microorganisms indigenous to soil of organic compounds initially present in such NAPLs as creosote (1), coal

tar (2), and pure organic solvents (3). This slow degradation of components of NAPLs may be increased by slurrying the contaminated soil, by introduction of specialized microorganisms, and by addition of inorganic nutrients and synthetic surfactants (4-6). The stimulation of microbial activity by slurrying suspensions of soil or by surfactants may result from an enhancement in the rate of partitioning of the microbial substrate from NAPL to water, although other explanations are possible, such as an increase in the interfacial area and thus more extensive microbial colonization at the surface of the NAPL. Differences have been noted in the rates of transformation in soil slurries of biodegradable compounds present initially in different NAPLs, which may result from dissimilar partitioning rates or from interspecific microbial competition arising because of biodegradation of the NAPL (4).

Because of the potential importance of bioremediation for the decontamination of sites containing pollutants sequestered in NAPLs, a study was conducted to determine whether enhancement of partitioning to water of compounds in NAPLs would lead to a parallel increase in mineralization by microorganisms in soil slurries. Work in this laboratory has recently shown that biodegradation by individual microorganisms may be faster than the measured rates of partitioning (7). Phenanthrene was used as the test compound because it is a representative of the polycyclic aromatic hydrocarbons that are present in many NAPL-contaminated sites, and heptamethylnonane and dibutyl phthalate were selected as NAPLs because they are relatively nontoxic to microorganisms in soil degrading the test compound and because hydrophobic molecules initially in this phthalate are degraded slowly (4, 5).

Materials and Methods

2,2,4,4,6,8,8-Heptamethylnonane was purchased from Aldrich Chemical Co. (Milwaukee, WI), and dibutyl phthalate and phenanthrene were obtained from Sigma Chemical Co. (St. Louis, MO). Alfonic 810-60 [$C_nH_{2n+1}(OCH_2CH_2)_xOH$; $n = 8$–14, average $x = 4.5$], with a critical micelle concentration of 275 ± 25 $\mu g/mL$, was provided by Vista Chemical Co. (Houston, TX). [9-^{14}C]Phenanthrene (8.3 mCi/mmol) was obtained from Sigma Chemical Co.

Lima loam (pH 7.2, 7.8% organic matter, 21% moisture) from Aurora, NY, was used without drying. Edwards muck (pH 6.9%, 33% organic matter) was obtained from Montezuma, NY, and was dried in air to 6.7% moisture. A sample of aquifer solids (0.4% organic matter, 6.38% moisture) was obtained from Freeville, NY. The soils and aquifer solids were passed through a 2-mm sieve before use.

Mineralization of radiolabeled phenanthrene was determined in duplicate flasks at 22 ± 3 °C by measuring the formation of $^{14}CO_2$. Unless otherwise stated, 50 g of soil or aquifer solids was placed in 250-mL baffled flasks (Bellco Glass, Vineland, NJ), and 0.5 mL of a NAPL containing 50 000–80 000 dpm of radiolabeled phenanthrene and enough unlabeled phenanthrene to give 100 $\mu g/mL$ of NAPL was added to the soil and mixed for 1 min. A sterile solution containing 900 mg of KH_2PO_4, 100 mg of K_2HPO_4, 100 mg of NH_4NO_3, 100 mg of $MgSO_4 \cdot 7H_2O$, 100 mg of $CaCl_2 \cdot H_2O$, 10 mg of $FeCl_3$, and 2.0 μg each of $Na_2MoO_4 \cdot 7H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $CuSO_4 \cdot 5H_2O$

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TABLE 1

Effect of Volume and Identity of NAPL on the Partitioning and Mineralization of Phenanthrene

NAPL	NAPL vol (mL)	partitioning		mineralization		
		rate (ng mL^{-1} h^{-1})	equilibrium concn (ng/mL)	rate (ng mL^{-1} h^{-1})	time period (h)[a]	extent (%)[b]
heptamethylnonane	0.5	15.7A[c]	1.18A	7.81A	0–8.5 (2)	20.3A
heptamethylnonane:dibutyl phthalate (1:1)	0.5	13.4B	1.03A	4.81B	0–15.5 (4)	17.6A
dibutyl phthalate	0.5	10.9C	1.02A	4.15B	0–17.7 (5)	18.4A
dibutyl phthalate	2.5	6.80D	0.63B	1.17C	0–27.5 (9)	9.2B
dibutyl phthalate	5.0	5.90D	0.53B	0.68C	0–27.5 (4)	5.1B

[a] The values in parentheses are the number of points used to calculate mineralization rates. [b] Percent of substrate mineralized in 92 h. [c] Values in a column followed by the same capital letter are not significantly different ($P = 0.05$).

per liter of distilled water was added, 50 mL to Lima loam or aquifer solids, and 67 mL to Edwards muck. In tests of biodegradation in particle-free media, 60 mL of the salts solution was used. The slurries and particle-free media were then inoculated with a bacterium capable of using phenanthrene as a sole carbon and energy source for growth. The bacterium (strain M1) was provided by Dr. R. A. Efroymson of this laboratory. It was grown in 2.0-L flasks containing 1.0 L of a medium composed of 50 mg of phenanthrene and the inorganic salts solution. The flasks were incubated at 30 °C on a rotary shaker operating at 100 rpm. Cultures in the early stationary phase were passed through a 40-μm pore-size glass frit, and the filtrate was centrifuged at 8000g for 10 min at 4 °C. The pellets were washed twice and resuspended in salts solution to give a final density of approximately 2×10^9 cells/mL, and 1.0 mL of this suspension was used for inoculation.

The flasks were then closed with Teflon-lined stoppers, from which an 8-mL vial containing 2 mL of 0.5 M NaOH was suspended to trap $^{14}CO_2$. Unless otherwise stated, the flasks were incubated on an orbital shaker operating at 100 rpm. Periodically, the NaOH solution was removed and replaced by fresh alkali. The NaOH solution was mixed with Liquiscint scintillation fluid (National Diagnostics, Highland Park, NJ), and the radioactivity was determined with a liquid scintillation counter (Model LS7500; Beckman Instruments, Inc., Fullerton, CA). Mineralization rates were calculated as the slope of the regression line in each of two determinations.

Measurements of partitioning were carried out in triplicate under the same conditions used to determine mineralization except for the absence of an inoculum. The test period was usually 5 h. Although the soils were not sterilized, tests with uninoculated slurries showed that phenanthrene mineralization by indigenous microorganisms was not detectable in less than 100 h. The NAPL contained 1 100 000–1 300 000 dpm of radiolabeled phenanthrene and enough unlabeled phenanthrene to give 100 μg/mL of NAPL. After adding the salts solution to the mixture of soil and NAPL, 6.0-mL subsamples of the slurries were removed at intervals with a pipet having a large opening at the tip. The subsamples were clarified by centrifugation at 3000g for 1 min, and the supernatant was passed through a 0.45-μm pore-size nylon syringe filter (MSI, Westboro, MA). The filtrate was mixed with 10-mL portions of scintillation fluid in 20-mL scintillation vials, and the radioactivity was determined. In tests with particle-free media, the samples were not centrifuged. An F-test was used to analyze statistically the differences between treatments, and a t-test was used for comparisons of partitioning rates.

Results

The rates of partitioning of phenanthrene from the NAPL to the water phase in the soil slurries were rapid in the first 3 min, and the rates declined thereafter. Hence, the maximum partitioning rates were estimated from the first sample taken, which was at 3 min. It is thus possible that the actual maximum rates were somewhat higher. Equilibrium was reached within 60 min, and the concentrations at equilibrium were calculated from at least three determinations of phenanthrene in the aqueous phase after 60 min.

A study was conducted to determine how the volume and identity of NAPL would affect the partitioning of phenanthrene and the influence of these changes on biodegradation. For this purpose, 50 μg of the substrate was dissolved in the NAPLs, which were incubated in suspensions of Lima loam that were shaken at 100 rpm. The rate of partitioning and the equilibrium concentration of phenanthrene declined as the volume of dibutyl phthalate increased (Table 1). The rate was greater if the substrate was initially in heptamethylnonane or a 1:1 heptamethylnonane:phthalate solution than in the phthalate alone.

Mineralization was determined using the same conditions as partitioning. The rates of biodegradation in the slurries were affected in the same way as partitioning by NAPL volume and identity, namely, diminished by increasing the volume of the liquid and greater in heptamethylnonane (Table 1). In each instance, the rate of partitioning was statistically higher ($P = 0.05$) than the rate of mineralization, the differences ranging from 2- to 9-fold. Thus, mass transfer from NAPL to water did not limit microbial activity.

The possible effect of shaking and means of introducing the NAPL into the Lima loam slurries on partitioning was also investigated. The NAPL was dibutyl phthalate containing 100 μg of phenanthrene/mL. In some instances, the NAPL was added to the soil and immediately mixed with a spatula for 1 min, and the salts solution was then added to the NAPL–soil mixture to form the slurry (designated as initially mixed). In other instances, the salts solution was mixed vigorously for a few seconds with the soil in 250-mL flasks, and then the NAPL was added to the slurry (designated as NAPL suspended). Then, the flasks were either placed on a shaker operating at 100 rpm (designated as shaken) or allowed to stand without further shaking.

Shaking the soil slurries and different means of introducing the NAPL into the slurries influenced the partitioning rate and the phenanthrene concentration in the water phase at equilibrium. Thus, shaking the slurries increased both

TABLE 2

Effect of Mixing NAPL with Soil and Shaking Soil Slurries on the Partitioning and Mineralization of Phenanthrene Initially Present in Dibutyl Phthalate

treatment	partitioning		mineralization		
	rate (ng mL^{-1} h^{-1})	equilibrium concn (ng/mL)	rate (ng mL^{-1} h^{-1})	time period (h)[a]	extent (%)[b]
initially mixed					
shaken	10.9A[c]	1.02A	4.77A	0−9.5 (4)	13.5AB
			4.69A[d]	0−16.5 (5)	14.5AD[e]
not shaken	5.53B	0.62B	1.48B[d]	0−24 (8)	10.7B[e]
not initially mixed					
shaken	6.27B	0.64B	1.47B	0−9.5 (4)	3.5C
NAPL suspended, shaken	9.87A	0.86AB	3.13B	4−51 (10)	24.0D

[a] The values in parentheses are the number of points used to calculate mineralization rates. [b] Percent of substrate mineralized in 74 h. [c] Values in a column followed by the same capital letter are not significantly different. [d] Test of mineralization performed in a separate experiment. [e] Time interval of 138 h.

TABLE 3

Effect of Soil and Aquifer Solids on Partitioning to the Aqueous Phase and Mineralization of Phenanthrene Initially Present in Dibutyl Phthalate at 100 μg/mL

environmental sample	partitioning		mineralization		
	rate (ng mL^{-1} h^{-1})	equilibrium concn (ng/mL)	rate (ng mL^{-1} h^{-1})	time period (h)[a]	extent (%)[b]
none	1.17A[c]	1.20A	7.11A	0−28 (9)	50.2A
Lima loam	10.9B	1.02A	4.69B	0−16.5 (5)	19.4C
Edwards muck	10.1B	0.56B	2.02C	0−28 (9)	11.6C
aquifer solids	12.2B	1.14A	4.47B	0−16.5(5)	30.5B

[a] The values in parentheses are the number of points used to calculate mineralization rates. [b] Percent of substrate mineralized in 139 h. [c] Values in a column followed by the same capital letter are not significantly different ($P = 0.05$).

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the rate of partitioning, as expected, and the amount of phenanthrene in the aqueous phase at equilibrium (Table 2). However, the partitioning rate and the concentration in water at equilibrium were not significantly different if the NAPL was added directly to the whole slurry or was added to and mixed with the soil before addition of the salts solution. The rates of biodegradation were statistically slower ($P = 0.05$) than the measured rates of partitioning under all four of the foregoing conditions, again demonstrating that more substrate was entering the water than was being metabolized by the microorganisms. However, treatments that promoted partitioning enhanced mineralization even though the rate of mass transfer was not limiting the microorganisms, i.e., the microorganisms were stimulated for reasons other than the increases in availability of the substrate.

Partitioning of phenanthrene from 0.5 mL of dibutyl phthalate to the aqueous phase in the absence of soil was measured under the same conditions used for soil slurries. The rate of partitioning of the hydrocarbon, which was added to the NAPL to give 100 μg/mL, was linear but slow. The rate was appreciably faster when soil was present, possibly because the mechanical action of the soil particles reduced the droplet size and thus increased the NAPL−water interfacial area. The partitioning rates in the presence of the two soils and aquifer solids were not significantly different, but they were all higher than in the absence of soil (Table 3). The last value was calculated using six points on the linear portion of the partitioning curve. However, the concentration in the aqueous phase at equilibrium was much lower in the presence of the sample of muck, which probably results from extensive sorption of the compound by this organic soil. The rates of mineralization were significantly less ($P = 0.05$) than the rates of abiotic partitioning in the presence of soil or aquifer solids. In contrast, the rate of degradation by bacterium M1 significantly exceeded the measured partitioning rate in the absence of the soil or aquifer solids.

A study was also conducted to determine the influence of a surfactant (Alfonic 810-60) on partitioning and biodegradation. The addition of 0.05% Alfonic 810-60 to the slurry of Lima loam did not affect the partitioning rate or the concentration of phenanthrene in water at equilibrium (Table 4). The surfactant at 0.10% increased both the rate and the aqueous concentration at equilibrium. However, this increase in partitioning rate associated with the presence of the surfactant at this concentration was not correlated with a higher mineralization rate, rather the transformation was inhibited. Furthermore, although partitioning was especially rapid in the presence of 1.0% Alfonic 810-60, the microbial transformation was completely suppressed.

Discussion

The data show the influence of a number of variables on the rates of biodegradation of compounds initially present in NAPLs. Thus, mineralization is affected by the identity and volume of the NAPL, the mixing of the soil slurries with the NAPL containing the test compound, and the identity of the environmental sample. These observations are similar to those in other studies (4, 5). However, the present study was designed to evaluate whether the response to these treatments resulted solely from effects associated with partitioning rates, which was not part of the earlier investigations.

The measured rates of partitioning in these well-mixed slurries containing a NAPL and soil were high, and equilibrium was achieved in several minutes. The initial mixing of the soil with the NAPL and the subsequent shaking resulted in a large NAPL−water interfacial area that probably

TABLE 4

Effect of Alfonic 810—60 on the Partitioning and Mineralization of Phenanthrene Initially Present at 100 μg/mL of Dibutyl Phthalate in Slurries of Lima Loam

surfactant concn (%), v/v	partitioning		mineralization		
	rate (ng mL^{-1} h^{-1})	equilibrium concn (ng/mL)	rate (ng mL^{-1} h^{-1})[a]	time period (h)[a]	extent (%)[b]
0.00	10.9A[c]	1.02A	5.50A	0–10.5 (5)	18.2A
0.05	10.3A	0.97A	4.04AB	0–10.5 (5)	14.1B
0.10	25.9B	1.99B	2.96B	0–10.5 (5)	10.0C
1.0	1880C	3.13C[d]	0.00C	0–350 (24)	0.0D

[a] The values in parentheses are the number of points used to calculate mineralization rates. [b] Percent of substrate mineralized in 145 h. [c] Values in a column followed by the same capital letter are not significantly different. [d] Equilibrium concentration is the average of measurements made after 180 min, although two earlier measurements gave higher values.

was responsible for the rapid partitioning. These rates were enhanced by shaking, decreasing NAPL volumes, and increasing Alfonic 810-60 concentrations. Except in the absence of the soil or aquifer solids, in which case mineralization was faster than the measured rate of partitioning, the partitioning rates exceeded the mineralization rates, suggesting that mass transfer of substrate from NAPL to water did not limit biodegradation. The finding that a bacterium metabolized phenanthrene more rapidly in the absence of soil or aquifer solids than the measured partitioning rate has also been observed in tests with slow shaking and constant NAPL—water interfacial area (7). This more rapid degradation than the measured rate of partitioning could result from the microbial production of surface-active agents, as has been reported for the use of pure alkanes by bacteria (8, 9), or from the adhesion of the bacteria to the NAPL—water interface, as has been shown for *Arthrobacter sp.* degrading hexadecane and naphthalene dissolved in NAPLs (10, 11). Nevertheless, treatments inducing more rapid NAPL to water mass transfer in soil slurries led to faster biodegradation.

The concentration of O_2 may limit the rate of biodegradation of hydrocarbons in saturated soils (12, 13). Considering the solubility of O_2 in water, enough O_2 should have been present in solution for the complete oxidation to CO_2 of the 50 μg of phenanthrene initially present in the soil slurries, but O_2 would also be consumed in the decomposition of the soil organic matter. Thus, the increase in the rate of biological transformation that correlated with a decrease in NAPL volume and an increase with shaking speed may result from overcoming the O_2 limitation.

Two abiotic processes probably affected the phenanthrene concentration in the aqueous phase: partitioning from NAPL to water and simultaneous sorption by soil particles of the hydrocarbon in the water. Organic matter content is the major factor in soil determining the extent of sorption of hydrophobic compounds (14). The partitioning rates in slurries of soils with different contents of organic matter were statistically different, suggesting that the rate of partitioning from the NAPL was rapid compared to the initial rate of sorption. However, the lower aqueous concentration of phenanthrene at equilibrium in suspensions of the muck probably resulted from sorption to soil particles of the compound.

Although mass transfer from NAPL to the water did not limit biodegradation of phenanthrene, the data show that treatments that increased partitioning promoted microbial activity. This presumably results from the overcoming of some other limitation to biodegradation. Determining the identities of these limitations is thus essential in order to devise practical technologies for the bioremediation of pollutants in NAPLs.

Acknowledgments

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