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^{13}C NMR Analysis of Biologically Produced Pyrene Residues by *Mycobacterium* sp. KMS in the Presence of Humic Acid

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Abstract

Cultures of the pyrene degrading *Mycobacterium* sp. KMS were incubated with [4-^{13}C]pyrene or [4,5,9,10-^{14}C]pyrene with and without a soil humic acid standard to characterize the chemical nature of the produced residues and evaluate the potential for bonding reactions with humic acid. Cultures were subjected to a "humic acid/humin" separation at acidic pH, a duplicate separation followed by solvent extraction of the humic acid/humin fraction, and a high pH separation. ^{13}C NMR analysis was conducted on the resulting solid extracts. Results indicated that the activity associated with solid extracts did not depend on pH and that approximately 10% of the added activity was not removed from the solid humic acid/humin fraction by solvent extraction. ^{13}C NMR analysis supported the conclusion that the majority of pyrene metabolites were incorporated into cellular material. Some evidence was found for metabolite reaction with the added humic material, but this did not appear to be a primary fate mechanism.

Introduction

Bioremediation of hydrophobic organic contaminants results in contaminant mineralization, the persistence of sequestered contaminants or transformation products, and the produc tion of bound or humified residues.[1−8] While sequestered contaminants can often be extracted and chemically characterized, bound residues are defined by their resistance to extraction and are usually only detectable by the use of ^{14}C radiolabeled tracers. Compounds enriched with the stable ^{13}C isotope in conjunction with liquid- or solid-state nuclear magnetic resonance (NMR) or GC-MS analysis may also be employed to detect the presence and chemical nature of sequestered or bound contaminants. In the case of environmental NMR experiments, labeled compounds and relatively high contaminant concentrations are usually required because of the low natural abundance of ^{13}C in the environment (1.1%).[9]

Amounts of bound residues observed in laboratory studies of polycyclic aromatic hydrocarbon (PAH) degradation in soils usually range from 10 to 80% of the initially added parent compound and have been shown to both increase[4,7] and decrease[6] with microbial activity. While generally considered to be an acceptable endpoint to biological treatment, resistance to extraction makes their chemical nature difficult to assess and the mechanism of their ultimate incorporation into soil a subject of frequent conjecture.

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Significant research has been done to elucidate the binding mechanisms of phenolic, analinic, and nitro compounds to soils and humic materials.[10–13] ^{13}C NMR has been used to show the enzymatically mediated covalent binding of 2,4-dichlorophenol and phenol to aqueous humic acid[14,15] and the bonding of the herbicide analizine to humic materials in soils.[16] It is generally hypothesized that hydroxylated metabolites of heavier compounds such as PAHs may undergo similar oxidative coupling reactions.[17,18]

Chemical characterization of PAH derived residues in soil incubations have provided evidence of metabolite incorporation. GC-MS analysis of chemically degraded humic acids was used to show the presence of PAH metabolites that had not been removed by solvent extraction.[19] Metabolite association to humic acid through ester bonds, representing approximately 0.5% of the applied PAH, was shown after alkaline hydrolysis of extracted humic acid using $Na^{18}OH$.[6] Richnow et al.[20] also addressed the nature of bound anthracene residue formation in soil using ^{13}C-anthracene and GC-MS isotopic ratio determination, finding that soil residues increased in ^{13}C content with biological activity and alkaline hydrolysis of bound residue containing samples released ester bound metabolic products.

Guthrie et al.[21] employed flash pyrolysis-GC-MS and CPMAS ^{13}C NMR to evaluate the fate of ^{13}C labeled pyrene in sediments samples. They concluded that significant degradation of the added pyrene did not occur but that biological activity did alter the organic matter composition allowing for further sequestration of the parent [^{13}C]pyrene. A subsequent sediment study showed biological degradation of the added [^{13}C]pyrene and the presence of pyrene metabolites in solvent extracts.[22] CPMAS ^{13}C-NMR analysis of aged sediments incubated with [^{13}C]pyrene indicated that the ^{13}C label was incorporated into microbial biomass as the pyrene was degraded.

While laboratory studies have shown that free enzymes have the potential to catalyze pollutant binding reactions with humic materials and soil incubations have shown binding in soil systems, few studies have attempted to observe this reaction in a model system that includes capable bacterial cultures and isolated soil humic

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materials. In one such system, Seibel et al.[23] used liquid chromatography coupled with dissolved organic carbon detection to monitor bacterial PAH degradation in the presence of naturally occurring humic substances from a bog. They found that high and low molecular weight metabolites were formed but concluded that no humification or bound residue formation occurred between metabolites and humic materials.

A potential mechanism of contaminant binding in a soil system involves primary degradation of the parent compound by the microbial community, release of metabolic products either through active or passive efflux from the cell or after cellular decomposition, and subsequent reaction with soil components. Findings of active efflux mechanisms for PAHs,[24] the presence of metabolic products in PAH degrading cultures,[25] and the potential reactivity of xenobiotics with humic materials[10] support the possibility of this binding pathway occurring in a soil system. The objective of this study was to chemically evaluate this potential process of pyrene binding or humification in a model system containing ^{13}C labeled pyrene, pyrene degrading bacteria, and humic acid via ^{13}C NMR spectroscopy. Surrogate microcosms containing [^{14}C]pyrene were employed to track the fate of the added pyrene in the enriched cultures.

Materials and Methods

Chemicals.

Monolabeled [4-^{13}C]pyrene was synthesized in the Laboratory of P. G. Hatcher (The Ohio State University, Columbus, OH). Radiolabeled [4,5,9,10-^{14}C]pyrene (95% purity, specific activity = 56 mCi/mmol) was purchased from Amersham International (Buckinghamshire, England). [^{12}C]Pyrene (>99%) was purchased from Sigma-Aldrich. Analytical reagent grade potassium hydroxide and sodium hydroxide pellets were purchased from Mallinckrodt Baker Inc. (Paris, KY). A.C.S. grade acetone, hexane, acetic acid, and HCl were purchased from Fisher scientific. IHSS soil humic acid standard was purchased from the International Humic Substance Society (IHSS, St. Paul, MN). Luria broth (LB) was composed of 10 g/L of NaCl, 10 g/L of Bacto Tryptone, and 5 g/L of yeast extract (Difco, Becton Dickson, Sparks, MD). Dilute

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LB was a 1/10 dilution of LB broth with basal salts medium. Basal salt medium contains (per liter) the follow ing: 2.376 g of $(NH_4)_2SO_4$, 0.278 mg of $FeSO_4*7H_2O$, 10.69 mg of $CaCl_2*7H_2O$, 0.2464 g of $MgSO_4*7H_2O$, 0.4973 g of NaCl, 1.42 g of Na_2HPO_4, and 1.36 g of KH_2PO_4 and has a pH of 6.5. Dimethyl sulfoxide-d_6 (DMSO-d_6) was purchased from Acros Organics (Geel, Belgium). Acetate buffer was prepared by titrating 11.5 mL of acetic acid in 200 mL of deionized water to pH 4.0 with 0.5 N NaOH.

Incubations.

Bacterial incubations consisted of pyrene and pyrene degrading *Mycobacteria* sp. KMS (GenBank acquisition number AY083217) in a dilute LB medium with or without the presence of IHSS humic acid. A total of 16 cultures were incubated. Four repetitions of either the bacteria only (KMS) or humic acid treatments (KMS+HA) were set up with a mixture of [^{12}C]- and [^{14}C]pyrene to track biological activity through $^{14}CO_2$ evolution and the fate of the added pyrene in the enriched cultures. Duplicate incubations of each treatment were set up with either [^{13}C]pyrene or [^{12}C]pyrene for NMR analysis. All incubations were conducted in 125 mL Erlenmeyer flasks sealed inside of clean 1 quart mason jars with Teflon coated lids. Carbon dioxide traps consisting of 2.0 mL of 0.1 N KOH in a 7 mL scintillation vial were also included in all incubations.

Each flask received 10 mL of 3000 ppm pyrene (either ^{12}C or ^{13}C) dissolved in methanol to provide excess pyrene for the duration of the incubation. Radiolabeled treatments also received 100 µL of a 2500 ppm solution of pyrene in methanol with [^{14}C]pyrene activity of approximately 500 000 DPM (0.225 µCi). The methanol was then allowed to evaporate leaving the crystallized pyrene on the flask surface. Bacteria only treatments received 59.4 mL of dilute LB medium and were inoculated with 0.6 mL of a *Mycobacterium* sp. KMS culture grown in the presence of pyrene. Humic acid treatments received 57 mL of dilute LB medium, 1.5 mL of 3000 ppm IHSS soil humic acid standard dissolved in 0.5 N NaOH, 0.9 mL of acetate buffer to adjust pH to approximately 6.4 (the pH of the dilute LB medium), and 0.6 mL of KMS inoculum. Cultures were incubated in the dark at 30 °C on a rotary shaker at 125 rpm for 56 days. $^{14}CO_2$ evolution was

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monitored over the incubation period by scintillation counting of the KOH traps. Nonradiolabeled cultures also included CO_2 traps that were counted along with those from labeled cultures. All ^{14}C activities were determined by liquid scintillation counting with a Beckman LS 6000 liquid scintillation counter and Beckman Ready Gel scintillation cocktail (Beckman Instruments Inc., Fullerton, CA).

One gram incubations of contaminated soil from land treatment unit 2 at the Libby Groundwater Superfund site (Libby, MT) were incubated with 10 mg of [^{13}C]pyrene added in 100 µL of methylene chloride. ^{14}C Labeled and poisoned surrogates were also incubated. The soil microcosms were incubated in the dark for 1128 days at 30 °C after which humic acid was extracted with a total of 21 mL of 0.5 N NaOH (over three consecutive extractions), precipitated with 2.0 mL of concentrated HCl, centrifuged (10 000*g* for 10 min), and analyzed using ^{13}C NMR. Two biologically active microcosms and one poisoned microcosm containing either [^{13}C]- or [^{14}C]pyrene were incubated and analyzed.

Humic Separations.

A schematic of the humic separation procedure is shown in Figure S-1 of the Supporting Information. All flasks containing either ^{12}C-, ^{13}C-, or ^{14}C-labeled pyrene were subjected to the same separation. At the conclusion of the 56-day incubation, each flask was mixed briefly by hand, and a 0.4 mL sample was removed for ^{14}C counting in radiolabeled flasks and bacterial counting in nonradiolabeled flasks. The pH was then lowered to below 2.0 with 750 µL of HCl, and the cultures were allowed to shake at 75 rpm overnight at room temperature to drive evolved CO_2 out of the cultures and into KOH traps which were then counted for radioactivity. The pH was then adjusted to a range of 11.2−11.5 by addition of 1.0 mL of 13 N NaOH to simulate a humic material extraction. Flask headspace was immediately flushed with nitrogen and allowed to shake in the dark at 75 rpm for 2 h at room temperature. After shaking, 0.45 mL of each flask was subsampled again for radiolabel counting, and the contents of each flask were divided into three 20 mL aliquots that were placed in 40 mL Teflon centrifuge tubes for further

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separation. Empty flasks were rinsed with methanol three times to account for residual radioactivity.

After being split into three 20 mL aliquots, the contents of each flask were subjected to three different separations to evaluate [^{14}C]pyrene fate (radiolabeled incubations) and to isolate enriched samples for NMR analysis (^{12}C and ^{13}C incubations). The first involved separation of humic acid/humin and fulvic acids by addition of 400 μL of concentrated HCl to lower the pH to <2.0. Each flask was then equilibrated for 1 h to precipitate the combined "humic acid/humin" fraction which was then separated by centrifugation at 10 000*g* for 10 min and allowed to air-dry. The fulvic acid fraction was decanted and sampled for ^{14}C counting.

The second treatment followed the same humic separation described above, but after allowing the humic acid/humin fraction to air-dry until no free-standing water was observed, it was solvent extracted with 10 mL of acetone:hexane (1:1) with shaking for 10 min. The humic material was then separated from the solvent by 10 min of centrifugation and allowed to air-dry for analysis. The solvent extract was sampled for ^{14}C counting.

Finally, the third aliquot of each flask was centrifuged for 10 min (10 000*g*) at the previously elevated pH (11.2−11.5) to remove insoluble cellular residue and associated humic material. This insoluble fraction correlates to the "humin" component of a traditional humic extraction. The supernatant was decanted, sampled for ^{14}C counting, acidified with 300 μL HCl, equilibrated for 45 min, and centrifuged to separate the humic acid fraction from the fulvic acid. The remaining fulvic acid was decanted and sampled for ^{14}C counting. This final extraction most closely simulates a traditional humic acid extraction and separation from a soil system with the first insoluble residual representing the soil humin and the second representing the isolated humic acid.

NMR Analysis.

Air-dried solid samples derived from incubations with only [^{12}C]- or [^{13}C]pyrene were dissolved in 400 μL of DMSO-d_6 and placed in 5

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mm NMR tubes for ^{13}C NMR analysis. The proton decoupled ^{13}C NMR spectrum was obtained on a Bruker ARX-400 MHz spectrometer with a ^{13}C resonant frequency of 100.61 MHz. Spectra were recorded with a 45° pulse angle and a 0.4 s pulse delay with a total of 30 600 scans per sample at a temperature of 298 K. Chemical shifts of observed signals were referenced to the center peak of the DMSO septuplet (indicated by * in Figures 1−4) that was set to 39.4 ppm, and exponential line broadening at 1 Hz was applied.

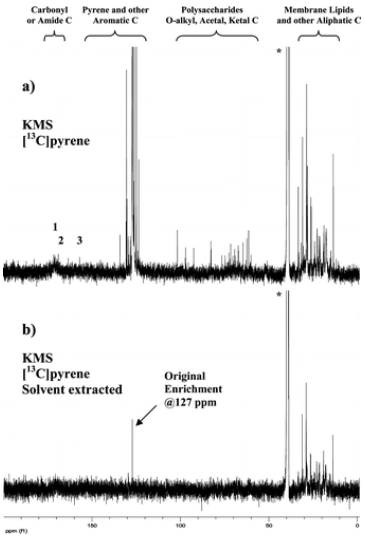

Figure 1 ^{13}C NMR spectra of humic acid/humin fraction from *Mycobacteria* sp. KMS incubated with [^{13}C]pyrene (a) without and (b) with solvent extraction. *DMSO septuplet set to 39.4 ppm. Refer to Table 2 for assignments of numbered signals.

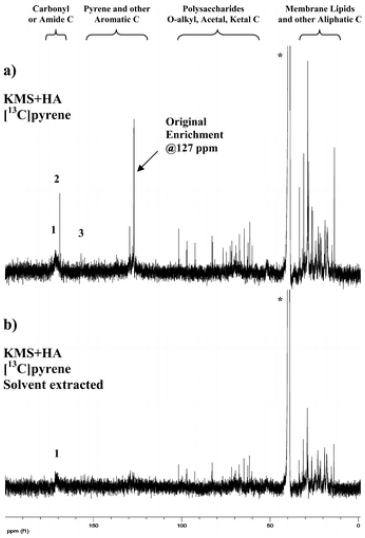

Figure 2 $^{1}3C$ NMR spectra of humic acid/humin fraction from *Mycobacteria* sp. KMS and IHSS soil humic acid incubated with [^{13}C]pyrene (a) without and (b) with solvent

extraction. *DMSO septuplet set to 39.4 ppm. Refer to Table 2 for assignments of numbered signals.

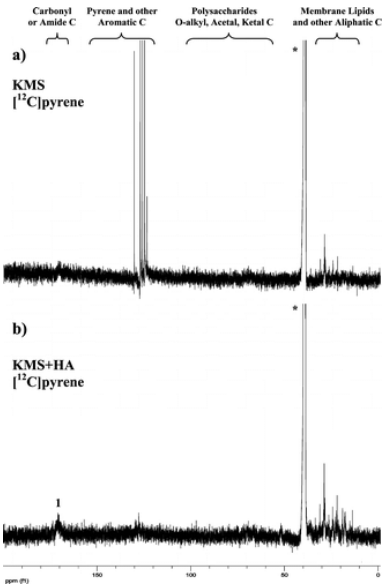

Figure 3 ^{13}C NMR spectra of humic acid/humin fraction from (a) *Mycobacteria* sp. KMS incubated with [^{12}C]pyrene and (b) *Mycobacteria* sp. KMS and IHSS soil humic acid incubated with [^{12}C]pyrene. *DMSO septuplet set to 39.4 ppm. Refer to Table 2 for assignments of numbered signals.

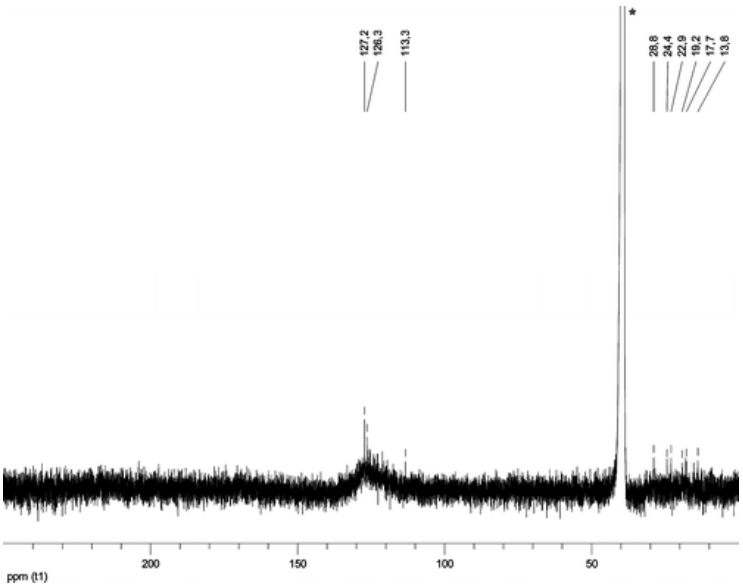

Figure 4 ^{13}C NMR spectrum of humic acid extracted from a 1 g sample of Libby LTU soil incubated with 10 mg of [^{13}C]pyrene for 1128 days at 30 **°C (repetition 2).** The percentage of applied ^{14}C associated with the humic acid of duplicate radiolabeled surrogate microcosms was 3.1 and 3.4%. The percentage of ^{14}C trapped in $^{14}CO_2$ traps during the incubation period was 38 and 69%, respectively. *DMSO septuplet set to 39.4 ppm.

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Results and Discussion

Radiolabeled Pyrene.

Over the 56-day incubation, all radiolabeled treatments produced $^{14}CO_2$ indicating significant biological activity. All cultures contained approximately 1×10^8 cells/mL based on plate counts. Mass balance results are presented in Table 1. ANOVA analysis of replicate treatments (n = 4, α = 0.5) indicated significant differences between KMS and KMS + HA treatments, and subsequent t-tests showed cultures with only bacteria had statistically higher $^{14}CO_2$ recovery (29.6% vs 25.1%), solution phase activity (23.7% vs 20.5%), and total recovery (55.9% vs 45.9%) than the cultures containing humic acid. Relatively low total recoveries were likely the result of inefficient CO_2 trapping given the large headspace, small volume of the CO_2 trap, and repeated opening of the system to change the traps. Preliminary sorption studies with humic acid and [^{14}C]pyrene had recoveries greater than 90% (data not shown). It should be noted that the $^{14}CO_2$ trapping was used primarily as an indicator of biological activity and that characterization of the approximately 20% of added label remaining associated with the enriched aqueous culture was the focus of the study.

Table 1. Mass Balance Results from Separations[a] of Radiolabeled Cultures Containing Either Bacteria Only (KMS) or Bacteria and Humic Acid (KMS + HA)

	KMS	KMS + HA	p-value (α = 0.5)
Mass Balance			
recovered as CO_2[a]	29.6	25.1	0.007
recovered in flask[e]	2.6	0.3	**>0.05**
in solution at pH 11.5[b]	23.7	20.5	0.037
total recovery	55.9	45.9	0.003
HA Separation (pH < 2)			
fulvic acid[d]	7.9	7.3	**>0.05**
humic acid/humin[c]	15.8	13.2	**>0.05**
HA Separation and Solvent Extraction			
fulvic acid[d]	7.7	7.1	**>0.05**
humic acid/humin[c]	16.0	13.4	**>0.05**
humic acid/humin, nonextractable[d]	9.4[†]	9.8	**>0.05**

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High pH Separation (pH > 11)			
humin[c]	16.7	11.7	0.013
supernatant[d]	6.9	8.8	0.013
fulvic acid[d]	7.9	7.8	**>0.05**
humic acid[e]	−1.0	1.0	0.001

[a] The three separations conducted on aliquots from each culture were a humic acid/humin separation, a humic acid/humin separation followed by solvent extraction of the humic acid/humin fraction, and a high pH centrifugal separation followed by humic acid separation of the supernatant. All numbers represent percentages of the initial ^{14}C activity. *p*-Values are reported where statistically significant differences between treatments were observed ($\alpha = 0.05$, $n = 4$). Significant differences between percentages of ^{14}C recovered under different separations are indicated by superscript roman letters in the table body. Different letters indicate significant differences. [†]$n = 3$.

Distribution of the radiolabel remaining in the cultures under the three separation scenarios is reported in Table 1. The results indicated that approximately 13−16% of the initial radiolabel was associated with the acid insoluble humic acid/humin fraction of the culture regardless of the presence of additional humic acid. Table 1 also shows that the percentage of radioactivity associated with the aqueous (fulvic acid or supernatant) and solid (humic acid/humin or humin) phase after centrifugation was statistically the same regardless of the pH during centrifugation, suggesting that the activity was primarily associated with the operationally defined humin component of the culture. When the culture was separated at high pH to keep the humic acid in solution, simulating a base humic/fulvic acid extraction from soil, the supernatant of the KMS + HA culture contained more activity compared to the KMS culture (8.8% vs 6.9%). Subsequent acid precipitation showed that approximately 1.0% of the original ^{14}C was associated with the humic acid fraction from the KMS + HA culture, indicating that some metabolites may have reacted with the added humic acid.

To evaluate unextractable or bound residues, the solid-phase humic acid/humin fraction isolated in the acidic separation was extracted with a mixture of acetone/hexane (1:1). Approximately 10% of the initial activity was found to be unextractable. The presence of added humic acid in the culture did not have a statistically significant effect on the unextractable fraction.

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^{13}C NMR.

Samples analyzed by ^{13}C NMR included the humic acid/humin fraction of KMS and KMS + HA cultures. Humic acid/humin fractions that had undergone solvent extraction were also analyzed. Spectra of unextracted and extracted humic acid/humin fractions are shown in Figures 1 and 2 for KMS and KMS + HA treatments, respectively. Cultures incubated with [^{13}C]pyrene were compared to cultures incubated with unenriched pyrene to assess the potential for incorporation of the pyrene carbon into cell biomass or into humic material. Spectra from humic acid/humin fractions isolated from these unenriched cultures of KMS and KMS + HA are shown in Figure 3. Numbered signals correspond to entries in Table 2 that lists the observed signals in each sample and proposed signal assignments. Expansions of all spectra with detailed signal assignments are shown in Figures S-2−S-5 of the Supporting Information with signal comparisons given in Table S-1.

Table 2. Observed ^{13}C NMR Signals and Proposed Signal Assignments[a]

signal no.	KMS-^{13}C	KMS+HA-^{13}C	KMS-^{12}C	KMS+HA-^{12}C	KMS-^{13}C extracted	KMS+HA^{13}C extracted	signal assignment[b]
1	171.4	171.5		170.7		171.5	carbonyl or amide carbon
2	169.0	169.0					
3	156.6	156.9				aromatic carbon	
4		134.2					
5		130.7					
6		130.5	130.4				pyrene
7		130.2					
8		129.5	129.5			129.0	
9		128.3					
10			127.9	127.8			
11		127.4					*d*
12[c]		127.2	127.1	127.1	127.2		pyrene[c]
13		126.9					*d*
14		126.5					
15		126.3					
16		126.0	126.0				pyrene
17		124.9	124.8				pyrene
18		123.6	123.5				pyrene

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signals 19−33 from 101 to 60 ppm	yes	yes	not obsd	not obsd	not obsd	yes	O-alky, acetal, and ketal carbon polysaccharides
signals 34−52 from 34 to 14 ppm	yes	yes	yes	yes	yes	yes	methyl and alkyl carbon membrane lipids, waxes, and other aliphatics

[a] A complete listing of observed signals is found in the Supporting Information, Table S-1.[b] Based on ref *26*. Interpretation of pyrene signals is based on standards of [^{12}C]- and [^{13}C]pyrene sorbed to humic acid (Supporting Information, Figures S-11 and S-12.) [c] Signal from enriched pyrene.[d] Probable spinning sidebands based on size and symmetry.

Unenriched samples displayed a total of 19 distinct signals, 5 of which were from pyrene, while 52 signals were observed in enriched samples. This indicates that the carbon originally associated with pyrene was incorporated into a wide variety of products with distinct chemical structures and does not suggest that a primary reaction mechanism was dominant with regard to pyrene degradation and the fate of the associated carbon. The majority of enriched signals fell into two groups having chemical shifts of 13−34 ppm and 60−102 ppm, likely associated with aliphatic carbons in bacterial membranes and polysaccharides, respectively, as indicated in Table 2.[26] Similar signals were observed in ^{13}C NMR spectra for biofilms of *Pseudomonas aeruginosa* by Mayer et al.[27] indicating the presence of carboxyl (~175 ppm), hydroxyl (~80 ppm), acetal (~110 ppm), and various methyl and methylene groups (~10−40 ppm). Baldock et al.[28] also associated the increase in O-alkyl (45−120 ppm) and alkyl (0−45 ppm) signals in CP/MAS ^{13}C NMR analysis of a soil enriched with [^{13}C]glucose with an increase in bacterial polysaccharide and lipid structures observed in the soil as the added glucose was degraded. Pyrene metabolites as observed by Guthrie-Nichols et al.[22] may have also contributed to the observed signals.

Enriched signals in cultures incubated with added humic acid were very similar to those observed in cultures with only bacteria (compare Figures 1(a) and 2(a)), with the exception of three small signals at 127.9, 27.2, and 22.4 ppm found in the KMS + HA incubation but not in the KMS incubation (Supporting Information, Table S-1). This suggests that many products of catabolic reactions within the cell were not available for reaction with humic acid components, which could be possible if pyrene metabolites were

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actively excreted from the cell. Instead, these data suggest that pyrene associated carbon was incorporated into cellular material regardless of the presence of humic acid in the culture.

Signals observed in the 170 ppm range for unextracted ^{13}C enriched incubations (Figures 1 and 2) and the ^{12}C incubation containing humic acid (Figure 3) are likely associated with carbonyl carbons in carboxyl, ester, or amide bonds as proposed by Mayer et al.[27] or with pyrene metabolites as observed by Guthrie-Nichols et al.[22] Signal 2 (Table 2) at 169 ppm was prominently observed in KMS + HA incubations (Figures 2(a) and also S-7(b)) and could indicate reaction of pyrene metabolites with the humic acid via covalent mechanisms as suggested by Richnow et al.[19] and Bortiatynski et al.[15] The significant presence of this signal in the humic acid fraction isolated from the high pH supernatant (Figure S-7(b)) despite the low level of radioactivity associated with this fraction (1.0% in Table 1) supports the conclusion that reaction with the added humic acid may have occurred; however, this signal was not observable in the solvent extracted sample isolated from the KMS + HA culture (Figure 2(b)).

With regard to the potential for bound residue formation, the solvent extracted sample of the humic acid/humin fraction from the KMS culture (Figure 1(b)) displayed fewer signals than the unextracted sample (Figure 1(b)). Noticeably absent were the unenriched carbon signals from the added pyrene centered at approximately 127 ppm with only the signal from the ^{13}C enriched carbon at 127.2 ppm being observed. Signals in the 60−102 ppm range were apparently removed by the extraction, while the majority of signals in the 13−34 ppm range were present in the extracted sample. Extraction of the KMS + HA humic acid/humin fraction likewise resulted in a reduction of the number of observed signals and the disappearance of the pyrene signal, but the majority of signals present in the unextracted KMS + HA sample were still observable (Figure 2). As noted above, one significant exception was the prominent signal at 169 ppm (Figure 2(a)) that was present in the KMS + HA sample but absent in the extracted sample indicating that the associated pyrene degradation product may not have contributed to the bound fraction under these experimental conditions. Pyrene metabolites associated with signals that were present in the extracted humic acid/humin of KMS + HA, but

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not in that of KMS treatments, are likely minor contributors to the total bound carbon based on results of the ^{14}C incubations. Overall, the general persistence of the enriched signals in the humic acid containing cultures and the approximately 10% of initially added radioactivity that was unextractable, regardless of the presence of humic acid, indicate a significant potential for the enriched cellular material to contribute to the residual or bound carbon fraction after biodegradation.

Results of this study suggest that there is little evidence to show that pyrene metabolites became associated with the IHSS humic acid through any preferential reactions mechanisms under our experimental conditions, although evidence was found of metabolite association with humic acid in both the radiolabeled fractionation study and the ^{13}C NMR analysis. Instead, the humic acid/humin fraction that was isolated from the final cultures appears to have chemical characteristics similar to bacterial biomass supporting the idea that the pyrene had been incorporated into cellular material. This may be a primary mechanism for bound residue formation with hydrophobic, persistent, organic pollutants such as pyrene.

Although many studies have shown the potential for enzymatically catalyzed reactions resulting in coupling with humic material,[10] it is not known whether these reactions play a significant role in the binding of metabolic products of PAHs in natural soil systems. Richnow et al.[19,20] did find evidence of ester bond formation from bacterial anthracene degradation in a soil system, and Weber and Huang[18] found evidence of phenanthrene-phenol coupling in an in vitro system containing free enzymes. Ressler et al.[29] also reported the formation of a humic acid like organic acid fraction during the liquid culture degradation of a mixture of PAHs by an undefined enrichment culture from contaminated soils, supporting the potential for residue forma tion via extracellular metabolite reactions.

Because the bacterial enzymes responsible for PAH degradation are most likely intracellular, based on the increased oxidizing capacity of cell-free extracts,[30] it is likely that many intracellular oxidation products remain in the cell and enter subsequent anabolic and catabolic cycles. This conclusion is supported by the finding that only

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2−3% of ^{13}C labeled acenaphthene metabolites from degradation by single and mixed cultures could be identified in solvent extracts of whole cultures with some cultures showing no extractable product accumulation.[31] Richnow et al.[32] also found that 0.9% (11% of the bound residue) of ^{13}C added as phenanthrene was "bound" as amino acids indicating that incorporation into cellular biomass may be a significant mechanism of bound residue formation. Baldock et al.[28] reported on a similar mechanism by which ^{13}C[glucose] was incorporated into humic material via apparent microbial anabolism. Likewise, Mayaudon and Simonart[33] described the incorporation of ^{14}C labeled *Azotobacter* and *Aspergillus* into humic and fulvic acid fractions and humin of a pasture soil indicating that bacterial and fungal anabolism may lead to humified residues in soil systems. Finally, Guthrie-Nichols et al.[22] also concluded that [^{13}C]pyrene incubated in an aerobic sediment system was incorporated into biomass polymers.

Our data support the hypothesis that a significant amount of the insoluble residual carbon remaining after PAH degradation may be in the form of cellular biomass or biomass associated metabolites that are subject to subsequent humification reactions as opposed to a more direct coupling mechanism of extracellular PAH metabolites with soil humic material. The ^{13}C NMR spectrum of humic acid isolated from a PAH contaminated Superfund site soil after 1128 days of incubation with [^{13}C]pyrene also showed signals in the 13−34 ppm region (Figure 4) indicating the potential for a similar bound residue producing mechanism in field contaminated soils. These signals were not observed in the humic acid of a poisoned soil microcosm (Figure S-10). This mechanism may be specific to bacterial degradation of hydrophobic PAHs by organisms capable of complete mineralization which is thought to be the primary mechanism of PAH oxidation in engineered land treatment systems.

The potential for bound residue formation via cellular incorporation supports the continued acceptance of bound residues as alternative endpoints of the bioremediation process. These data support previous research indicating that the humification or binding process is an active contributor to contaminant and toxicity reduction in contaminated soil systems. Further research is needed to characterize degradation and residue formation mechanisms with other

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contaminants, bacteria, and humic materials and to further define the contributing roles of cellular incorpora tion and extracellular metabolite reaction in the humification process for PAHs and other hydrophobic contaminants.

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Supporting Information Available

Expanded NMR spectra, detailed signal assignments, ad ditional spectra, and a schematic of the experimental design are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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