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Effect of Aging of Chemicals in Soil on Their Biodegradability and Extractability

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A study was conducted to determine whether the time that a compound remains in a soil affects its biodegradability and the ease of its extraction. Phenanthrene and 4-nitrophenol were aged in sterilized loam and muck, and bacteria able to degrade the compounds were then added to the soils. Increasingly smaller amounts of phenanthrene in the muck and 4-nitrophenol in both soils were mineralized with increasing duration of aging. Aging also increased the resistance of phenanthrene to biodegradation in nutrient-amended aquifer sand. The rate of mineralization of the two compounds in both soils declined with increasing periods of aging. The amount of phenanthrene and 4-nitrophenol added to sterile soils that was recovered by butanol extraction declined with duration of aging, but subsequent Soxhlet extraction recovered phenanthrene from the loam but not the muck. The extents of mineralization of phenanthrene previously incubated for up to 27 days with soluble or insoluble organic matter from the muck were similar. Less aged than freshly added phenanthrene was biodegraded if aggregates in the muck were sonically disrupted. The data show that phenanthrene and 4-nitrophenol added to soil become increasingly more resistant with time to biodegradation and extraction.

Introduction

Sorption of organic chemicals to soils and sediments often entails an initially rapid and reversible process followed by a period of slow sorption occurring over weeks, months, or perhaps years, and the slow sorption leads to a chemical fraction that then resists desorption (*1*−*3*). The desorption-resistant fraction is often persistent in natural environments. Polychlorinated biphenyls (*4*, *5*), pesticides (*6*, *7*), and halogenated aliphatic hydrocarbons (*8*, *9*) have been found to exist in soils and sediments partially in a strongly sorbed, resistant form, and the size of this desorption-resistant fraction may increase dramatically with time as the chemical remains in the soil or sediment. For example, the quantity of trichloroethylene resistant to desorption from a silty-clay soil increased from 10 to 45% of that initially added after 2.5 and 15.5 months, respectively (*10*). Similarly, appreciably more picloram was desorbed from a sandy loam just amended with the compound than from the soil amended 200 days earlier (*11*). The processes by which organic compounds become increasingly desorption-resistant in soils and sediments, sometimes termed chemical aging, are poorly understood.

Several mechanisms have been described for the aging of chemicals in soils and sediments. The term aging does not include reactions that alter the structure of the molecule; for example, polymerization or covalent binding to humic substances. Partitioning into humic matter may be important in the sorption of nonionic organic chemicals and could be a mechanism of their aging in soils and sediments (*12*, *13*). The aged or desorption-resistant fraction of organic compounds may result from the slow diffusion of these molecules within some components of solid organic matter in soils (*14*, *15*). A second hypothesis for aging suggests that chemicals slowly diffuse into and become entrapped within small pores in soil aggregates (*9*). Diffusion of chemicals from these micropores may be retarded by the tortuous path through the pores and by partitioning of the chemical between pore water and organic matter on pore walls (*9*, *15*, *16*). It is also possible that the formation of strong bonds between organic compounds and soil or sediment constituents may account for their resistance. For example, Isaacson and Frink (*17*) proposed that a desorption-resistant fraction of phenol and some substituted phenols in a sediment is partially the result of hydrogen bonding of these compounds to humic materials.

Except for two recent studies of pesticides (*7*, *18*), the importance of aging to the environmental fate of organic compounds is largely unexplored. As a result, a study was initiated to determine the effect of aging time in soils upon the biodegradability and extractability of organic compounds. Phenanthrene and 4-nitrophenol were chosen as test molecules because upon initial addition to soil they are readily biodegradable but differ markedly in hydrophobicity.

Materials and Methods

Environmental Samples. The environmental samples were Lima loam (pH 7.2, 4.0% organic matter) from Aurora, NY; Edwards muck (pH 6.9, 19.3% organic matter) from Montezuma, NY; and an aquifer sand (pH 7.4, 2.3% organic matter) from Freeville, NY. Soil aggregates of 0−4 or 2−4

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mm in diameter were used. The aquifer sand was passed through a 2-mm sieve. The solids were air dried.

Aging of Chemicals. Unlabeled phenanthrene and [9-^{14}C]phenanthrene (either 8.3 or 13.1 mCi/mmol, >98% purity) were purchased from Sigma Chemical Co., St. Louis, MO. [2,6-^{14}C]4-Nitrophenol (12.2 mCi/mmol, 98% purity) was obtained from California Bionuclear Co., Sun Valley, CA. Unlabeled 4-nitrophenol was provided by Eastman Organic Chemicals, Rochester, NY.

Dry sieved soils and sand were sterilized with 2.5 Mrad of γ-irradiation from a ^{60}Co source, and 10-g portions were added to sterilized 50-mL screw cap test tubes. To each tube was added 0.5 mL of a solution containing 100 μg of unlabeled phenanthrene and approximately 1.0×10^5 dpm of [^{14}C]phenanthrene in CH_2Cl_2. The liquid was added dropwise to bring the phenanthrene concentration to 10 μg/g of soil or sand. In some experiments, 5 g of soil was used, and it was amended with 0.5 mL of a solution containing 5×10^4 dpm of [^{14}C]phenanthrene and 50 μg of unlabeled phenanthrene to give a concentration of 10 μg of phenanthrene/g of soil. The tubes were placed in a hood for 1 (soil) or 1.5 h (aquifer sand), and the samples were shaken or, in a few experiments, stirred every 15 min to allow the dichloromethane to evaporate and to ensure thorough mixing of phenanthrene with the soil. Autoclaved deionized water was then added to bring the moisture level of Lima loam and aquifer sand to 20% and of Edwards muck to 40% (w/w). In experiments with 5 g of muck, the soil was stirred after addition of the water; in all other instances, the water was allowed to percolate through the samples without stirring. The tubes were sealed with sterile screw caps fitted with silicone-backed Teflon liners and incubated in the dark at 20 ± 1 °C. Phenanthrene was added at predetermined times to additional tubes by the same method to give soil or aquifer sand with the test compound aged for several periods of time.

A solution (2.0 mL) containing 1.0×10^5 dpm of labeled and 10 or 100 μg of unlabeled 4-nitrophenol in sterile deionized water was added to 10-g samples of sterile Lima loam or Edwards muck to give final concentrations of 1 or 10 μg/g of soil. An additional 2.0 mL of water was added to the muck samples. The samples were incubated in the dark at 20 ± 1 °C in 50-mL test tubes sealed with Teflon-lined screw caps.

To determine whether the soils and aquifer sand remained sterile while the compounds were aging, in many experiments, a sample from each tube was added to plates of Trypticase-soy agar (BBL Microbiology Systems, Cockeysville, MD), which were incubated at 29 °C. No growth appeared on agar inoculated with environmental samples in which phenanthrene was aged. Growth also was not evident in most samples in which 4-nitrophenol was aged, but bacteria appeared on the medium to which a few samples of Lima loam were added. However, when these bacteria were isolated and added to an inorganic salts solution containing 4-nitrophenol, degradation of the compound (as assessed by the disappearance of its yellow color) was not observed.

Mineralization of Chemicals Aged in Sterilized Soils. Soil and aquifer samples containing phenanthrene or 4-nitrophenol aged for various lengths of time were transferred from the tubes to sterile 60-mL glass jars. The samples were inoculated with either *Pseudomonas* strain R (provided by R. A. Efroymson of this laboratory), if the sample contained phenanthrene, or isolate WS-5 (provided by W. S. Steffensen of this laboratory), if the sample contained 4-nitrophenol. The inoculum provided between 10^5 and 10^7 cells/g of soil and sufficient water to bring the moisture level of the soil samples to 95–120% field capacity (55% for Edwards muck and 27% for Lima loam) and the aquifer sand to just above saturation.

Each jar was sealed with a Teflon-lined silicone stopper, through which was placed a 16-gauge steel cannula and a 18-gauge hypodermic needle. A small vial containing 1.5 mL of 0.5 N NaOH, which trapped $^{14}CO_2$ released in mineralization, was suspended by a wire from the cannula. The NaOH in the trap was periodically removed through the cannula and replaced with fresh solution. The alkali was mixed with 4.5 mL of Liquiscint scintillation cocktail (National Diagnostics, Inc., Atlanta, GA), and the radioactivity of the sample was determined with a liquid scintillation counter (Model LS 7500; Beckman Instruments, Inc., Irvine, CA).

Bacteria. *Pseudomonas* strain R was grown at 29 °C in a medium containing phenanthrene in excess of its water solubility and 0.10 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeCl_3$, 0.10 g of $MgSO_4 \cdot 7H_2O$, 0.10 g of NH_4NO_3, 0.20 g of KH_2PO_4, and 0.80 g of K_2HPO_4/L of distilled water. The pH was 7.0. In some instances, the medium contained 0.90 g of KH_2PO_4 and 0.10 g of K_2HPO_4/L to give a pH of 5.7. After 5–7 days, the culture was passed through a 40-μm pore-size glass frit to remove remaining phenanthrene crystals, and the culture was centrifuged at 10400*g* for 12 min. The cells were resuspended in sterile distilled water. Isolate WS-5 was grown at 29 °C in the salts solution supplemented with 20 μg of 4-nitrophenol/mL. After 4 days, the culture was centrifuged, and the cells were resuspended in sterilized distilled water. The number of cells of each bacterium was determined by plating on Trypticase-soy agar.

Extraction. In the initial extraction, 20 or 25 mL of *n*-butanol was added to the tubes containing soil or aquifer sand in which the compounds were aged. The contents of the tubes were thoroughly mixed for 2 min, and the resulting slurry was then passed through Whatman No. 1 filter paper. The test tubes were washed with an additional 10 mL of butanol, which was also passed through the filter paper. The radioactivity in the filtrate was then determined. After butanol extraction, the phenanthrene-amended samples of soil or aquifer solids remaining on the filters were extracted for 8–10 h in a Soxhlet apparatus using dichloromethane as the solvent. The extracts were brought to near dryness using a Buchi Rotavapor evaporation apparatus (Buchler Instruments, Inc., Fort Lee, NJ), and the radioactivity in the extract was measured.

Initially, butanol and Soxhlet extracts of [^{14}C]phenanthrene from Edwards soil were also analyzed by high-pressure liquid chromatography. The Soxhlet extracts were initially passed through 0.20-μm syringe filters (Millex-FG_{13}, Millipore Co., Bedford, MA) to remove particulate matter. A liquid chromatograph (Hewlett-Packard Series 1050, Hewlett-Packard Co., Avondale, PA) fitted with a Spherisorb ODS-2 octadecyl-bonded silica column (Hewlett-Packard; 5μ, 250 × 4 mm) was used with acetonitrile–water (86:14) as the mobile phase at a flow rate of 0.8 mL/min. Phenanthrene was detected by its UV absorbance at 254 nm. The amounts of phenanthrene in the soil extracts as determined by liquid chromatography were the same as determined by measurements of radioactivity. Because all of the radioactivity in the extracts appeared to be phenanthrene, subsequent analysis involved only measurements

of radioactivity. In one experiment, butanol extracts of 4-nitrophenol aged at 10 μg/g of Lima loam were analyzed by HPLC using the silica column. The mobile phase was a solution containing 2.92 g of Na_2CO_3 and 1.89 g of $NaHCO_3$/L of water (pH 10.0) and methanol (70:30) at a flow rate of 0.7 mL/min. After adding a small quantity of the carbonate solution to raise the pH of each sample, 4-nitrophenol was detected by its absorbance at 402 nm. The quantities of extracted 4-nitrophenol determined by HPLC were the same as those determined by measuring radioactivity, and all other analyses were based only on measurements of radioactivity.

Aging of Phenanthrene in Humin and Soluble Organic Matter. Sterilized Edwards muck (30 g dry wt) was extracted for 43 h at room temperature with 300 mL of 0.1 N NaOH under N_2 to give soluble organic matter and insoluble humin (*19*). The samples were kept on a rotary shaker operating at 110 rpm during the extraction. The suspension was centrifuged at 16000*g* for 10 min, the supernatant was decanted, and the solids were resuspended in 100 mL of distilled water. The centrifugation was repeated, and the two supernatants were combined. The remaining solids were suspended in 300 mL of distilled water, and this humin fraction and the soluble organic matter (in the supernatant) were brought to pH 6.0 with 2 N HCl and autoclaved for 30 min.

Portions (25 mL) of these two fractions were placed in sterile, acid-washed 125-mL glass bottles, and radiolabeled phenanthrene in 20 μL of dichloromethane was added to each bottle to give 1.0 μg/mL and approximately 1.5×10^5 dpm of [^{14}C]phenanthrene. The bottles were sealed with Teflon-lined screw caps and incubated in the dark. Phenanthrene was added periodically to the samples so that when *Pseudomonas* strain R was added in each bottle to give 6.3×10^6 cells/mL, phenanthrene had been aged in triplicate samples of each type for 0, 14, and 27 days. A sterilized test tube containing 2 mL of 0.5 N NaOH was placed upright within each bottle to trap $^{14}CO_2$ produced by mineralization. The dimensions of the test tubes (76 mm high, 10 mm diameter) precluded them from overturning within the bottles. The bottles were incubated on a rotary shaker operating at 120 rpm, and the NaOH was periodically replaced with fresh solution. The radioactivity in the alkali was then measured.

Mineralization of Aged Phenanthrene after Disruption of Soil Aggregates. Radiolabeled phenanthrene added to give 10 μg/g was aged in 5-g samples of Edwards muck for 300 days. Phenanthrene was also freshly added to other samples of the soil. The samples were transferred to sterile 60-mL glass jars, and 10 mL of sterile salts solution (pH 5.7) was added to each jar. Three jars containing soil with either aged or freshly added phenanthrene were then exposed to sonic oscillation for 10 min using a Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY) set at 50 W power. Triplicate soil samples containing aged and unaged phenanthrene were not subjected to sonic treatment. Each jar then was inoculated with 6.5×10^7 cells of *Pseudomonas* strain R contained in 1 mL of distilled water. The jars were sealed with Teflon-covered silicone stoppers and incubated at 20 ± 1 °C on a rotary shaker operating at 140 rpm. Mineralization of phenanthrene was measured by trapping $^{14}CO_2$ in a vial of 0.5 N NaOH suspended within each jar.

Data Analysis. The maximum rate of mineralization was determined by conducting a linear regression analysis on the points that formed the steepest section of the mineralization curve. Three or more data points were used for each regression. The regression coefficient (r) was usually greater than 0.99.

To compare the amount of phenanthrene or 4-nitrophenol extracted from soil after various aging times and to compare rates or extents of mineralization, an analysis of variance was conducted. If the F-statistic from the analysis of variance showed a significant difference ($P = 0.05$), Fisher's least-significant-difference test was used to determine which samples differed.

Results

Mineralization of Aged Phenanthrene. Phenanthrene was aged in 10-g samples of sterilized muck, loam, and aquifer sand for 0, 13, 27, or 84 days. *Pseudomonas* strain R was inoculated at 3.0×10^5 cells/g of soil (dry wt) after the aging period. Mineralization of the hydrocarbon was not detected in uninoculated soil or aquifer sand. The rate of biodegradation in the muck declined as the time of its residence in the sterilized soil increased (Figure 1). The maximum rates of mineralization for aging times of 0, 13, 27, and 84 days were 11.5, 8.3, 7.2, and 6.5%/day, respectively. The degradation of freshly added phenanthrene was significantly faster than the aged compound ($P = 0.05$). The extents of mineralization were similar for phenanthrene aged for 13, 27, and 84 days, but the extents were significantly lower than that for freshly added phenanthrene. The rates of biodegradation in the loam also declined with time of aging. The rates of mineralization for aging times of 0, 13, 27, and 84 days were 17.6, 14.4, 13.3, and 11.2%/day, respectively. The rates were significantly different ($P = 0.05$), except the rates for 13 and 27 days of aging, which were statistically indistinguishable. The extents of mineralization were the same regardless of the aging period.

In the aquifer sand, the rates of biodegradation were unaffected by aging (Figure 1). The mineralization of freshly added phenanthrene was much slower in the aquifer sand than in the soils even though the inoculum sizes were identical, possibly the consequence of a limitation in inorganic nutrients or oxygen resulting from standing water over the sand. The limitation of nutrients or oxygen may have obscured the effect of aging in the aquifer sand, as the mineralization rate would likely be controlled by the availability of the limiting inorganic nutrient rather than organic substrate.

To determine whether an inadequate supply of inorganic nutrients or oxygen limited biodegradation in the aquifer sand, an experiment similar to that described above was conducted, but one set of samples was amended with the inorganic salts solution at the time *Pseudomonas* strain R was added, and all samples were shaken at 150 rpm to increase oxygen availability. Samples of aquifer sand (10 g) were aged with phenanthrene at 10 μg/g for 0 or 327 days, and *Pseudomonas* strain R was added to give 5.4×10^5 cells/g. The rate of mineralization of freshly added substrate in slurries of aquifer sand was twice that previously observed with nonshaken samples of aquifer sand (2.6 and 1.3%/day, respectively), and the addition of inorganic nutrients further increased the rate to 4.1%/day (Figure 2). Although biodegradation of aged phenanthrene was also stimulated by the inorganic nutrients, the rate was still appreciably lower than that for freshly added substrates.

Phenanthrene was aged in sterilized 5-g samples of muck for 0, 204, or 315 days, and *Pseudomonas* strain R was inoculated to give 9.8×10^6 cells/g soil. Both the rate and

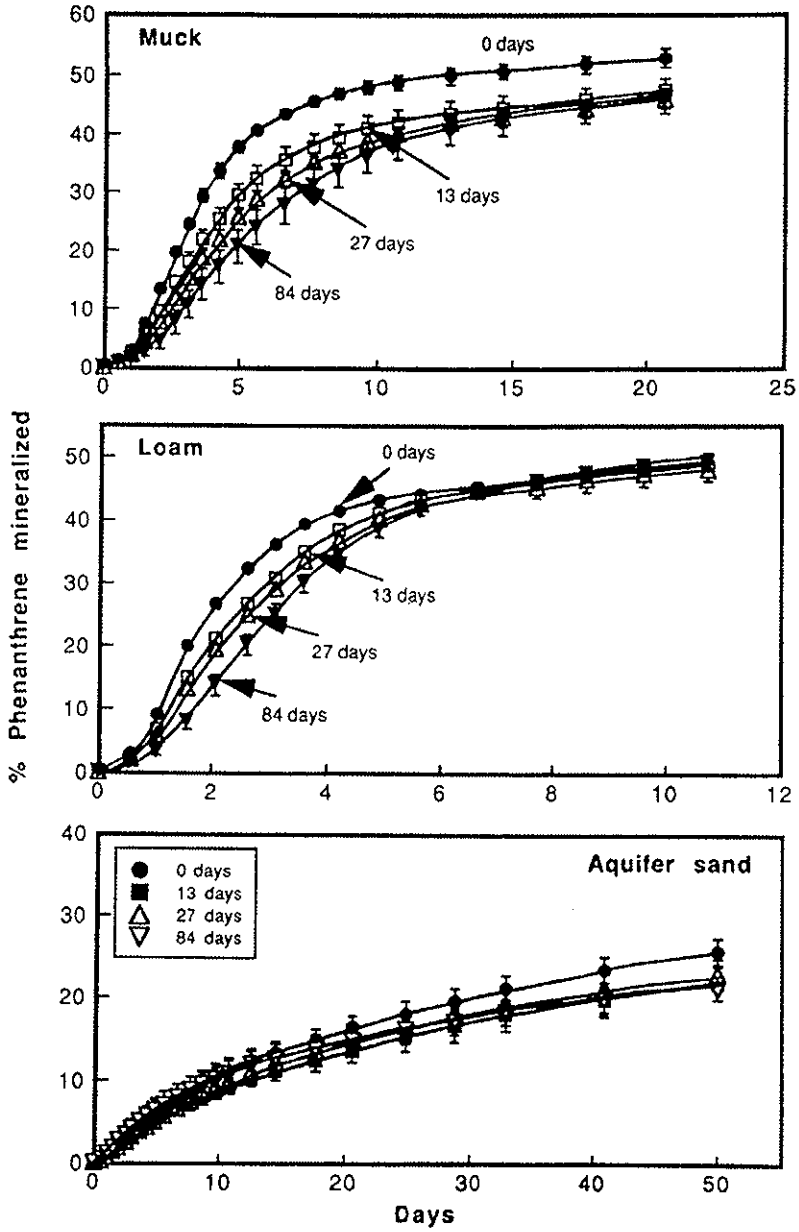

FIGURE 1. Mineralization by *Pseudomonas* strain R of phenanthrene aged for 0, 13, 27, and 84 days in two soils and an aquifer sand. The error bars represent the standard deviation of three or four replicates. If error bars are not evident, they are obscured by the points.

extent of biodegradation declined in muck soil during these longer periods of aging (Figure 3). The maximum rates of mineralization were 19.1, 6.3, and 2.7%/day, and 59.0, 49.8, and 42.2% were mineralized in 33 days in samples with phenanthrene aged for 0, 204, and 315 days, respectively. The rates and extents of mineralization were significantly different at each aging time ($P = 0.05$).

Extraction of Aged Phenanthrene. The soil samples were mixed for 2 min with 20 or 25 mL of *n*-butanol and then filtered, a procedure that extracted 96 ± 2% of the compound freshly added to Edwards muck. The quantity extracted from Edwards muck with butanol declined from 94.5% for unaged phenanthrene to 67.0% when the compound was aged in soil for 13 days, and the value was not significantly changed with further aging (Table 1). Nearly all of the phenanthrene aged in Lima loam for 0 and 13 days was extracted with butanol, but the percentage was somewhat less as the compound aged for 27 and 84 days. Almost all of the phenanthrene aged for 0 and 84 days in the aquifer sand was extracted, but for unknown reasons, only about 80% was extracted after 13 and 27 days of aging.

The butanol-extracted soil was then extracted for 8 h with CH_2Cl_2 in a Soxhlet apparatus. Of the approximately 35% of phenanthrene that was not extracted from the muck with butanol, approximately two-thirds was recovered by Soxhlet extraction, but approximately 10% of the total phenanthrene was not recovered by the two extractions. The approximately 10% of the added phenanthrene that was not removed from Lima loam by butanol was completely recovered by Soxhlet extraction. Recovery of the phenanthrene was also complete in all of the samples of aquifer sand.

Phenanthrene was aged in the muck for longer periods of time, and then the samples were extracted. The quantity of phenanthrene extracted with butanol declined from 95.1% after 1 day of aging to 81.8% after 6 days and remained <80% thereafter (Table 2). The amount of the hydrocarbon extracted from the muck with butanol in this experiment

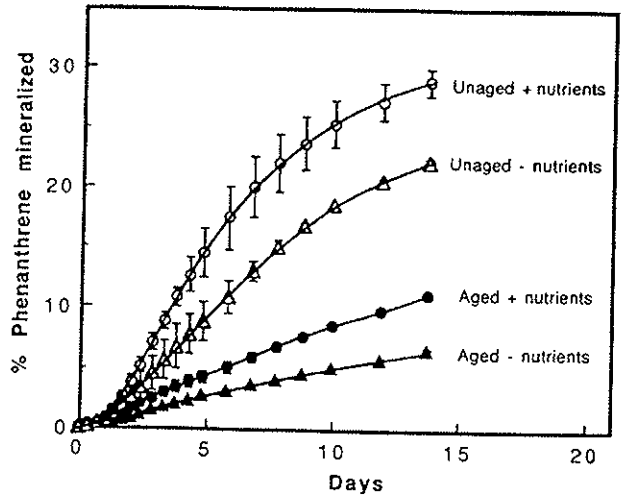

FIGURE 2. Effect of inorganic nutrients on the mineralization in slurries by *Pseudomonas* strain R of phenanthrene aged 0 or 327 days in aquifer sand. The error bars represent the standard deviation of duplicates. If error bars are not evident, they are obscured by the points.

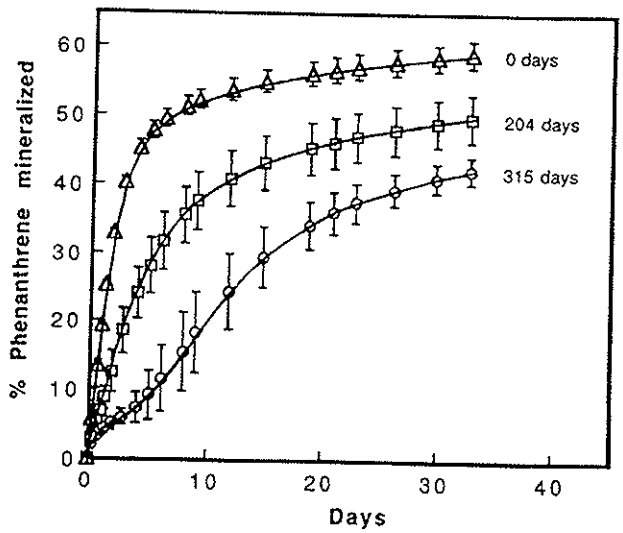

FIGURE 3. Mineralization by *Pseudomonas* strain R of phenanthrene at 10 μg/g aged for 0, 204, or 315 days in Edwards muck. The error bars represent the standard deviations of triplicates.

was somewhat higher than that reported above, perhaps because 5 rather than 10 g of soil was used in this instance. Some but not all of the compound aged for 315 days was recovered by a 10-h Soxhlet extraction. The results of mineralization in muck samples with phenanthrene aged for 0, 204, and 315 days were reported in Figure 3.

Mineralization and Extraction of Aged 4-Nitrophenol. 4-Nitrophenol was added to sterilized 10-g samples of Lima loam and Edwards muck at 10 and 1 μg/g and allowed to age in the dark for 0, 40, or 103 days. Bacterium WS-5 was then added to give 2.6×10^6 cells/g soil, and mineralization of the compound was determined. The rate and extent of mineralization of 4-nitrophenol in Edwards muck declined with residence time (Figure 4). The decline was most pronounced at the lower 4-nitrophenol concentration. Similarly, the rate and extent of mineralization of this compound in Lima loam declined with aging time, especially at the lower concentration.

A statistical analysis of the rates and extents of degradation is presented in Table 3. From these data, it is evident

TABLE 1

Extraction of Phenanthrene from Edwards Muck, Lima Loam, and Aquifer Sand

environmental sample	aging time (days)	% extracted[a]		
		butanol	Soxhlet	total
Edwards muck	0	94.5 ± 3.9b	9.0 ± 1.7b	103.5 ± 3.6b
	13	67.0 ± 7.2a	24.8 ± 5.5a	91.8 ± 1.8a
	27	63.8 ± 6.2a	24.5 ± 4.1a	88.2 ± 2.2a
	84	61.2 ± 5.5a	25.9 ± 2.8a	87.1 ± 3.7a
Lima loam	0	98.6 ± 1.8b	6.1 ± 1.3a	104.7 ± 3.1ab
	13	96.0 ± 3.5b	11.3 ± 0.3b	107.3 ± 3.8b
	27	88.8 ± 0.1a	10.7 ± 0.1b	99.5 ± 0.3a
	84	87.4 ± 3.1a	12.7 ± 3.3b	100.1 ± 1.3a
aquifer sand	0	98.2 ± 0.9b	4.2 ± 0.9a	102.5 ± 1.1a
	13	79.5 ± 2.1a	22.0 ± 2.1b	101.5 ± 0.6a
	27	82.8 ± 9.0a	20.4 ± 6.9b	103.2 ± 3.1a
	84	96.2 ± 4.5b	5.2 ± 2.3a	101.4 ± 2.8a

[a] Values are the means and standard deviations of triplicate samples except Lima loam at 13 and 27 days of aging, which are duplicate samples. For each environmental sample, values in a column followed by the same letter are not significantly different.

TABLE 2

Extraction of Phenanthrene from Edwards Muck after Various Periods of Aging

aging time (days)	% extracted[a]		
	butanol	Soxhlet	total
0	97.7 ± 2.3b	7.4 ± 0.8a	105.2 ± 3.1b
1	95.1 ± 1.5b	ND[b]	ND
6	81.8 ± 1.0a	ND	ND
28	79.9 ± 5.4a	ND	ND
56	74.2 ± 6.6a	ND	ND
204	74.3 ± 12.7a	20.1 ± 5.4b	94.4 ± 7.7a
315	72.3 ± 4.5a	14.3 ± 1.4b	86.6 ± 3.5a

[a] Values are the means and standard deviations of triplicate samples. Values in a column followed by the same letter are not significantly different. [b] ND, not determined.

that the reduction in extent of degradation is statistically significant at each sampling time and at each concentration in both soils. Moreover, the rates of mineralization of both concentrations of 4-nitrophenol incubated in both soils for 103 days were significantly lower than the rate of mineralization of the freshly added compound.

The soils in which 4-nitrophenol was aged for 0, 40, or 103 days were also extracted with *n*-butanol. Preliminary tests showed that more unaged 4-nitrophenol was removed from soil samples by butanol than by water, methanol, or ethanol. The quantity of 4-nitrophenol extracted by butanol from the sterile soils declined as aging time increased (Table 4). The amount extracted from Lima loam declined further with aging time, especially at the lower concentration. In Edwards muck, in contrast, the amount extracted declined initially but not after 40 days regardless of the concentration.

Phenanthrene Aged with Organic Matter. Soluble organic matter affects the biodegradation of organic compounds (*20*). Thus, an experiment was conducted to determine if phenanthrene mineralization would be influenced by aging in the presence of soluble soil organic matter as well as humin. The rates of mineralization of phenanthrene that was and was not aged with soluble organic matter were rapid (Figure 5). In the presence of organic matter, the maximum rates of mineralization of phenanthrene aged for 0, 14, and 27 days were 49.8, 37.2,

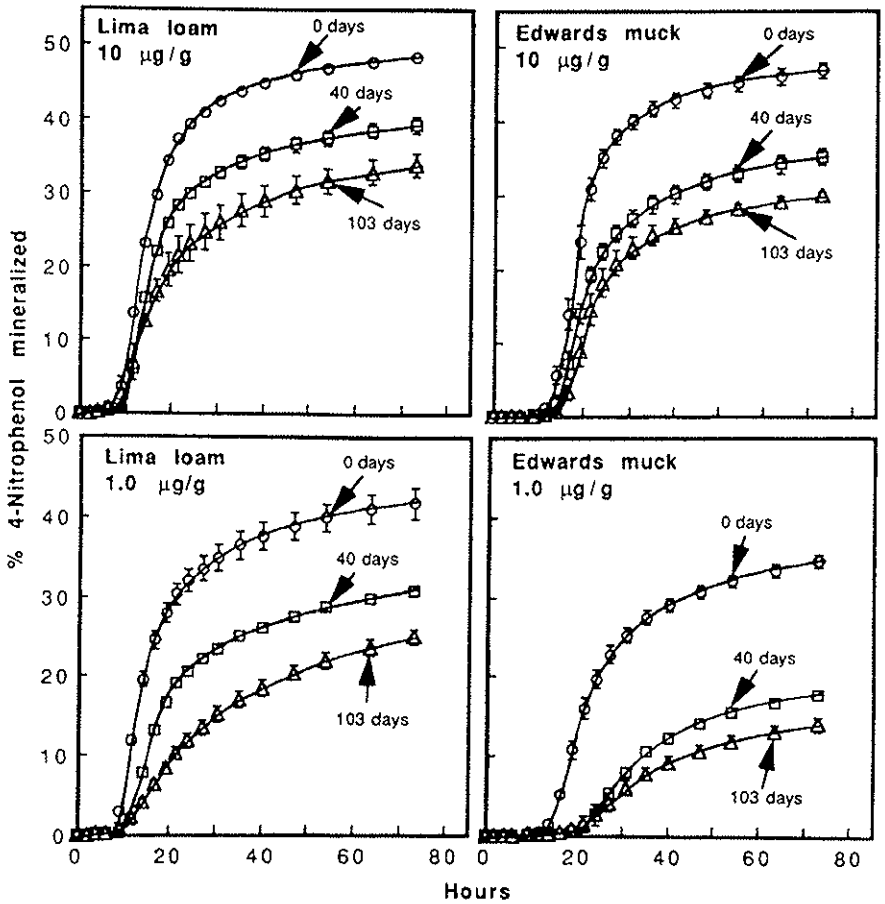

FIGURE 4. Mineralization by bacterium WS-5 of two concentrations of 4-nitrophenol aged in two soils for 0, 40, or 103 days. The error bars represent the standard deviations of triplicates.

TABLE 3

Maximum Rate and Extent of Mineralization by Isolate WS-5 of 4-Nitrophenol Aged in Two Soils

soil	4-nitrophenol added (µg/g of soil)	aging time (days)	maximum rate[a] (%/h)	extent at 73 h (%)
Lima loam	1	0	3.33 ± 0.20 c	41.8 ± 1.9c
		40	2.23 ± 0.08b	30.7 ± 0.6b
		103	0.82 ± 0.13a	25.0 ± 0.9a
	10	0	3.92 ± 0.14b	48.5 ± 0.2c
		40	3.18 ± 0.16b	39.3 ± 1.1b
		103	2.11 ± 0.64a	33.9 ± 1.5a
Edwards muck	1	0	2.19 ± 0.18c	34.6 ± 0.8c
		40	0.82 ± 0.03b	17.9 ± 0.1b
		103	0.56 ± 0.02a	14.1 ± 1.0a
	10	0	3.60 ± 0.21b	46.8 ± 1.1c
		40	2.58 ± 0.18a	35.1 ± 1.2b
		103	2.26 ± 0.21a	29.9 ± 0.4a

[a] Values are the means and standard deviation of triplicate samples. For each soil and at each chemical concentration, values in a column followed by the same letter are not significantly different.

TABLE 4

Butanol Extraction of 4-Nitrophenol Aged in Two Soils

soil	4-nitrophenol added (µg/g)	aging time (days)	4-nitrophenol extracted (%)[a]
Lima loam	1	0	78.4 ± 4.0c
		40	54.2 ± 1.2b
		103	37.6 ± 2.7a
	10	0	81.9 ± 2.7c
		40	61.8 ± 0.9b
		103	55.9 ± 4.2a
Edwards muck	1	0	74.9 ± 1.5b
		40	35.8 ± 0.6a
		103	33.5 ± 4.2a
	10	0	75.3 ± 2.7b
		40	49.4 ± 2.1a
		103	45.4 ± 1.2a

[a] Values are the means and standard deviation of triplicate samples. For each soil and at each concentration, values followed by the same letter are not significantly different.

and 38.1%/day, respectively; although the rate at 0 days is statistically higher than the latter two ($P = 0.05$), the contribution to aging probably is not great given the fast rates of mineralization. At 19 days of incubation, 55.1, 50.4, and 53.1% of the compound was mineralized after aging periods of 0, 14, and 27 days, respectively (data not shown); although the value at 14 days is significantly different from the values at 0 and 27 days, the differences are quite small.

In the presence of humin, the maximum rates of mineralization were 62.1, 49.8, and 46.0%/day for phenanthrene aged for 0, 14, and 27 days, respectively. The rates were rapid in the presence of humin, and the first value is significantly higher than the latter two ($P = 0.05$). After 19 days, the extents of mineralization were similar for all samples.

Mineralization of Phenanthrene after Sonic Disruption of Aggregates. The rates of mineralization of both aged and unaged phenanthrene were significantly enhanced ($P = 0.05$) by sonication (Figure 6). The mineralization rates in aggregated and sonicated samples containing unaged

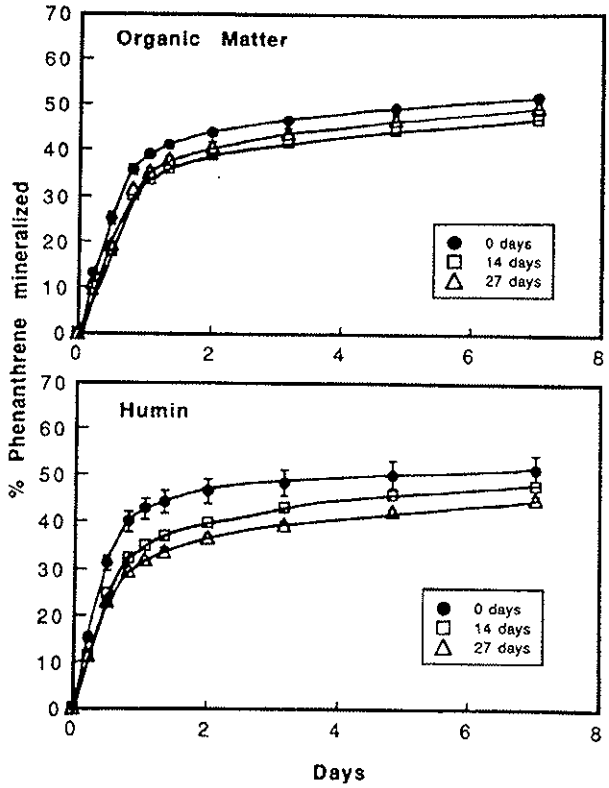

FIGURE 5. Mineralization by *Pseudomonas* strain R of phenanthrene aged for 0, 14, or 27 days in extracted soil organic matter or humin. The error bars represent the standard deviations of triplicates.

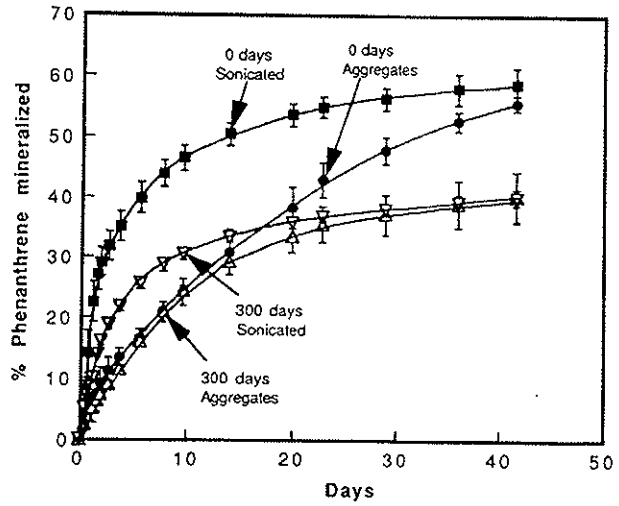

FIGURE 6. Mineralization by *Pseudomonas* strain R of phenanthrene aged for 0 or 300 days in Edwards muck that had been either dispersed by sonic disruption or left as aggregates prior to inoculation. The error bars represent the standard deviations of triplicates.

chemical were 5.8 ± 1.0 and 22.7 ± 2.5%/day, respectively. The rates of mineralization of aged phenanthrene in aggregates and sonic-treated soil were 4.3 ± 0.1 and 8.9 ± 0.3%/day, respectively. The extents of mineralization of aged phenanthrene in aggregated and sonicated soils were similar, as were the values for unaged chemical in the aggregated and sonic-treated soils.

Discussion

Our data show that the extent of mineralization of phenanthrene in the muck and of 4-nitrophenol in the muck and loam soils decreased significantly with aging time. Such reductions in mineralization suggest that an ever greater percentage of each of the compounds becomes less bioavailable and more resistant to biodegradation with time. Studies of samples from contaminated field sites also show marked declines in biodegradability with aging. Thus, Steinberg et al. (*9*) found that 1,2-dibromoethane present in soil in a tobacco field 3 years after its application was resistant to microbial degradation, although the compound freshly added to the same soil was quickly metabolized. Similarly, freshly added simazine was degraded in a field soil in which the herbicide had persisted (*7*).

The maximum rate of mineralization of phenanthrene and 4-nitrophenol also declined with increasing aging time in the loam and muck. The duration of the processes responsible for the decline in bioavailability is evident in the finding that the rate of mineralization of phenanthrene aged in muck for 315 days was less than half the rate in samples aged for 204 days. A reduction in mineralization rate suggests that the concentration of compound available to the bacterium that degrades it is declining (*21*). The concentration of an organic substrate available to bacteria, and thus the rate of its metabolism, may be controlled by the rate of transfer of the compound from an unavailable to an available form.

It has been hypothesized that aging involves diffusion into soil micropores, partitioning into soil organic matter, strong surface adsorption, or a combination of these processes (*9, 15, 16*). Hence, the rate of biodegradation of the aged compounds may be limited by the rates of diffusion from the micropores, partitioning out of the organic matter, or desorption from surfaces (*21*). Diffusion, partitioning, and sorption have each been shown to reduce rates of biodegradation. For example, Scow and Alexander (*22*) reported that slow diffusion of glutamate and 4-nitrophenol from porous clay beads reduced the rate and extent of their biodegradation by bacteria, and partitioning from solid alkanes, waxes, and polymers can reduce the rate of mineralization of some compounds (Hatzinger and Alexander, unpublished data). Sorption of various compounds to suspended sediments (*23*) and humic materials (*20*) also may reduce the rates of their biodegradation.

The concentration of a chemical available for biodegradation in soil also may be reduced by chemical oxidation and reduction, photolysis, covalent binding to soil constituents, and polymerization. Such reactions transform the parent compound, often irreversibly, to one or several new compounds and therefore do not represent aging of the parent molecule itself. To avoid biological transformations of the test compounds during the aging period, the soils and aquifer sand were initially sterilized. Abiotic reactions, including photolysis, hydrolysis, and oxidation are not important mechanisms of degradation of polycyclic aromatics, such as phenanthrene, in soils (*24*). Accordingly, HPLC analysis of butanol and Soxhlet extracts from soil containing aged [^{14}C]phenanthrene revealed that the compound had not been altered during aging in the sterilized soil.

Sorption of substituted phenols, which occurs primarily by hydrophobic sorption and hydrogen bonding (*17, 25*), as well as partitioning into soil organic matter or diffusion into soil micropores may account for the diminished extractability and biodegradability as 4-nitrophenol remains in soil. However, substituted phenols may form free radicals under oxidizing conditions or in the presence of oxidases and subsequently couple or polymerize (*26, 27*), and certain

phenols may thus form covalent linkages with components of humic materials (*28*). However, because of the electron-withdrawing nitro group, 4-nitrophenol is not readily converted to a free radical, even in the presence of phenol oxidase (*27*) or a chemical oxidizing agent (*29*) and thus, in contrast with other phenols, neither polymerizes nor irreversibly binds by covalent linkages to granular activated carbon (*30*). Microorganisms may reduce the nitro group to an amino group (*31*), and the amino moiety could subsequently bind to humic substances. Such a reduction of 4-nitrophenol is unlikely in sterile soil. Nevertheless, because the 4-nitrophenol-amended soils were not subjected to Soxhlet extraction, the identity of the compound remaining after butanol extraction is not known.

The simple butanol extraction presumably removed labile phenanthrene and 4-nitrophenol in the soils and aquifer sand. The material removed in this way may include the compound in aqueous solution and that sorbed to accessible surfaces that come into contact with the extractant. Karickhoff (*1*) used a similar extraction procedure with hexane to study the desorption of polycyclic aromatic hydrocarbons from river sediments. The amounts of 4-nitrophenol and phenanthrene readily extractable from the muck and loam soils with butanol decreased with aging time, but the amount extracted from the muck was relatively constant after the initial period of aging. The rapid initial decline in the amounts extracted is consistent with the greater resistance to biodegradation with duration of aging.

The rate of mineralization of phenanthrene in aquifer sand was initially found to be slow and did not change appreciably with time of aging. However, the addition of inorganic nutrients combined with shaking to increase oxygen availability stimulated the biodegradation rate of the unaged hydrocarbon, but degradation of the aged compound remained slow. It is probable that the effect of aging was not initially observed in the aquifer sand because the availability of oxygen and inorganic nutrients limited the biodegradation rate rather than the availability of substrate. The occurrence of aging in aquifer sand is not surprising because slow, nonequilibrium sorption of organic compounds does occur in aquifer solids, possibly caused by slow diffusion into the microporous sand grains or possibly surface adsorption (*3*, *32*). Retarded diffusion through organic matter also cannot be ruled out as a mechanism of aging in the aquifer sand because the sand contained a relatively high percentage of organic matter for an aquifer solid.

If sequestration of organic molecules entails entrapment within the soil structure, disruption of the structural integrity might increase the bioavailability of these compounds. Pignatello (*33*) and Steinberg et al. (*9*) were able to extract aged halogenated aliphatic compounds only after crushing the contaminated soil in a ball mill. In this study, aggregates of Edwards muck containing aged and freshly added phenanthrene were dispersed by sonic disruption. Such a procedure converts soil aggregates to primary particles without appreciable dissolution of organic or inorganic materials (*34*). Disruption of soil structure increased the rate of mineralization of the aged and the freshly added phenanthrene by 2- and 4-fold, respectively, compared to the soil with aggregates, suggesting that part of the compound sequestered in the aggregates was released by sonic treatment. However, much of the phenanthrene in the aged samples was not made available to *Pseudomonas* strain R by sonic disruption as indicated by the lower rate and extent of mineralization of aged compared to freshly added chemical after sonic treatment. It is possible that the aged compound that is not bioavailable is associated with stable microaggregates and that the failure of the sonic treatment to increase bioavailability of the aged phenanthrene results from the lack of disruption of the stable microaggregates containing the aged hydrocarbon.

Sorption of nonionic compounds in soil may result from surface adsorption rather than partitioning into soil organic matter (*35*), and the formation of aged compounds may involve strong adsorptive interactions rather than diffusion within aggregates or organic matter. Strong adsorption might also explain why sonic disruption did not make aged phenanthrene available to *Pseudomonas* strain R. To test the importance of adsorption to aging, phenanthrene was incubated for various lengths of time with soluble organic matter and humin from Edwards muck. These fractions do not have the aggregate structure of soil, so surface interactions are more likely to be of importance. Short-term sorption of organic compounds to humic materials reduces their mineralization in soil (*36*) and culture (*20*), but the importance of these materials to aging is unknown. The rate of phenanthrene mineralization in the presence of extracted soil organic matter was rapid, and the extent of mineralization was not greatly affected by incubation time. The rate of mineralization in the presence of humin was also fast, and although the increased residence time with humin reduced somewhat the rate of biodegradation, the extent of mineralization was not affected. In a similar time course of aging in the whole muck soil, the rate and extent of phenanthrene mineralization declined significantly. The absence of a decrease in phenanthrene bioavailability upon incubation with extracted organic materials supports the view that aging of chemicals in soil results from slow diffusion, partitioning, or a combination of both.

The effects of aging on the fate of chemicals in soils, sediments, and aquifer materials are poorly understood. The results of this investigation as well as studies of soils from field sites with aged pesticides (*7*, *9*) suggest that the biodegradability of organic chemicals decreases significantly with time of aging. These findings raise questions about the possible effectiveness of bioremediation of sites containing compounds that may have aged and the utility of existing models of the fate of chemicals at field sites. Clearly, more research is necessary to determine not only the importance of aging to chemical bioavailability but also the mechanisms involved and the factors that affect it.

Acknowledgments

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