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# Reduced Pyrene Bioavailability in Microbially Active Soils

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The influence of microbial activity on pyrene biodegradation and fate was assessed by quantifying the distribution of $^{14}$C-label and pyrene among soil organic matter fractions (SOM) in soils that contained varied microbial communities. A southern, hardwood forest soil was incubated with pyrene in aerated chambers for 9 months. Soil was incubated in three sets: soil alone, soil augmented with a pyrene-degrading community, and soil inhibited with sodium azide ($NaN_3$). After 9 months, pyrene mineralization was observed only in the degrader-amended soil set. Most of the pyrene and [$^{14}$C]pyrene in the $NaN_3$-amended soil set was recovered by solvent extraction (>75%). The [$^{14}$C]pyrene extracted from the $NaN_3$-amended soil set was mineralized by a microbial community shown to mineralize pyrene. Extractable pyrene from nonamended soil and degrader-amended soil sets decreased with time due to biodegradation. Extracted label from these soils was not mineralized to the same extent as $NaN_3$-amended soil. Nonextractable label in humic/fulvic acid and humin fractions was greater in these soils than in the $NaN_3$-amended soil set. Pyrene degradation products were detected in biologically active soils by HPLC. Over time, the extractability of pyrene and pyrene products in biologically active soils decreased to a greater extent than $NaN_3$-amended soil.

## Introduction

Studies of polycyclic aromatic hydrocarbons (PAH) fate have shown that microbial mineralization of PAHs, especially PAHs with four or more benzene rings, decreases with increasing contaminant residence time in soils (*1*−*3*). Decreased microbial mineralization is often attributed to PAH association with the soil organic matrix (SOM) (*2*, *4*). Proposed interactions between PAH and SOM include adsorption and absorption (*5*, *6*), chemisorption (*7*), partitioning (*8*), and covalent binding to the soil matrix (*9*, *10*). Sorptive and partitioning processes reduce PAH mineralization by slowing PAH desorption from SOM into soil aqueous phases where biodegradation is believed to occur (*11*−*14*). Nonsorptive interactions may inhibit complete PAH degradation by slowing desorption of PAH transformation products.

The type of PAH−SOM interaction will significantly affect long-term contaminant fate and bioavailability (*8*). Irreversible binding of pesticide residues in soil, a result of either biological or abiotic oxidative coupling reactions, has been proposed to limit residue desorption and transport (*9*, *10*). Recent evidence suggests that a significant fraction of bound pesticide residues may not irreversibly bind to soil but may sorb to soil via cation and hydrophobic interactions that do not necessarily limit residue mobility (*11*). Both covalent and noncovalent interactions can contribute to nonlinear, nonequilibrium distributions of contaminants in aqueous and solid phases of soils (*6*, *15*, *16*). For nonionic, recalcitrant compounds such as DDT or higher molecular weight PAHs, adsorption and partitioning within SOM or soil micropores is considered a primary mechanism for association with SOM (*6*, *8*). These associations primarily involve noncovalent interactions between pollutant and SOM (*17*). Several studies have noted higher than expected sorption values, as defined by distribution coefficients [$K_D$] and partition coefficients [$K_{oc}$], for soils and sediments contaminated with PAH for extensive periods of time (*18*−*21*).

If sorption and partitioning mechanisms dominate PAH fate in soils, then the PAH remaining in SOM should be primarily parent PAH compounds that are sorbed to organic surfaces. Slow rates of desorption become the primary limitation for biodegradation; however, the presence of adapted PAH-mineralizing communities in contaminated soils suggests that PAH desorption occurs at sufficient rates over time to establish and maintain adapted microbial communities (*22*−*24*). PAH biodegradation appears to proceed, albeit at much slower rates than predicted or desired (*16*, *21*, *22*).

To date, the bioavailability of aged PAH to microorganisms has been studied in soils aged under sterile conditions (*2*). Studies of this design are important in determining the affects of long-term partitioning and sorptive interactions in soil with limited or absent microbial activity. In situ evaluations of PAH-contaminated soils and sediments likewise use sterilized soils to measure aqueous and nonaqueous phase PAH concentrations (*6*, *16*, *21*). It should be noted, however, that contaminated soils and sediments usually contain active PAH-degrading microbial communities; therefore, soils and pollutants have been exposed to biological activity prior to experimental analyses (*22*−*24*).

Previous research has shown that contaminant biodegradation by specific microorganisms can alter desorption rates of contaminants from sorbed surfaces (*25*−*28*). For pesticides, biodegradation has been shown to contribute to significant residue accumulation in soil at rates much greater than surface sorptive interactions (*9*). The primary focus of this study was to assess how biological activity influenced interactions of pyrene and pyrene products with soil organic matter.

The first objective of this study was to determine how microbial activity influenced associations between pyrene and particular SOM fractions over extended periods of time (9 months). Experiments were then conducted to determine if pyrene−SOM associations altered pyrene bioavailability (solvent extractability and microbial degradation). Experiments were designed to follow the fate of pyrene in a consistent soil matrix with and without microbial activity. Pyrene degradation and association with SOM were quantified by systematic removal and analysis of gas phase traps and soil subsamples from aerated soil chambers. The soil matrix was extensively fractionated to separate soluble SOM (lipids, carbohydrates, and humic/fulvic acids) and insoluble SOM (humin). SOM extracts were analyzed by HPLC and liquid scintillation counting (LSC) to determine residual pyrene concentrations and the formation of intermediate products. The $^{14}$C activity in soils and SOM fractions after 270 days was assayed for bioavailability by incubating soils

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**TABLE 1**

**Section A: Description of Soils**

| soil | location | soil type | [PAH][a] | pH[b] | CEC[c] | % OC[d] |
|---|---|---|---|---|---|---|
| SF soil | Raleigh, NC | mineral soil | | 4.8 | 3.4 | 3.5 |
| MN soil[e] | St. Louis Park, MN | sand (90%) | ≈1571 | 8.0 | 21.6 | 1.5 |

**Section B: Nomenclature of Soils and Soil Amendments**

| abbreviation | soils and soil amendment description | type of microbial community (pyrene mineralization) |
|---|---|---|
| SF | Schenck Forest Soil | natural community |
| SF+Azide | Schenck Forest Soil + 0.5% $NaN_3$ | metabolically inhibited |
| | (soil with no known exposure to PAHs) | (no) |
| | (control soil with no known exposure to PAHs) | (no) |
| SF+MN | Schenck Forest Soil + PAH−contaminated soil (20:1 w/w) | PAH-adapted community |

[a] Total PAH contamination, mg/kg (*18*). [b,c] Measured by North Carolina Department of Agriculture, Raliegh, NC. [c] Cation exchange capacity (mequiv/100 $cm^3$). [d] Percent organic carbon (% OC) in soils, analyzed by Huffman Labs, Golden, CO (SW-846 Method 9060). [e] MN soils were repeatedly contaminated (+70 yr) with wood-treating wastes containing mixtures of PAHs.

or soil fractions with a microbial community shown to mineralize pyrene (MN soil) in static microcosms and measuring the amount of evolved $^{14}CO_2$ efflux over time. Comparisons were made between soils with and without microbial activity to determine the extent of biological influence on pyrene−SOM interactions and pyrene biodegradation with time.

## Experimental Section

**Soils.** Surface soil from Schenck Research Forest (SF) was collected to a depth of 10 cm from a stand of hardwood trees (North Carolina State University, Raleigh, NC). Creosote-contaminated soil, containing a pyrene-mineralizing microbial community (MN), was collected from the Reilly Tar and Chemical Company Superfund Site (St. Louis Park, MN). All soils were sieved (2 mm) and stored at 4 °C in the dark prior to use. Schenck Research Forest soil was used within a 2-week period after collection. Physical characteristics of soils are shown in Table 1, Section A.

SF soil has had no prior history of significant PAH contamination. Some SF soil was amended with either sodium azide ($NaN_3$) or MN soils prior to the addition of pyrene and [$^{14}$C]pyrene. These amendments provided three soil groups with distinct microbial communities: (1) the natural, nonadapted microbial community (SF); (*2*) the natural, nonadapted microbial community inhibited with $NaN_3$ (SF+Azide); and (*3*) the natural, nonadapted microbial community augmented with a known pyrene-mineralizing microbial community (SF+MN) (Table 1, Section B). Previous work has demonstrated that MN soils contain an active pyrene-mineralizing community (*24*). MN soils contain active heterotrophic microbial communities as determined by acridine orange counts, phospholipid fatty acids analyses, and colony forming unit counts. This microbial community was shown to actively degrade pyrene and other PAHs in batch slurry microcosms (*24*).

**Chemicals**. Pyrene and [4,5,9,10-$^{14}$C]pyrene (>98% purity, specific activity = 32 mCi/mmol) were purchased from Sigma Chemical Company (St. Louis, MO). Pyrene and [$^{14}$C]pyrene stock solutions were maintained in 95% ethanol/water solutions and were diluted in 95% methanol (500 $\mu$L) prior to the addition to soils. Sodium azide ($NaN_3$) was purchased from Sigma Chemical Company (St. Louis, MO). Sodium hydroxide (NaOH) and sodium pyrophosphate ($Na_4P_2O_7$) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Hydrofluoric acid (HF), hydrochloric acid (HCl), and potassium hydroxide (KOH) were purchased from Fisher Scientific (Pittsburgh, PA). Deionized, carbon filtered, sterile-filtered water was used for all solutions and soil rinses. Solvents [methylene chloride (DCM), methanol (MeOH), and acetonitrile (ACN)] were of the highest quality available. Scintisafe scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was used for liquid scintillation counting (LSC) analyses and to trap organic volatiles in aerated chambers.

**Soil Preparation Prior to Incubation.** Bulk SF soil was homogenized, divided into 100-g aliquots, and mixed with soil amendments, if necessary, prior to the addition of pyrene and [$^{14}$C]pyrene. Three replicates were prepared for each soil series at each time point. Pyrene (100 $\mu$g/g) and [$^{14}$C]-pyrene (0.01 $\mu$g/g) were added to soils via a methanol carrier (500 $\mu$L). Sterile, distilled, deionized water was added to soils to attain a soil moisture content of 30% (wet weight basis). Soils were mixed well to distribute water and to volatilize methanol. To control biological activity in SF+Azide soil, a 0.5% solution of $NaN_3$/sterile $H_2O$ (*29*, *30*) was added following pyrene addition (instead of water alone) to attain the desired moisture content. All containers were left open for 4 h to evaporate the methanol. After evaporation, soils were mixed again, and a 10-g subsample was removed to determine the amount and distribution of $^{14}$C activity at the beginning of incubations ($t$ = 4 h).

**Aerated Systems for Soil Incubations.** All soils were incubated in glass jars (250 mL) with Teflon/silicone septa-lined polypropylene caps and 0.015 mm Teflon tubing for connections to chambers and volatile trapping solutions (Scientific Specialties Service, Inc, Randallstown, MD). The headspaces of chambers (147 $cm^3$ with soil) were continuously aerated with a Whisper 100 aquarium pump (Oakland, NJ) at 25 mL/min, representing approximately 10 air exchanges per hour. Air was filtered through activated charcoal, glass wool, and 1 N KOH prior to introduction to chambers. Soil moisture was maintained by periodic addition of sterile water (0.5% $NaN_3/H_2O$ for control soils) to each individual chamber via a water inlet and a syringe. Soils were incubated for 270 days. Over the course of incubations, trapping solutions (1 N KOH and Scintisafe cocktail solutions) were periodically removed and analyzed for $^{14}CO_2$ and $^{14}$C-labeled volatile products, respectively. In prior experiments, the efficiency of trapping volatilized compounds (phenanthrene and trichloroethylene) with Scintisafe cocktail solutions was greater than 92 ± 4.4%.

## Analytical Procedures

**Fractionation of Soil Organic Matter**. Soil subsamples (10 g) were periodically removed from aerated jars with clean, stainless steel spatulas and weighed into 40-mL glass screw cap EPA vials with Teflon septa caps. Soil aliqouts were fractionated to determine the distribution of $^{14}$C activity in water-soluble extracts, an organic solvent extract (DCM), humic and fulvic acids fractions, and nonextractable material in residual soil (humin) according to a modified version of

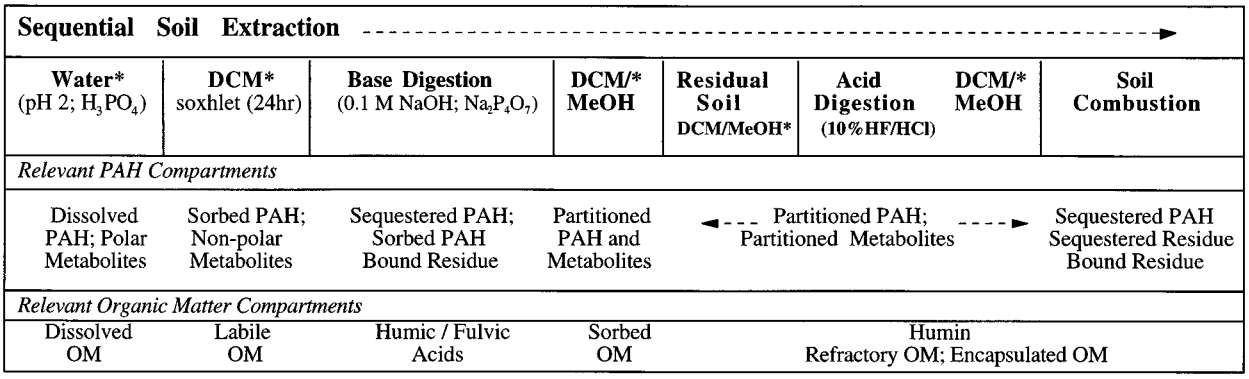


**FIGURE 1. Soil organic matter fractionation protocol. An asterisk (*) indicates that the extracts were analyzed by HPLC fluorescence.**

**TABLE 2. Distribution (%) of $^{14}C$ Activity among SOM Fractions for SF+Azide Soils[a,b]**

| soil fractons | day 0 | day 30 | day 150 | day 270 |
|---|---|---|---|---|
| $^{14}CO_2$ | | 0.8 ± 0.2 | 0.5 ± 0.2 | 0.7 ± 0.2 |
| $^{14}C$-labeled $H_2O$ extracts | 1.0 ± 0.5 | 0.8 ± 0.2 | 0.5 ± 0.2 | 0.7 ± 0.2 |
| $^{14}C$-labeled DCM extracts | 72 ± 0.8 | 54 ± 2.9 | 58 ± 7.1 | 78 ± 3.5 |
| $^{14}C$-labeled humic/fulvic acids | 6.8 ± 0.4 | 5.5 ± 0.1 | 6.2 ± 1.4 | 8.0 ± 0.4 |
| $^{14}C$-labeled residual soil | 21 ± 0.9 | 39 ± 2.8 | 35 ± 5.9 | 14 ± 2.5 |

[a] Percentages are means ($n = 3$) ± 1 SD. Percent values are the amount of $^{14}C$ activity in each fraction divided by the total $^{14}C$ activity recovered in each soil sample (10 g). Mean values were extrapolated to 100 g of soil. [b] Samples were analyzed at 4 h to allow for evaporation of MeOH carrier.

the soil organic matter fractionation proposed by Schnitzer and Schuppli (*31, 32*) (Figure 1).

To liberate pyrene or pyrene products strongly associated with the soil matrix, residual soil fractions were further digested with HF/HCl solution and then extracted with DCM/MeOH prior to HPLC analysis (Figure 1). To determine the affect of base solutions and HF/HCl solutions on pyrene stability and structure, pyrene standards were added to solutions used for base extraction and acid digestion of residual soil. After 24-h incubation, the solutions were extracted with DCM/MeOH and then analyzed by HPLC. No pyrene degradation was observed (data not shown).

All fractions were analyzed for $^{14}C$ activity by mixing aliquots (1 mL) of samples with 9 mL of Scintisafe scintillation cocktail (Fisher Scientific, Pittsburgh, PA) and liquid scintillation counting (LSC) on a Packard 1900TR scintillation counter (Downers Grove, IL). Residual soil fractions were combusted with a Harvey biological oxidizer (R. J. Harvey Instrument Corporation, Hillsdale, NJ) to produce $^{14}CO_2$ that was trapped in 1 N KOH and quantified by LSC. Recovery of $^{14}CO_2$ from biological oxidation was >95%.

**HPLC.** Aqueous and solvent extracts of soil fractions were analyzed by HPLC to quantify residual pyrene and to detect pyrene products in these fractions. Extracts of SOM fractions (water-washed soils, humic/fulvic acids, residual soil fractions, and residual soil fractions digested with HF/HCl) were prepared by shaking soil fractions with DCM/MeOH solvents (4:1 v/v) for 24 h and then allowing the samples to stand for 1 week prior to solvent removal and filtering for HPLC analysis. The extracts of soil fractions were analyzed by HPLC chromatography on a Waters 600E system controller and 717 autosampler and a Supelco LC−PAH column (250 mm × 4.6 mm, i.d., Bellefonte, PA) using an elution gradient from 65% acetonitrile/35% 0.1% trifluoracetic acid to 100% acetonitrile at 18 min with a flow of 1 mL/min. Pyrene and transformation products were detected by fluorescence (Waters 470 scanning fluorescence [260 excitation; 374 emmission (nm)]) and UV absorbance (Spectroflow 757 absorbance detector [254 nm], (Kratos Analytical)). HPLC data was analyzed with Millennium 2010 software (Millipore Corporation, Milford, MA).

**Bioavailability of Residual $^{14}C$ Activity after 270 Days. Static Microcosms.** After 270 days, the bioavailability of the $^{14}C$ activity remaining in soils or SOM fractions was determined by quantifying $^{14}CO_2$ efflux following the addition of a known pyrene-mineralizing microbial community (MN soils). Only soils and SOM fractions collected at day 270 were used in these experiments. These incubations were conducted in 40-mL EPA glass vials (Fisher Scientific, Fairlawn, NJ) with caps and Teflon/silicone septa fitted with Teflon buckets. Chromatography paper, soaked in 1 N KOH, was placed in Teflon buckets to trap evolved $^{14}CO_2$. Positive control vials containing MN soil (1 g); sterile, distilled deionized water (10 mL); and [$^{14}C$]pyrene (1.0 ng/g) were prepared and incubated to verify pyrene mineralization activity in MN soils.

DCM extracts from soils, humic/fulvic acid fractions, and residual soils were mixed with a soil slurry containing MN soil (1 g) and sterile, distilled deionized water (10 mL). Prior to incubation, aliquots of soils, SOM fractions, or SOM extracts were removed to determine initial $^{14}C$ activity. DCM extracts of incubated soils were reduced to a volume of 100 $\mu$L with a stream of nitrogen gas before mixing with the MN slurry. A 10-mL sample of humic and fulvic acid fractions was mixed directly with the MN slurry and adjusted to pH 8 (0.1 M HCl), the natural pH of MN soils. Residual soil fractions (2 g) were mixed with the MN slurry. After 4 weeks, all vials were acidified with 0.5 mL of 20% v/v $H_3PO_4$ solution and shaken for 24 h to liberate $^{14}CO_2$. The 1 N KOH-saturated chromatography paper was removed from the vial and analyzed by LSC to determine the amount of $^{14}CO_2$ evolved.

**Mass Balance Recoveries of [$^{14}C$]Pyrene from Incubated Soils.** Each soil series contained three replicates. Mass balances are summed averages of volatile $^{14}C$-labeled products and $^{14}C$ activity recovered from soil subsamples (for four time periods) plus the average amount of $^{14}C$ activity remaining in unfractionated soils. Summations of fractionated and unused soils were divided by the total amount of [$^{14}C$]pyrene initially added.

## Results

For clarity, the three soil groups will be referred to in the text, figures, and tables as forest soil alone (SF), soil amended

**TABLE 3. Distribution (%) of $^{14}C$ Activity among SOM Fractions for SF Soils[a]**

| soil fractions | day 0 | day 30 | day 150 | day 270 |
|---|---|---|---|---|
| $^{14}CO_2$ | | 0.9 ± 0.7 | 2.1 ± 1.1 | 2.4 ± 1.0 |
| $^{14}C$-labeled $H_2O$ extracts | 0.5 ± 0.3 | 1.5 ± 0.2 | 0.6 ± 0.3 | 0.8 ± 0.1 |
| $^{14}C$-labeled DCM extracts | 57 ± 12 | 28 ± 6.6 | 32 ± 8.1 | 28 ± 3.9 |
| $^{14}C$-labeled humic/fulvic acids | 7.8 ± 4.6 | 16 ± 5.2 | 23 ± 3.5 | 26 ± 2.9 |
| $^{14}C$-labeled residual soil | 35 ± 9.4 | 55 ± 4.0 | 43 ± 6.6 | 44 ± 3.9 |

[a] Values are means ($n = 3$) ± 1 SD. Percent values are the amount of $^{14}C$ activity in each fraction divided by the total $^{14}C$ activity recovered in each soil sample (10 g). Mean values were extrapolated to 100 g of soil.

**TABLE 4. Distribution (%) of $^{14}C$ Activity among SOM Fractions for SF+MN Soils[a]**

| soil fractions | day 0 | day 30 | day 150 | day 270 |
|---|---|---|---|---|
| $^{14}CO_2$ | | 25 ± 3.3 | 26 ± 3.5 | 27 ± 2.9 |
| $^{14}C$-labeled $H_2O$ exracts | 0.7 ± 0.4 | 1.4 ± 0.2 | 0.5 ± 0.1 | 0.5 ± 0.1 |
| $^{14}C$-labeled DCM extracts | 21 ± 8.9 | 9.6 ± 1.2 | 31 ± 13 | 11 ± 2.7 |
| $^{14}C$-labeled humic/fulvic acids | 6.6 ± 3.9 | 7.5 ± 2.3 | 24 ± 9.5 | 14 ± 6.2 |
| $^{14}C$-labeled residual soil | 73 ± 7.1 | 58 ± 5.9 | 29 ± 18 | 49 ± 3.1 |

[a] Values are means (n = 3) ± 1 SD. Percent values are the amount of $^{14}C$ activity in each fraction divided by the total $^{14}C$ activity recovered in each soil sample (10 g). Mean values were extrapolated to 100 g of soil.

with $NaN_3$ (SF+Azide), and soil amended with pyrene degraders (SF+MN) as noted in Table 1, Section B. Soil fractionation with NaOH + $Na_2P_4O_7$ or HF/HCl will be referred to as base and acid digestions of soils, respectively (Figure 1).

**Mineralization of [$^{14}C$]Pyrene.** As shown in Tables 2−4, significant [$^{14}C$]pyrene mineralization ($^{14}CO_2$) was not observed for SF+Azide (Table 2) nor SF soil (Table 3) during the incubation period. SF+MN soil mineralized [$^{14}C$]pyrene (25%) within 2 weeks (data not shown) with no subsequent increase in mineralization activity (Table 4). This observation was consistent with previous results using this soil and verified the presence of an adapted PAH-mineralizing community in SF soil augmented with MN soils (*24*). If [$^{14}C$]pyrene mineralization was the sole end point considered, these results suggested that [$^{14}C$]pyrene was not metabolized by the microbial community in SF soil.

**Distribution of $^{14}C$ Activity in SOM Fractions.** Mass balance recoveries of added [$^{14}C$]pyrene to SF+Azide, SF, and SF+MN soil were 112 ± 11.3, 99.0 ± 8.1, and 109 ± 8.4%, respectively, at the end of 270 days incubation. The mass balance recoveries of [$^{14}C$]pyrene for each soil series demonstrate good recovery from both aerated chamber incubations and SOM fractionation protocols.

The distribution profiles of $^{14}C$ activity in SOM fractions for the three soil groups are also shown in Tables 2-4. For control soils (SF+Azide soil), most of the $^{14}C$ label was recovered in DCM extracts at all sample times (Table 2). After day 30, distributions of $^{14}C$ activity in DCM extracts of biotic soils [SF, SF+MN] were significantly different ($p < 0.05$ *t*-test comparison) than SF+Azide soil (Tables 3 and 4). Decreased amounts of $^{14}C$ activity in DCM extracts of SF and SF+MN soil relative to SF+Azide soil suggest the onset of pyrene degradation. Although SF soil did not mineralize pyrene, label distribution in SF soil was similar to SF+MN soil. These results, in contrast to mineralization data, provided initial evidence that [$^{14}C$]pyrene degradation did occur in SF soil.

Microbial transformation of [$^{14}C$]pyrene in SF and SF+MN soil was accompanied by a higher percent of label in humic/fulvic acids fractions and residual soil fractions (Tables 3 and 4). Label accumulation of similar magnitude was not observed for SF+Azide soil (Table 2). After 270 days of incubation, most of the label in residual soil fractions of SF and SF+MN soil was not extractable with DCM even after base and acid digestion of soils (Figure 2). In SF+Azide soil,

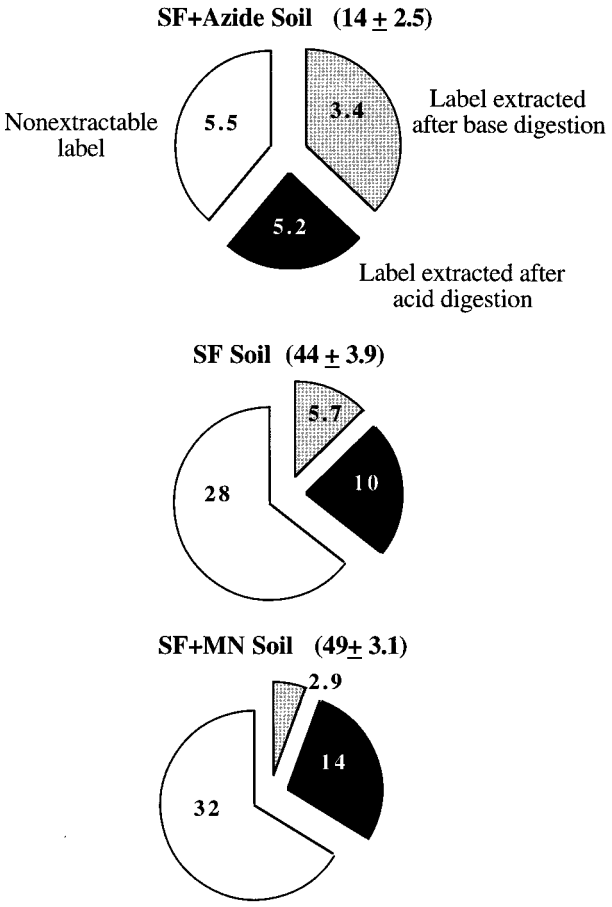


**FIGURE 2. Amount of $^{14}C$-bound residue (%) recovered in DCM/MeOH extracts of residual soil fractions after base digestion (gray) and acid digestion (black). Soils were incubated with [$^{14}C$]pyrene for 270 days. Nonextractable label (white) was recovered by combustion of soil after base and acid digestion.**

more than half of the label in residual soil fractions (8.6% of 14%) was extracted with DCM after base and acid digestion (Figure 2).

It was important to distinguish what portion of label remaining in SOM fractions was the parent compound and what portion constituted biotransformed products. The

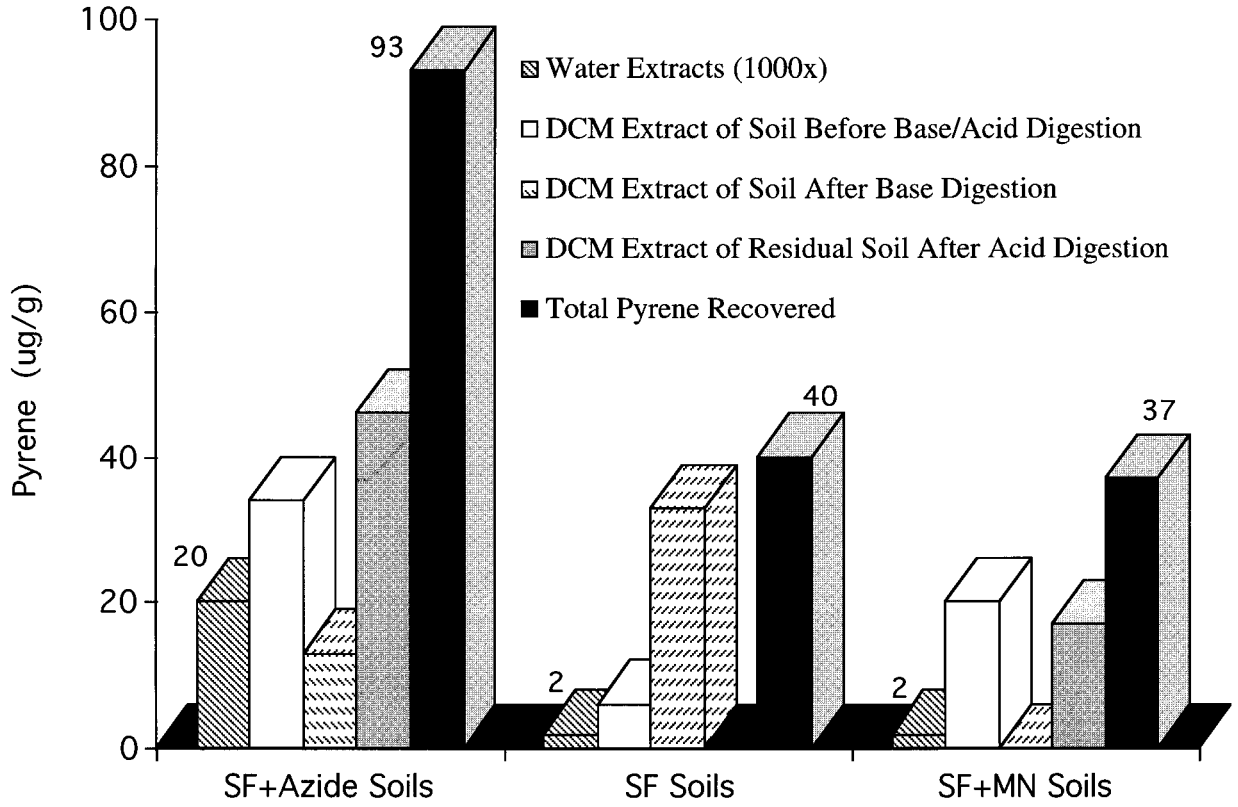


**FIGURE 3. Concentrations of pyrene in solvent extracts of SOM fractions after 270 days. Missing values are indicative of samples without pyrene or samples with pyrene concentrations below detection limits of fluorescence detection. Samples were analyzed by HPLC.**

concentrations of pyrene in water and DCM extracts of SOM fractions (HPLC analysis) are shown in Figure 3 for all three soils at day 270. Total pyrene concentrations (93 $\mu$g/100 $\mu$g) from solvent extractions of SOM fractions (Figure 3) corresponded well with label recoveries (Table 2) for SF+Azide soil. These results indicate that pyrene was not significantly transformed nor irreversibly bound in SF+Azide soil after 9 months incubation.

Pyrene recovery from SF and SF+MN soil was significantly less than pyrene recovery from SF+Azide soil (Figure 3). These results provided further evidence that microbial transformation of pyrene did occur in both SF and SF+MN soil. Unlike SF+Azide soil, pyrene concentrations did not correspond with the amounts of label in SOM fractions. These inconsistencies suggested the presence of labeled transformation products. Products ($P_1$–$P_7$) were detected (HPLC–UV) in DCM extracts of SF and SF+MN soil (Figure 4). The number of products was greater in SF soil than SF+MN soil. Decreased efficiency of pyrene transformation (mineralization versus incomplete degradation) resulted in greater accumulations of pyrene products in SF soil as compared to SF+MN soil. These products were not observed or detected at similar concentrations in SF+Azide soil.

For both SF and SF+MN soil, polar products that were apparent at day 150 ($P_1$ and $P_2$) in initial DCM extracts of both SF and SF+MN soil were not detected in the same DCM extract on day 270. Whether these products were further metabolized or incorporated into SOM cannot be determined by this experimental design. However, products with similar retention times ($P_6$ and $P_7$) were detected after base and acid digestion of both soils at day 270.

**Bioavailability of Residual $^{14}$C Activity in Static Microcosms after 270 Days.** Understanding the influence pollutant–SOM interactions exert on biotoxicity and ultimate biodegradation is critical for pollutant fate assessment. We report here the influence of biologically mediated and abiotic pyrene–SOM associations on pyrene bioavailability for further biodegradation by a microbial community shown to mineralize pyrene (MN soil). Biotoxicity results will be reported in a subsequent manuscript with substantially aged [$^{14}$C]pyrene soils.

After 270 days, DCM extracts, humic/fulvic acids, and residual soil fractions were incubated in static microcosms to determine the availability of $^{14}$C activity for mineralization by the microbial community in MN soil. Maximum [$^{14}$C]-pyrene mineralization in positive control microcosms (28% ± 4.5) was similar to aerated chambers with SF+MN soil (Table 4); thus verifying the pyrene-mineralization activity of MN soils used in bioavailability experiments.

As shown in Figure 5, mineralization of label in DCM extracts of SF+Azide soil was greatest (20 ± 6.1%). DCM extracts of SF+Azide soil contained significant amounts of pyrene (34 $\mu$g/g) and [$^{14}$C]pyrene (78% ± 3.5) by HPLC (Figure 3) and LSC data (Table 2). These results indicated that the [$^{14}$C]pyrene extracted from SF+Azide soil after 270 days was mineralized (20% ± 6.1) to approximately the same extent as freshly added [$^{14}$C]pyrene (27% ± 3.5). DCM extraction of [$^{14}$C]pyrene (78% ± 3.5) after 9 months was similar to published recoveries of aged phenanthrene in sterile soils after 3.7 months incubation (*2*). Base and acid digestion of soil liberated an additional 8.6% [$^{14}$C]pyrene from soil (Figure 2). These results provide further evidence that most of the spiked pyrene (≈86.6%) did not irreversibly associate with SOM after 9 months when incubated under microbially inhibited conditions.

Although significant amounts of label were detected in DCM extracts and SOM fractions from SF and SF+MN soil (Tables 3 and 4), subsequent mineralization of this label did not occur (Figure 5). Mineralization of label remaining in DCM extracts of SF soil was lowest. HPLC analyses had shown that SF and SF+MN soil contained mostly pyrene products that were detected at day 150 but were more difficult to extract by day 270 (Figure 4). These results suggest that the label in DCM extracts of SF soil was not available for mineralization

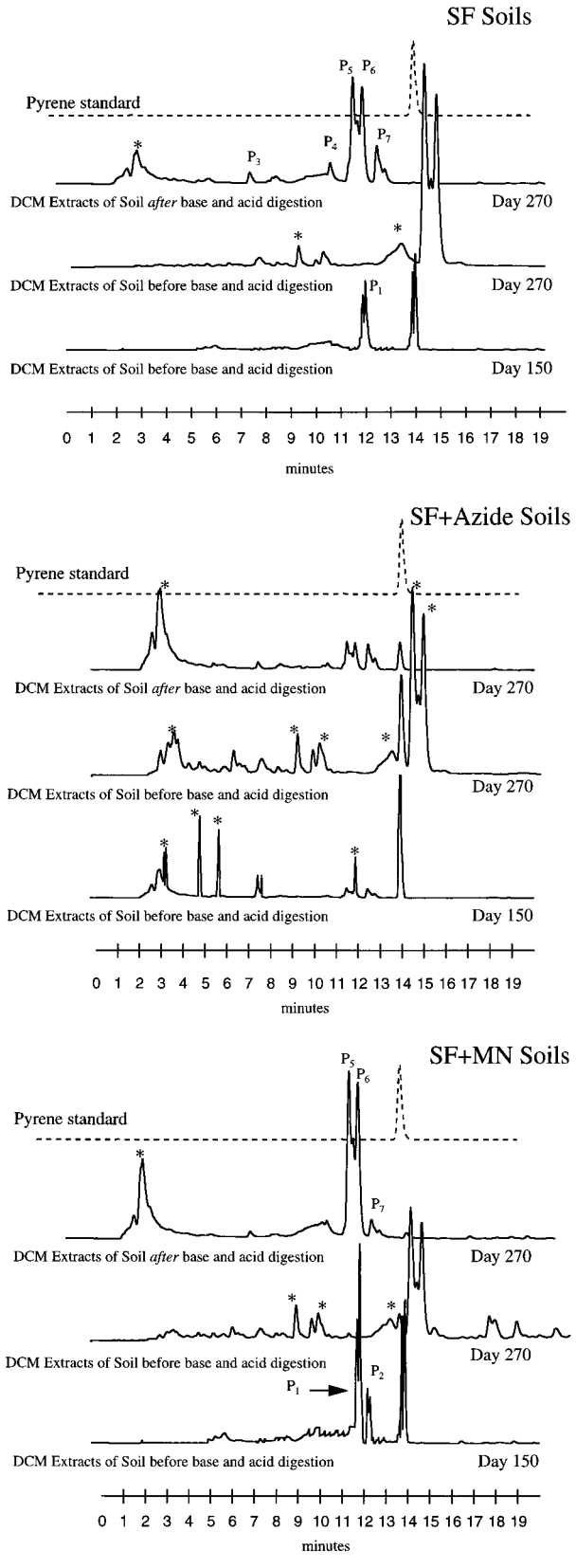


**FIGURE 4. Chromatographs of UV detector output (254 nm) for SF+Azide, SF, and SF+MN soils. Each chromatograph shows DCM extracts of soils at day 150, DCM extracts of soils at day 270, DCM extracts of soils after acid and base digestion on day 270, and DCM extracts of standard pyrene solution. Polar products are noted as $P_1$−$P_7$. The asterisk (*) peaks refer to soil constituents not degraded by microorganisms over time.**

and that label bioavailability declined with time due to association of pyrene products with SOM. Mineralization of label in residual soil fractions from SF soil (10%) and SF+MN soil (8%) (Figure 5) most likely reflects mineralization of pyrene products liberated from the SOM by base and acid digestion of the SOM matrix ($P_5$−P6; Figure 4). For biologically active soils, pyrene mineralization appeared more dependent on SOM associations with pyrene products than pyrene.

In short, a major fraction of the label originally added to biotic soils resided in the residual soil fractions after 270 days. Most of this label was resistant to solvent extraction and mineralization by a microbial community known to mineralize pyrene. Like pyrene, pyrene products became more difficult to recover by solvent extraction with time. In metabolically inhibited soils (SF+Azide), 14% of the spiked [$^{14}$C]pyrene was not solvent extractable after 270 days. Of that 14%, only 5% appeared irreversibly bound to SOM. The label extracted from SF+Azide soil was readily mineralized when incubated with MN soil that contains a microbial community shown to mineralize pyrene.

## Discussion

Irreversible sorption is often defined as the resistance of bound compounds to extraction by solvents or solvent mixtures (*11*). In this study, pyrene and pyrene products are considered irreversibly bound if they are not removed by solvent extraction after extensive base and acid digestion of soils. Acid digestion of soils can protonate intermediate products as well as hydroxylated moieties in soils and minerals (*31*), thereby releasing ionized or hydrophobically bound compounds. Once protontated, compounds associated with SOM via weak electrostatic, H-bonding, or ionic interactions become available for subsequent nonpolar solvent extractions as shown by data in Figure 4.

Because pyrene mineralization did not increase between 150 and 270 days, the disappearance of pyrene and metabolic products with time (Figure 4) appeared to result from interactions of pyrene and pyrene products with SOM. Previous research has demonstrated that pollutants and their intermediates can bind to SOM via covalent and noncovalent interactions (*2, 10, 11, 15, 27*). Recent studies have begun to assess mechanisms of residue accumulation in tandem with bioavailability (*2, 3, 11, 38*−*40*). For pesticides, both covalent bonding (*9, 28, 40*) and sorption (*2, 11*) appear to reduce pesticide residue bioavailability; however, it remains unclear the degree to which either mechanism can be manipulated to control bioavailability (*9*).

Recently, Burgos et al. (*27*) correlated irreversible binding of naphthalene in sandy soils to biologically catalyzed oxidation reactions of naphthalene to more polar intermediate products. Oxidized pyrene intermediates, primarily pyrene *cis*-4,5-dihydrodiols, have been observed in both pure culture studies (*33*−*36*) and recently in estuarine sediments (*39*). We cannot offer a specific mechanism for intermediate product−SOM interactions until products are identified. It is attractive to consider that products observed in this study, perhaps initially similar in structure to the aforementioned pyrene metabolites, were more resistant to solvent extraction due to oxidative coupling reactions with SOM. We also speculate that some of the products shown in Figure 4 represent (a) pyrene products biodegraded to microbial cellular constituents associated with soil or (b) extracted pyrene intermediate products that had sorbed to or been entrained within micropore or organic matrixes (*9, 11, 17*). Because the majority of bound residue was not extractable and thus not identified in biological soils (Figure 2), it is possible that bound label composition includes pyrene sorbed or partitioned in SOM as well as covalently bound pyrene products (*8, 11, 20*).

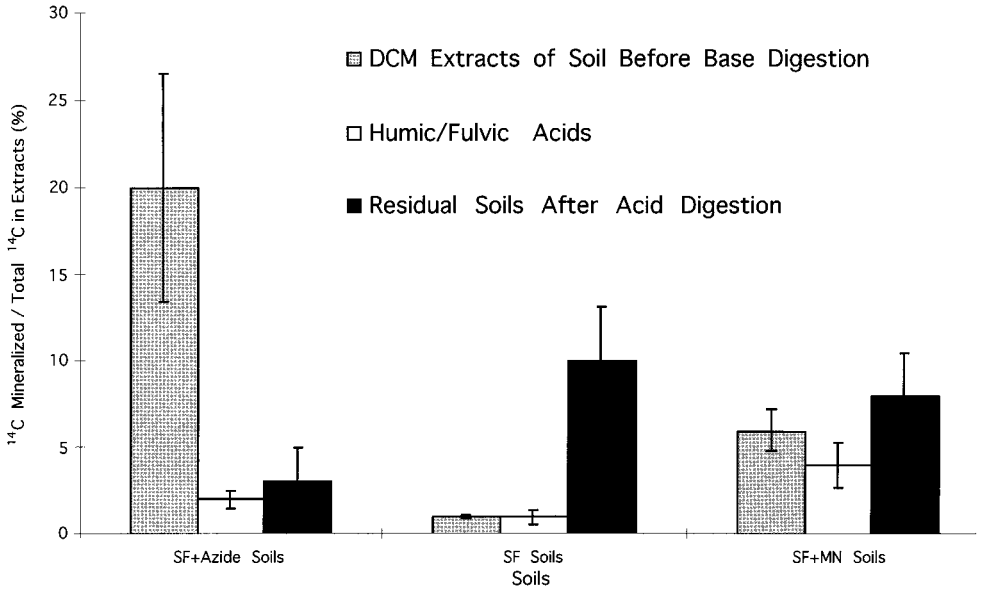


**FIGURE 5. $^{14}$C activity (%) mineralized by a known pyrene-mineralizing community in DCM extracts, humic/fulvic acids, and residual soils from soils previously incubated for 270 days with pyrene and [$^{14}$C]pyrene. Aliquots of DCM extracts (100 $\mu$L), humic/fulvic acids (10 mL), and residual soils (2 g) were incubated for 28 days in soil slurries containing 1 g of MN soils and 10 mL of $H_2O$. Values are reported as percent means $\pm$ 1 SD ($n = 3$).**

Differentiation of pollutant or transformed pollutant product interactions with SOM is dependent on compound identification and a fundamental understanding of binding behavior between targeted compound and soil (*9*, *11*, *27*). As shown in these experiments, radiolabeled studies provide important data about pollutant biodegradation, association with SOM, and the fate of intermediate products with time. However, these studies often cannot provide relevant information concerning the identity of bound label, types of interactions between contaminant and soil, or molecular changes to contaminant−soil interactions (*17*).

Partition coefficients have also been used to characterize and predict PAH bioavailability in soils and sediments (*16*, *17*, *41*). It is possible to calculate distribution coefficients, $K_D$, and partition coefficients, $K_{oc}$, from the data presented in this study. However, it should be noted that soil organic matter heterogeneity may diminish the accuracy of these calculations and that calculations of $K_{oc}$ values should involve soils samples of variable organic matter content (*20*, *21*). Distribution coefficients, $K_D$, were determined by using HPLC data for pyrene concentrations in water extracts ($C_w$) and soils ($C_s$) (Figure 3). The following equations were used for calculation where $K_D$ is mL/g of soil, $C_s$ is $\mu$mol/g, $C_w$ is $\mu$mol/mL, log $K_{oc}$ is mL/g of organic carbon, and $f_{oc} = 0.035$:

$$K_D = (C_S)/(C_W)$$

$$K_{oc} = (K_D)/(f_{oc})$$

Log $K_{oc}$ values based on literature data, HPLC data, and LSC data are shown in Table 5. Log $K_{oc}$ values calculated from HPLC data were based on measured pyrene concentrations (HPLC) after 270 days as shown in Figure 3. The mean of log $K_{oc}$ values (day 0, 30, 150, and 270) calculated from LSC data are shown only for SF+Azide soil because $^{14}$C activity corresponded to [$^{14}$C]pyrene only in this soil. Log $K_{oc}$ values were higher for biologically active soils than for SF+Azide soil and were similar to reported measurements for pyrene from in situ contaminated sediments and soils (*19*−*21*, *24*).

The larger $K_{oc}$ values (HPLC) observed for all three soils in this study may suggest that only a fraction of pyrene sorbed to soils was available for equilibrium partitioning and desorption (*20*). Higher than expected $K_{oc}$ values have been measured and attributed to PAH origin and formation (*21*), physical changes to SOM configuration (*16*), climatic perturbations (*7*), retarded intraparticle diffusion (*8*), and biodegradation (*26*). In this study, differences between biologically active soils and biologically inhibited soils suggest that biological activity may have reduced pyrene desorption over the 270-day period. However, it is difficult to determine if biological alterations to soil structure or soil chemistry altered pyrene−SOM interactions within the experimental parameters of this study (*15*).

**TABLE 5. Log $K_{oc}$ Values for Pyrene in Soil after Day 270**

| soil | log $K_{oc}$ |
|---|---|
| literature value[a] | 4.79 |
| SF+Azide | 5.02[b], 4.78[c] |
| SF | 5.75[b] |
| SF+MN | 5.72[b] |

[a] Ref 16. [b] Log $K_{oc}$ values from HPLC data used summed concentrations of pyrene in aqueous and organic solvent extracts of soils as shown in Figure 3. [c] LSC data refer to log $K_{oc}$ values calculated from concentrations of [$^{14}$C]pyrene in aqueous and organic solvent extracts of SF+Azide soils where $^{14}$C activity equals [$^{14}$C]pyrene. LSC data shown are reported as the mean $\pm$ 1 SD for four time periods (0, 30, 150, and 270 days).

Results from this study suggest that long-term contaminant assessments should consider diagenetic processes in relation to contaminant interactions with organic matter. If true, biological activity may beneficially affect biotoxicity, contaminant immobilization, and risk reduction, but at the same time limit or slow bioremediation goals of complete contaminant degradation. To fully evaluate the validity and consequences of biological activity, future studies must address abiotic and biological changes to SOM configurations in tandem with product identification and characterization of contaminant and contaminant product partitioning behavior over time. To accurately assess contaminant fate, it is important to understand aging and SOM−pollutant interactions with respect to both parent compound, contaminant intermediate products, and the soil organic matrix.

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