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Effect of Concentration on Sequestration and Bioavailability of Two Polycyclic Aromatic Hydrocarbons

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A study was conducted to determine the effect of concentration on sequestration and bioavailability of phenanthrene and pyrene in soil. The compounds at 1.0, 10, and 100 mg/kg of soil became increasingly resistant to a mild solvent extraction and progressively less bioavailable to earthworms (*Eisenia foetida*) as a result of aging for 120 days. Aging also resulted in both compounds at 1.0 and 10 mg/kg and phenanthrene but not pyrene at 100 mg/kg becoming more resistant to microbial degradation. Increasing the concentration led to an increase in the percentages of the unaged and aged compounds that were susceptible to microbial degradation. Some of each of the two compounds was still available to earthworms following biodegradation. The data show that sequestration of the polycyclic aromatic hydrocarbons occurs at both low and high concentrations.

Introduction

Persistent organic compounds in soil often become increasingly less available to a variety of organisms and to nonvigorous extraction by organic solvents. This time-dependent reduction in availability results from the molecules becoming sequestered by mechanisms not fully understood. Sequestration has been observed to occur with several insecticides (*1*), 4-nitrophenol (*2*), phenanthrene, pyrene, anthracene, fluorene (*3*), and 1,2-dibromoethane (*4*). The persistent organic compounds with increasing time become less toxic to insects (*5*) and plants (*6*), less available for biodegradation (*2, 4, 7*), more resistant to removal by mild extractants (*2, 4*), and less readily assimilated by earthworms (*8*).

Polycyclic aromatic hydrocarbons (PAHs) are contaminants in many urban and agricultural soils. Their concentrations in contaminated sites may sometimes be quite high, but often the level of contamination is low. The sequestration and bioavailability of PAHs at individual concentrations has been estimated by extraction procedures (*2, 4*), assessments of biodegradation (*2, 4, 7*), or uptake by earthworms (*8*). However, it is not known how the concentration of PAHs affects sequestration or bioavailability. The behavior of organic compounds is known to differ at different concentrations, as indicated by studies of the persistence of atrazine (*9*) and the rates of degradation of toluene and trichloroethylene (*10*).

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Therefore, a study was conducted of the effect of concentration on sequestration and bioavailability of two PAHs, namely, phenanthrene and pyrene. After each compound had aged or been subjected to microbial degradation after aging, the extent of sequestration was measured by the change in recovery by mild extraction and by earthworm uptake. The term sequestration refers to a loss in availability of a compound, and the term aging refers to the time required.

Materials and Methods

Aging. Phenanthrene (>96% pure) and pyrene (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich Chemical (Milwaukee, MI), respectively. Previously untreated Lima loam (pH 7.34, 7.71% organic carbon, 21.5% clay, 44.3% silt, and 34.2% sand) was air-dried, passed through a 2-mm sieve, sterilized with 2.5 Mrad of γ-irradation from a ^{60}Co source, and stored for 2 weeks before use. The soil did not contain detectable levels of phenanthrene or pyrene. Ten-gram portions of the sterile soil were added to sterile 50-mL screw cap tubes, and phenanthrene or pyrene in 200 μL of methylene chloride was added to give final concentrations of 1.0, 10, or 100 mg/kg of dry soil. The tubes were vigorously mixed for 10 s every 30 min for 2 h to allow the methylene chloride to volatilize and mix the chemical with the soil. The uniformity of distribution was addressed by analyzing a series of 2-g portions taken from the chemically amended soils. Sterile deionized water was then added to bring the moisture level of the soil to approximately 80% of field capacity at 1/3 bar. The tubes were tightly capped with silicone-backed Teflon liners and kept in the dark at 21 ± 1 °C.

The aging of samples for 0, 20, and 120 days was started at different times so that all aged samples were available on the same day for analysis. Sufficient samples were aged at each concentration to provide three samples for each extraction and five samples for measurements of earthworm uptake at each aging period. Another set of samples was prepared and aged in the same way for extraction of the soil and for measurement of earthworm uptake after bioremediation. Another group of samples was also aged for assessing the concentrations of each compound remaining in soil during the biodegradation.

Biodegradation. After the compounds had aged for 0, 20, and 120 days, three or five replicate samples were inoculated with a phenanthrene-degrading bacterium (strain P5-2) or a pyrene-degrading bacterial enrichment (PAH01) to give 1×10^8 cells/g of soil. The organisms were grown in 250-mL Erlenmeyer flasks containing an inorganic salts solution (*2*) and 500 mg of phenanthrene or pyrene/L; the flasks were incubated at 30 °C on a rotary shaker operating at 120 rpm. After 60 h, the culture was passed through a glass frit (40-μm pore) to remove the remaining crystals of phenanthrene or pyrene. The cells were collected by centrifugation at 10400*g* for 10 min, suspended in sterile 0.85% NaCl solution, and again centrifuged. The cells were suspended in 2.0 mL of a solution (pH 7.0) containing 200 μg each of KH_2PO_4 and NH_4NO_3 to give 1×10^8 cells/g of soil. The samples were kept at 21 ± 1 °C for 30 days (phenanthrene) or 45 days (pyrene).

The concentration of each compound remaining in the soil during the biodegradation was determined weekly by removing triplicates from the samples prepared for measuring the concentrations of each compound remaining in the soil, and analysis was by 8-h Soxhlet extraction and high-performance liquid chromatography. Abiotic degradation was not observed. Biodegradation was considered to be complete when the concentration did not decrease further; this usually

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TABLE 1. Effect of Concentration and Aging of Phenanthrene on Extractability and Availability to Earthworms

initial concn (mg/kg)	aging time (days)	% extracted		assimilated by worms[a] (%)
		Soxhlet	ethanol−water	
1.0	0	104.2A[b]	70.6A	8.58A
	20	99.1B	50.0B	4.21B
	120	93.7C	33.3C	3.34B
10	0	102.3A	68.4A	8.29A
	20	101.7A	52.3B	4.67B
	120	96.5A	32.1C	3.78B
100	0	103.1A	69.6A	4.03A
	20	103.7A	69.3A	2.94AB
	120	97.4A	43.8B	2.52B

[a] Based on amounts added to soil. [b] For any one concentration, values in a column followed by the same letter are not statistically significant ($P < 0.05$).

required an incubation period of approximately 30 days. The samples then were subject to extraction, and earthworm uptake was measured.

Earthworm Uptake. After completion of aging or after the aged compounds had been subjected to biodegradation, six earthworms (*Eisenia foetida*) (obtained from Carolina Biological Supply, Burlington, NC) were added to the top of the soil, which had been well mixed. The tubes were covered with aluminum foil bearing five holes for aeration, and they were kept in a moist chamber in the dark at 21 ± 1 °C. After 10 days, the worms were placed on wet filter paper in a Petri dish for 24 h to allow for depuration. The worms were frozen at −20 °C and weighed. After homogenizing by grinding with about 30 g of anhydrous Na_2SO_4, the tissues were subjected to Soxhlet extraction. The average weight of worms added for each treatment ranged from 1.79 to 1.98 g. Phenanthrene and pyrene were not degraded in the period the worms were in the soil unless the appropriate bacteria were added.

Analysis. For Soxhlet extraction, 2.0 g of soil or worm tissue was transferred to a cellulose extraction timble and extracted with 120 mL of methylene chloride for 8 (soil) or 10 h (worms). The extracts were concentrated to near dryness with a rotary evaporator, and the resulting material was dissolved in ethanol (phenanthrene) or butanol (pyrene).

Mild extraction was performed by placing 2.0 g of soil in a 50-mL Teflon centrifuge tube, and 5 mL of 75% ethanol in water (phenanthrene) or 20 mL of butanol (pyrene) was added to each tube. The tube was mixed with a Vortex mixer for 5 s (phenanthrene) or 10 s (pyrene) and centrifuged at 7600*g* for 10 min.

The supernatants were analyzed by means of a high-performance liquid chromatograph (Hewlett-Packard series 1050, Hewlett-Packard Co., Avondale, PA) fitted with a Spherisorb ODS-2 octadecyl-bonded silica column (Hewlett-Packard). Acetonitrile:water (86:14) and acetonitrile:water (90:10) were the mobile phases for phenanthrene and pyrene, respectively. Phenanthrene and pyrene were detected by their absorbance at 254 and 240 nm, respectively.

Least-significant differences and Duncan's multiple range test were used to evaluate the statistical significance of differences among values.

Results

Phenanthrene at three concentrations was aged for 0, 20, and 120 days. The recovery of the compound by Soxhlet extraction appeared to decline somewhat with aging time, but the small decrease was only statistically significant in soil receiving the lowest concentration (Table 1). The data represent the percentage recoveries of the amounts initially added. In contrast, the percentage of the hydrocarbon that was extracted by 75% ethanol declined markedly with aging

TABLE 2. Influence of Biodegradation of Phenanthrene Aged at Several Concentrations on Subsequent Extractability and Availability to Earthworms

initial concn (mg/kg)	aging time (days)	% extracted		assimilated by worms(%)	
		Soxhlet	ethanol−water	before bioremediation[b]	after bioremediation[c]
1.0	0	34.1A[a]	13.9A	6.24A(A)	18.0A
	20	53.6B	13.6A	5.21A(B)	9.30B
	120	70.3C	14.2A	3.27B(C)	4.12C
10	0	4.28A	1.87A	1.27A(A)	33.6A
	20	13.9A	3.42B	0.895A(AB)	6.75B
	120	48.1B	3.01AB	0.774A(B)	1.55C
100	0	1.06A	0.32A	0.046A(A)	3.33A
	20	2.10B	0.58B	0.067A(A)	3.33A
	120	5.03C	0.53B	0.052A(A)	0.87B

[a] For any one concentration, values (not in parentheses) in a column followed by the same letter are not statistically different ($P < 0.05$). [b] Percentage of the amount initially added. Letters in parentheses designate differences significantly different at $P < 0.1$. [c] Percentage of the amount after biodegradation determined by Soxhlet extraction.

time, and the recoveries after 120 days were 47% at the two lowest concentrations and 63% at the highest concentration of the quantities extracted at 0 days. It is noteworthy that although the percentages of unaged phenanthrene extracted by 75% ethanol were essentially the same in soil receiving the three concentrations, the percentage after 120 days of aging was higher in the soil receiving 100 mg/kg than 10 or 1.0 mg/kg. If the amount not extracted is a reflection of sequestration, the percentage sequestered diminished as the concentration increased.

Measurements of phenanthrene uptake by *E. foetida* showed that a decline in bioavailability occurred at all three phenanthrene concentrations (Table 1). That decrease occurred largely in the first 20 days. The percentages assimilated at the three test periods were similar at 1.0 and 10 mg/kg but were lower at 100 mg/kg. Because the Soxhlet extractions showed that aging did not appreciably diminish the total concentration, the decline in percentage assimilated after 0 and 120 days indicates a 61.0, 54.4, and 37.5% decline in bioavailability with time; the values represent the differences between the percentages at 0 and 120 days divided by the value at 0 days.

Some of the samples were inoculated with a phenanthrene-degrading bacterium. After the soils had been incubated for 30 days to allow for biodegradation, some were extracted, and earthworms were added to others. Analysis of the Soxhlet extracts of the soils after biodegradation showed that the compound became more resistant to biodegradation as a result of aging (Table 2). This decline in bioavailability to the bacterium associated with increasing residence time proceeded for >20 days because the percentages of phenanthrene remaining were higher after 120 than 20 days. When the data are considered in terms of the amount of phenanthrene metabolized (100% less the percentage extracted), it is evident that higher percentages of the substrate were degraded with increasing substrate concentrations. Extraction of the soil after biodegradation showed an increase in the amount removed by 75% ethanol as a result of aging at the two higher concentrations. However, more relevant to a consideration of what fraction is utilized by bacteria is a calculation of the amount of phenanthrene remaining after biodegradation that is extracted by the milder solvent (i.e., the ratio of percentage extracted by 75% ethanol to that evident by the Soxhlet procedure). Such calculations show a consistent decrease with aging at all concentrations—from 30.2−43.7% for the unaged compound to 6.3−20.2 after 120 days aging; thus, the bacteria appear to be more readily using the fraction removed by the milder solvent.

TABLE 3. Influence of Concentration and Aging of Pyrene on Extractability and Availability to Earthworms

initial concn (mg/kg)	aging time (days)	% extracted		assimilated by worms[b] (%)
		Soxhlet	butanol	
1.0	0	74.6A[a]	66.2A	6.24AB[c]
	20	65.2A	31.7B	6.67A
	120	47.5B	26.0B	4.24B
10	0	88.3A	72.5A	12.4A
	20	70.0B	41.6B	10.2B
	120	62.9B	40.1B	6.41C
100	0	103.2A	82.1A	22.8A
	20	86.8B	53.7B	18.5B
	120	78.6B	54.5B	12.5C

[a] For any one concentration, values in a column followed by the same letter are not significantly different ($P < 0.05$). [b] Based on the amount added to the soil. [c] Significantly different at $P < 0.1$ from the value after 120 days of aging.

TABLE 4. Influence of Biodegradation of Pyrene Aged at Several Concentrations on Subsequent Extractability and Availability to Earthworms

initial concn (mg/kg)	aging time (days)	% extracted		assimilated by worms (%)	
		Soxhlet	ethanol–water	before bioremediation[a]	after bioremediation[b]
1.0	0	15.9A	3.90A	ND[c]	ND
	20	18.5AB	4.52A	ND	ND
	120	22.0B	5.38A	ND	ND
10	0	8.33A	2.01A	ND	ND
	20	10.7B	2.47B	ND	ND
	120	11.7B	2.48B	ND	ND
100	0	5.45A	0.870A	0.084A	1.86A
	20	5.45A	1.34AB	0.093A	1.73A
	120	5.37A	1.44B	0.097A	1.64A

[a] Percentage of the amount initially added. [b] Percentage of the amount after bioremediation determined by Soxhlet extraction. [c] ND, not detected.

In agreement with the previous finding, the data in column 5 of Table 2 show that aging of phenanthrene in unbioremediated soil results in progressively smaller amounts of the compound assimilated by the worms, at least at the two lowest concentrations. The data in the last column show that a portion of the phenanthrene remaining following biodegradation also was available to the worms. Following biodegradation, a higher percentage of the compound initially added at 1.0 mg/kg was assimilated by the worms than when added at 10 mg/kg, and the percentages were still lower at an initial concentration of 100 mg/kg.

Pyrene at 1.0, 10, and 100 mg/kg was also aged for 0, 20, and 120 days. In contrast with the data for phenanthrene, Soxhlet extraction did not lead to essentially complete recovery of the freshly added compound (Table 3). Thus, analysis by this presumably vigorous procedure revealed that appreciable amounts of the hydrocarbon had became sequestered within 120 days. Sequestration was also evident by the results of extraction with butanol, and up to 74% of the pyrene could not be removed at the lowest concentration by butanol. The extraction procedure showed that sequestration occurred at each concentration, but the extent was greatest at 1.0 mg/kg.

Concentration of pyrene also had a marked effect on earthworm uptake (Table 3). The larger the amount added to the soil, the higher was the percentage of the compound assimilated (Table 3). The same relationship was evident if the values for percentage assimilated are calculated from the amounts of pyrene found in the soil by Soxhlet extraction (data not shown). Moreover, sequestration was evident at each concentration, but the effect was more pronounced at the two higher levels. A comparison of the data for 0 and 120 days suggests that aging at these concentrations resulted in approximately a 2-fold decline in availability of pyrene to *E. foetida*. Considering the values for the same aging times, the percentage assimilation increased with increasing initial concentrations; this had not been observed with phenanthrene.

Some of the soil samples were inoculated with a mixed culture containing pyrene-utilizing bacteria. After extensive degradation had occurred, the soils were either extracted or earthworms were introduced to assess bioavailabilty. Analysis of the Soxhlet extracts revealed that pyrene aged at 1.0 and 10 mg/kg became more resistant to microbial degradation as a result of aging; i.e., more of the compound remained after biodegradation of the 120-day aged than of the unaged pyrene (Table 4). No such effect was evident at 100 mg/kg. On the basis of the amounts of the pyrene that was degraded, a larger percentage of the compound was metabolized at the higher concentrations. As noted with phenanthrene, aging resulted in an increase in the amount removed by a mild extractant, in this case, butanol—an effect that was statistically significant only at the two higher concentrations. Although the values at 1.0 mg/kg suggest a similar effect of butanol extraction, the analytical precision at this low concentration was poor. In contrast with the findings with phenanthrene, calculation of the ratio of the percentage of that which was butanol-extractable to the percentage extracted by the Soxhlet procedure revealed little or no change at 1.0 and 10 mg/kg and an increase at 100 mg/kg after 120 days of aging. Therefore, the microorganisms do not appear to be more readily using the butanol-extractable fraction than the total pool of pyrene.

The quantity of pyrene assimilated by the earthworms in soil initially amended with 1.0 and 10 mg/kg and then subjected to biodegradation could not be determined because the quantity present was less than the sensitivity of the analytical method. At the highest concentration, some of the pyrene remaining after biodegradation was assimilated by *E. foetida*.

Discussion

The concentration of a compound often affects its behavior in soil. For example, the frequent existence of nonlinear sorption isotherms for organic compounds introduced into soil (*11*) suggests the saturation of sorption sites. Such a saturation, if applicable to sequestration as well as to the adsorption that precedes sequestration, would be reflected in a declining percentage of the chemical that is sequestered as the concentration increases. In the present case, the amount of the two PAHs that became sequestered as measured by mild extraction increased, but the percentage decreased with increasing PAH concentration. The amount sequestered as measured by quantities degraded by bacteria similarly increased, but the percentage decreased with increasing concentration. Nevertheless, it is clear that sequestration occurred over the range of concentrations tested. It has been shown that concentration affects not only the ratio of sorbed to solution-phase pentachlorophenol in short time periods but also the onset of slow kinetics of sorption and the extent of short-term desorption (*12*).

Although a direct effect of concentration on sequestration has not been studied heretofore, evidence exists from separate studies that the process occurs at both low and high levels. For example, sequestration has been observed at concentrations as low as 1.0 mg/kg soil in the laboratory (*13*) and 4.5 kg/ha in the field (*6*). Conversely in a single soil, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) at as high a level as 4000 mg/kg as well as dieldrin at only 9.6 mg/kg were found to become sequestered when measured by declines in acute toxicity to insects (*14*).

Concentration effects on microbial processes have been extensively explored. In culture media, an increase in concentration may cause a shift from a range of no activity to a range with increasing rates of transformation to a range at which the rate of biodegradation is unaffected by changes in concentration (*15*). On the other hand, increasing concentration may result in slower rates of transformation, as shown in studies of toluene and trichloroethylene added to soil (*10*), and it may have different effects on the persistence of toxicants in soil, as shown in studies of atrazine (*9*). In activated sludge bioreactors, the percentage of several test compounds biodegraded differed according to whether the sludge was or was not adapted to biodegradation of test compounds (*16*). However, the percentage of glucose mineralized by a bacterial mixture was the same at C concentrations from 0.43 ng to 100 mg/L (*17*).

It is noteworthy that a portion both of phenanthrene and pyrene was still available following extensive biodegradation by bacteria. This was true whether the compounds were freshly added to soil or had been aged for 120 days. A similar observation has been made previously (*3*). This may have resulted from a slower rate of desorption than biodegradation in the test period (*18*). It is possible that the amount available for assimilation by the worms could be reduced or possibly eliminated if the soil had been reinoculated since reinoculation has been found to reduce the quantity remaining after the activity of the initial inoculum had largely ceased (*19*). Nevertheless, these data suggest that, even after the combined action of two processes—sequestration and bioremediation—that reduced the quantity of a chemical potentially bioavailable to a receptor species, the risk from low levels remains. An influence of concentration was evident in the amount of the chemical assimilated by *E. foetida*.

Concentration is only one of the variables whose effect on sequestration has not been investigated previously. At sites of contamination, soils typically containing many pollutants as well as other compounds, the sequestration of one compound is probably affected by the presence of others. Often, one or more compounds may be introduced long before others are applied to soil, and the sequence of introduction likely has an influence. Many chemicals in polluted soils are dissolved in nonaqueous-phase liquids, and it is not clear how the presence of such liquids or partitioning of target compounds from these nonaqueous liquids affects their sequestration. Because of the importance of sequestration and aging on the exposure of humans or ecological receptors to toxicants, it is essential that the role of these factors be evaluated.

Acknowledgments

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