[14C]Pyrene Bound Residue Evaluation Using MIBK Fractionation Method for Creosote-Contaminated Soil

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The fate of [14C]pyrene was evaluated in creosote-contaminated soil undergoing remediation in a prepared bed system at the Champion International Superfund site in Libby, MT. 14C-bound residue formation was evaluated using the methyl isobutyl ketone (MIBK) humic fractionation procedure, and it increased through 294 days of incubation in biologically active microcosms for humic acid, fulvic acid, bound humic acid, and mineral-associated organic carbon fractions. The relative affinity of the added pyrene and transformation products was highest for the humic acid fraction. Bound residue formation in PAH-contaminated soil was observed to be an important fate mechanism in the prepared bed system and may be an acceptable end point in the remediation of contaminated soil.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of environmental concern because of their potential carcinogenicity and their persistence in the soil environment (*1*). Laboratory studies have shown that significant fractions of 14C, added as 14C-bound PAHs, are not extractable by standard extraction methods (e.g., Soxhlet extraction). These bound residues may become associated with components of the soil matrix through several mechanisms including covalent bonding through biologically and abiotically mediated oxidative coupling reactions to humic substances (*2−4*) and intraparticle or intraorganic matter diffusion into organic soil components (*5*). Assessment of the nature and formation of PAH-bound residues and their associations with soil components is of interest with regard to the management of this process for soil detoxification and in the assessment of the ultimate fate and risk associated with PAH residues in soil.

Bound residues of pesticides have been defined as "that unextractable and chemically unidentifiable pesticide residue remaining in fulvic acid, humic acid, and humin fractions after exhaustive sequential extraction with nonpolar organic and polar solvents" (*6*). Laboratory studies have shown that bound residue formation of PAHs is a primary fate mechanism of PAHs (*7−10*). Guthrie and Pfaender (*10*) recently

showed that a major fraction (43−80%) of recovered 14C (added to soil as [14C]pyrene) was found in humic/fulvic acid extracts (6.6−26%) or in the residual humin (29−73%), regardless of the microbial ability of the soil to mineralize the compound. Poisoned controls showed less but significant amounts of residue formation (22−45% of recovered 14C). Sims and Abbott (*7*) found similar results with [14C]pyrene and [14C]benzo[*a*]pyrene and concluded that humification within humic/fulvic acid and humin fractions represented the most significant fate mechanism of each PAH in both previously contaminated and clean soils under biologically active and poisoned conditions with the soil humin serving as the dominant sink for PAH residues.

These studies utilized traditional base extraction techniques to separate humic and fulvic acids from the insoluble residual organic matter known as humin, which typically comprises more than 50% of the soil organic matter (*11*). Because it is insoluble at all pH values, humin has not been characterized as extensively as the more readily accessible humic and fulvic acids. To further characterize the organic makeup of humin, Rice and MacCarthy (*12*) developed a fractionation procedure utilizing methyl isobutyl ketone (MIBK) that separates soil humin into extractable lipid (removed by continuous solvent extraction) (*13*), bound lipid, bound humic acid, and insoluble mineral components (*12*). Variations of this method have recently been used to evaluate binding of various PAHs, PCBs, and pesticides to uncontaminated soil humin (*11, 14*), but the method has not been applied to a soil previously contaminated with a complex waste mixture from a field site.

The objectives of this study were to (i) characterize the bound residue formation process of a four-ring PAH ([14C]-pyrene) in a creosote-contaminated soil using the MIBK fractionation method and (ii) evaluate this process with respect to biological activity and time. Results of the study will add information to the mechanisms of PAH attenuation at the Champion International Superfund site.

Experimental Section

Soil. Contaminated soil from the prepared bed land treatment unit 2 (LTU 2) at the Champion International Superfund Site in Libby, MT, was used for this study. The soil had been previously contaminated with a mixture of creosote and pentachlorophenol used as a wood preservative at the site (*15, 16*). The soil was passed through a 1.7-mm sieve and homogenized by hand. The homogenized soil was classified as a loam (50% sand, 38% silt, 12% clay) with an organic carbon content of 1.43% (analysis by Utah State University Soil Testing Laboratory). The soil was stored in the dark at 4 °C until it was used.

Incubations. Soil samples were air-dried and then wet to a moisture content of 9.4% (dry weight basis) (90% field capacity) with deionized water. Eighty-gram (dry weight) samples were placed in biometer flasks (Bellco Glass, Vineland, NJ) and spiked with 2.14 μCi of [4,5,9,10-14C]pyrene with a specific activity of 56 mCi/mmol in 0.5 mL of methanol and mixed by hand with a glass pasteur pipet. The side tube of each biometer flask was then filled with 10 mL of 0.1 M KOH to serve as a trap for 14CO$_2$ evolved from mineralization of [14C]pyrene. The trap solution was periodically exchanged and analyzed for 14C. Polyurethane plugs (Baxter Diagnostics Inc, McGaw Park, IL), without precleaning, were placed within the biometer flasks to trap volatile 14C transformation products (*17*). Flasks were incubated in the dark at 20 °C, and a headspace oxygen concentration between 0 and 5%

was maintained according to the method of Hurst et al. (*8, 9*) to represent field conditions at the Libby site. Poisoned controls (2000 mg/kg $HgCl_2$) were also incubated. Replicate sets of flasks were sacrificed after 41 and 294 days of incubation.

Sequential Extraction. A sequential extraction/mass balance approach was employed to account for added ^{14}C. At the end of the incubation period, the entire content of each flask was extracted with 300 mL of a 5 mM solution of $CaCl_2$ for 24 h at pH 6.5. The soil was allowed to settle for 6 days, after which the aqueous phase was sampled and analyzed for ^{14}C. The aqueous phase was then drawn off, and the soil was allowed to air-dry. Dried soil was Soxhlet extracted with hexane:acetone (1:1) for 16 h. Solvent extracts were analyzed for ^{14}C by liquid scintillation counting and for pyrene by high-pressure liquid chromatography (HPLC). Polyurethane plugs were also Soxhlet extracted by the same method and analyzed for ^{14}C to account for volatile ^{14}C compounds. Solvent-extracted soils were then air-dried and ground. One-gram subsamples were combusted to account for solvent nonextractable ^{14}C residues. Ten-gram subsamples were fractionated by the MIBK method.

MIBK Fractionation Method. A modified form of the MIBK fractionation method of Rice and MacCarthy (*12*) was used to separate different components of the humic material remaining in the solvent extracted soil. Ten-gram soil samples were stirred in 200 mL of 0.5 M NaOH for 24 h to extract humic and fulvic acids. The entire mixture was then placed in a 2-L separatory funnel, and 100 mL of 0.5 M NaOH used to rinse the extraction flask was added. A total of 200 mL of MIBK was then added to the funnel, and the mixture was acidified with 16 mL of concentrated HCl and shaken vigorously by a mechanical mixer for 5 min. The mixture was allowed to separate for 45 min, and the aqueous phase containing residual mineral material and fulvic acid was discharged through the bottom of the flask. Humic acid, which remained suspended in the MIBK, was resolubilized by adding 300 mL of 0.5 M NaOH to the separatory funnel, which was shaken for 5 min and allowed to separate for 45 min. The aqueous phase containing residual mineral material and humic acid was discharged. Finally, 300 mL of deionized water was added to the funnel, shaken, and allowed to separate for 1 h. The aqueous phase, containing residual mineral material and bound-humic acid, was discharged, leaving the bound lipid fraction suspended in the MIBK phase.

Residual mineral material in all humic fractions was allowed to settle for 6 days, after which the aqueous phase of each fraction was sampled for ^{14}C analysis. The bound lipid extract was mixed vigorously before subsampling. Residual mineral material from all fractions was combined and combusted to determine ^{14}C associated with the mineral fraction. Total ^{14}C recovered in the MIBK fractionation for each sample was compared to the nonextractable ^{14}C determined by combustion.

Organic Carbon Analysis of Soil Fractions. To determine the amount of organic carbon present in each organic fraction isolated by the MIBK fractionation, three 80-g samples of soil were extracted sequentially by both solvent and MIBK method extractions. Solvent-extractable, bound humic acid, bound lipid, and mineral-associated organic carbon were determined by combustion with a LECO CHN-1000 analyzer (LECO Corp., St. Joseph, MI) after evaporation of all organic solvent or water from each fraction. Humic acids were precipitated by acidification and analyzed by the same method after being separated by centrifugation. Fulvic acid extracts were analyzed with a Dohrmann DC-180 dissolved organic carbon (DOC) analyzer (Rosemount Inc., Santa Clara, CA) after residual MIBK had been removed by evaporation. Residual MIBK was removed from triplicate distilled water

blanks, which were analyzed for DOC and subtracted from sample concentrations.

Analytical. 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). Correction for quenching was performed by the H-number routine of the scintillation counter software.

Total solvent-nonextractable and mineral-bound ^{14}C were determined by combustion with a Harvey biological oxidizer (R. J. Harvey Instrument Corporation, Hillsdale, NJ). Produced $^{14}CO_2$ was trapped using a mixture of Ready Gel, methanol, and monoethanolamine (50:40:10) (*18*).

Analysis of nonradiolabled pyrene in the solvent extracts was performed by high-pressure liquid chromatography using a Schimadzu 10A HPLC system with SIL-10A autosampler, SPD-10A UV detector at 254 nm (Shimadzu Scientific Instruments Inc., Columbia, MD), and Supelco (Bellefonte, PA) Supelcosil LC$-$PAH column (25.0 cm $\times$ 4.6 mm, 5 μm). An acetonitrile:water mobile phase program (40:60 isocratic to 4 min, gradient to 100% ACN at 30 min, 100% ACN to 40 min, gradient to 40:60 at 45 min) at a flow rate of 1.5 mL/min was used.

Chemicals. Radiolabeled [4,5,9,10-^{14}C]pyrene (95% purity, specific activity = 56 mCi/mmol) was purchased from Amersham International (Buckinghamshire, England). Analytical reagent grade potassium hydroxide (KOH) and sodium hydroxide (NaOH) pellets were purchased from Mallinckrodt Baker Inc. (Paris, KY). Reagent grade mercuric chloride was purchased from J. T. Baker Chemical Company (Phillipsburg, NJ). A.C.S. certified reagent grade methanol, methyl isobutyl ketone, monoethanolamine, and hydrochloric acid and HPLC grade acetonitrile, acetone, and hexane were purchased from Fisher Scientific (Pittsburgh, PA). Filtered (0.2 μm), deionized water was used for all solutions and HPLC analyses.

Results

Bound Residue Initial Distribution. Figure 1 shows the distribution of ^{14}C-bound residues among humic fractions isolated by MIBK fractionation in spiked samples that were immediately extracted (incubation time = 0 days). An average of 3.0 $\pm$ 0.1% (error = 95% confidence interval) of the total added ^{14}C was bound to the soil matrix. The MIBK fractionation showed that the majority of the solvent-nonextractable ^{14}C was associated with the bound lipid and humic acid fractions. Percentages of the radiolabeled carbon spike recovered in the solvent and water extracts were 85.1 $\pm$ 4.9% and 4.9 $\pm$ 4.5%, respectively.

Table 1 shows the percentage of solvent-nonextractable ^{14}C found in each MIBK fraction at incubation times of 0 and 294 days. The bound lipid and humic acid fractions contained the majority of the bound ^{14}C (58.1% and 20.9%, respectively) in time 0 treatments. To evaluate the tendency of the [^{14}C]-pyrene or ^{14}C transformation products to associate with each humic fraction, a relative affinity (RA) value similar to the relative affinity index used by Kohl and Rice (*14*) was calculated using the results of the carbon analysis presented in Table 1. The RA value was calculated by dividing the percentage of nonextractable ^{14}C associated with a fraction by the percentage of nonextractable organic carbon found in that fraction. An RA value greater than 1 indicates that the organic carbon fraction has an elevated tendency to accumulate ^{14}C over other fractions.

RA values for the 0 day incubation treatments are found in Table 1. The humic acid fraction had the highest RA value of 3.2, while other fractions had RA values equal to or less than 1.3, indicating an increased tendency for [^{14}C]pyrene or ^{14}C transformation product association with humic acid over other fractions. The increased association of pyrene or aromatic transformation products with the humic acid is
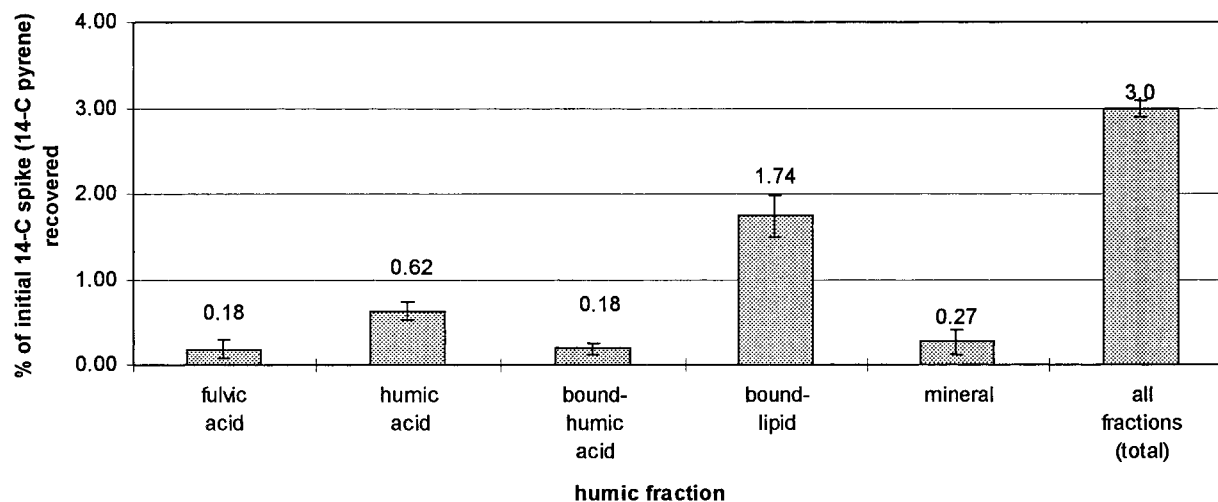
FIGURE 1. Initial percentages of added ^{14}C associated with humic fractions isolated by the MIBK method. Incubation time = 0 days (n = 3, 95% confidence interval shown).

TABLE 1. Percentage of Nonextractable [14]C Associated with Each Fraction Isolated by the MIBK Method at Incubation Times of 0 (n = 3) and 294 (n = 21) Days in Biologically Active Treatments[a]

	fulvic acid	humic acid	bound humic acid	bound lipid	mineral
% of nonextractable[14]C in fraction (t = 0 days)	6.0 (±3.8)	20.9 (±4.1)	6.2 (±2.7)	58.1 (±6.4)	8.9 (±4.8)
% of nonextractable[14]C in fraction (t = 294 days)	12.4 (±0.8)	38.8 (±1.1)	8.0 (±0.5)	20.1 (±1.7)	20.7 (±0.9)
% of nonextractable soil organic carbon in fraction	9.8 (±7.5)	6.6 (±1.5)	4.9 (±0.4)	43.6 (±6.6)	35.1 (±2.0)
RA value (incubation time = 0 days)	0.6	3.2	1.3	1.3	0.3
RA value (incubation time = 294 days)	1.3	5.9	1.6	0.5	0.6

[a] Percentages of nonextractable organic carbon in each fraction and calculated relative affinity (RA) values for each fraction at the two time periods are also shown (95% confidence interval).

likely caused by the high molecular weight and aromaticity commonly associated with humic acids (19).

The RA value of the bound lipid fraction of 1.3 indicates that it had a moderate affinity for the ^{14}C-bound residues in comparison to other fractions. Although the affinity of the bound lipid fraction was not high, it did contain the majority of bound ^{14}C in nonincubated treatments. This was most likely because it contained the highest percentage of non-extractable soil organic carbon (43.6 wt %, Table 1).

One of the fractions that contained the least amount of ^{14}C was the mineral fraction (Table 1), which is similar to the bound lipid fraction in nonextractable organic carbon percentage (35.1%). On the basis of organic carbon content, we would predict that the percentage of ^{14}C associated with the mineral fraction would be similar to that which was associated with the bound lipid fraction. Inaccessibility of the mineral-associated organic carbon or chemical variations among organic carbon fractions may have caused it to accumulate less ^{14}C than would be predicted.

Bound Residue Formation and Biological Activity. The amount and distribution of ^{14}C among carbon fractions in biologically active and poisoned microcosms after 294 days of incubation is shown in Figure 2. In biologically active soil, mineralization of added [^{14}C]pyrene was measured as 45.9 ± 3.02% after 294 days incubation (total ^{14}C recovery = 77.8 ± 1.3%). Poisoned microcosms accumulated less than 0.1% of the added ^{14}C in the CO_2 traps (total ^{14}C recovery = 76.1 ± 1.2%). Consistent with other studies (7, 8, 10), the amount of solvent-nonextractable ^{14}C increased as a result of biological activity.

In addition to the amount of bound residue formation, our results indicate that the distribution of ^{14}C was influenced by biological activity (Figure 2). A statistically significant increase in bound residue formation was observed for soil fractions including fulvic acid, humic acid, bound humic

acid, and residual mineral. The dominant fraction for accumulation of ^{14}C in biologically active soil was the humic acid fraction, which contained 38.8% of the nonextractable [14]C. Although the ^{14}C associated with the bound lipid fraction was significantly higher ($p < 0.05$, n = 21) under biotic conditions (Figure 2), the difference was small indicating that biological activity did not play a major role in bound residue formation within this fraction. Increased RA values for biologically active treatments extracted after 294 days of incubation (Table 1) indicate an increased role of all solvent-nonextractable humic fractions in bound residue formation except for the bound lipid fraction.

Unlike the results of the 0 day incubation analysis, analysis of biologically active microcosms after 294 days of incubation showed that the percentage of ^{14}C associated with the mineral-associated organic carbon was equal to the percentage found in the bound lipid fraction (2.2%) (Figure 2), which is consistent with the similar percentage of organic carbon found in each fraction. Diffusion of pyrene transformation products into organic matter intimately associated with the soil mineral component (20) may explain the increased role of this organic carbon fraction in the formation of bound residues.

Rates of Bound Residue Formation. Rates were calculated based on the changes in distribution of ^{14}C among soil fractions, determined by the MIBK fractionation method, at incubation times of 0, 41, and 294 days in biologically active microcosms (Figure 3). Significant increases in associated radioactivity over time were observed for all humic fractions except for the bound lipid fraction. Based on the data presented in Figure 3, a zero-order rate of 0.022%/day (8.4 × 10^{-9} mmol of pyrene/day) was calculated for the overall biological residue formation process. Zero-order rates of ^{14}C residue formation with bound humic acid, fulvic acid, mineral-associated, and humic acid organic carbon fractions
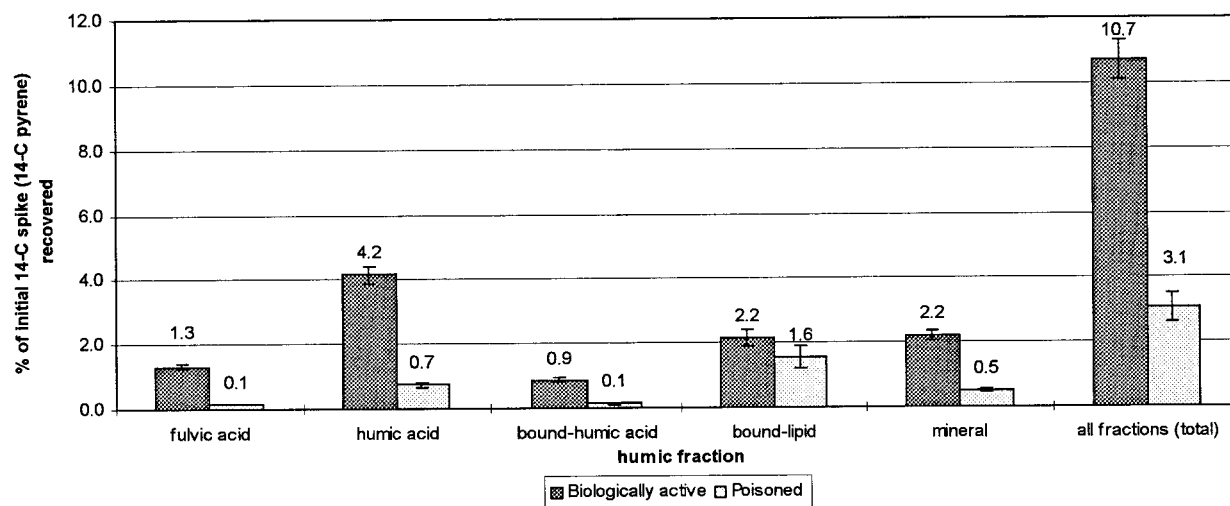
FIGURE 2. Average percentage of added ^{14}C found in each humic fraction in biologically active and poisoned microcosms after 294 days of incubation (95% confidence interval shown, $n = 21$).
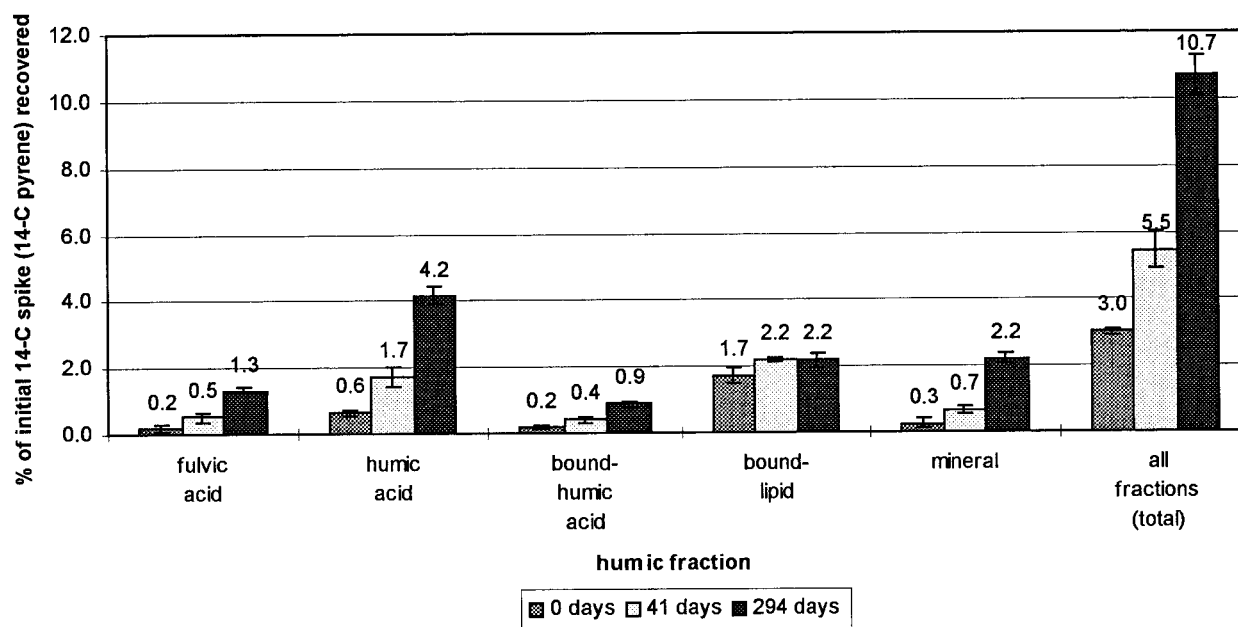

FIGURE 3. Average percentage of added ^{14}C found in each humic fraction after 0 ($n = 3$), 41 ($n = 21$), and 294 ($n = 21$) days of incubation in biologically active microcosms (95% confidence intervals shown).

were 0.002, 0.003, 0.006, and 0.010%/day, respectively. No rate was calculated for residue formation with the bound lipid fraction because incubation time did not significantly affect the percentage of ^{14}C associated with this fraction (Figure 3).

Rates of bound residue formation in poisoned microcosms were not significant because distribution of radioactivity in the humic fractions of poisoned microcosms after 41 and 294 days of incubation were consistent with the distributions at 0 days incubation as shown in Figure 4. This indicates that abiotic residue formation through intraorganic matter and intraparticle diffusion, which are suspected of decreasing substrate bioavailability over time (21), were not observable aging processes in this study. Pyrene or transformation products that had entered the soil matrix through these mechanisms were likely removed by the rigorous solvent extraction.

Discussion

This is the first study to examine bound residue formation of a hydrophobic PAH into soil fractions that include soil humin components of bound humic acid, bound lipid, and residual mineral-associated carbon in a field soil contaminated with a complex creosote waste. It is also the first application of the MIBK fractionation method to a complex waste-contaminated soil. If PAHs and their metabolites become less bioavailable and possibly less toxic because of associations with soil matrix fractions during the aging process (21–24), the approach described in this evaluation may be useful in characterizing bioavailability and toxicity associated with each soil fraction and changes that occur with treatment.

Abiotic Bound Residue Formation. The average ^{14}C found as bound residue in unincubated treatments (3.0%) and poisoned treatments after 294 days (3.1%) was unexpectedly low. Bound ^{14}C determined by combustion in poisoned treatments after 294 days (2.5 ± 0.3%) confirmed that bound residue formation without biological activity was minimal. Previous work with the Libby soil (from LTU 1) resulted in 8.3–12.5% of the added ^{14}C from [^{14}C]pyrene becoming bound to the residual soil (8). Other studies have shown significantly higher amounts of bound residue formation (20–30%) at

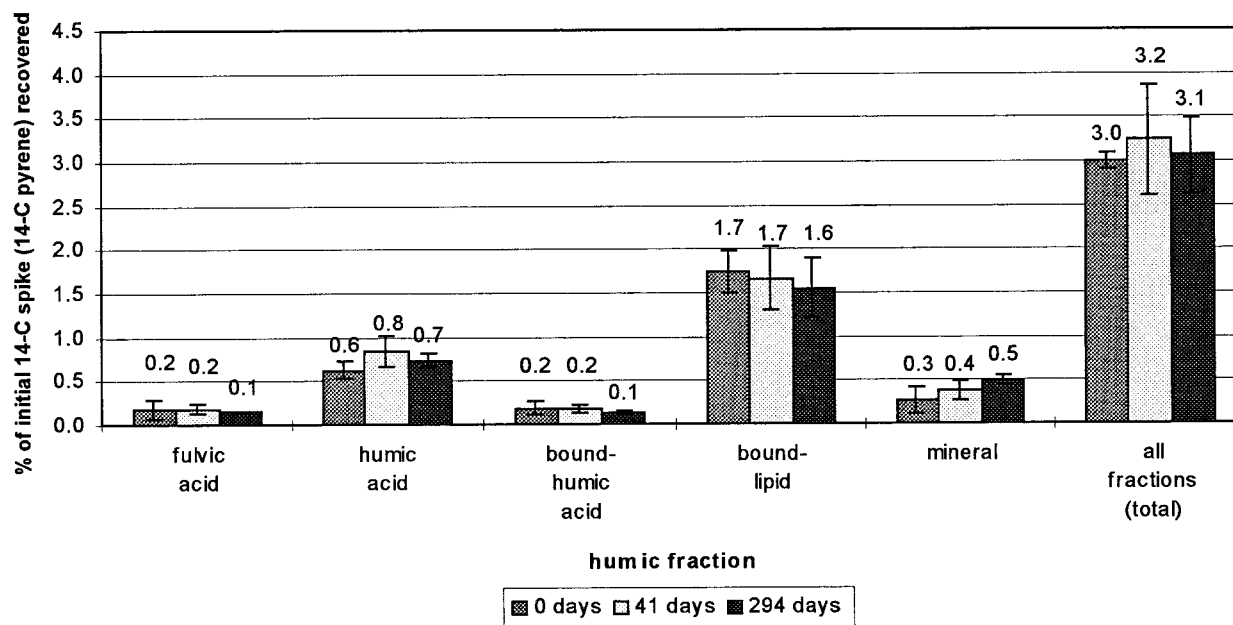FIGURE 4. Average percentage of added ^{14}C found in each humic fraction after 0 ($n = 3$), 41 ($n = 15$), and 294 ($n = 21$) days of incubation in poisoned microcosms (95% confidence intervals shown).

day 0 and at the end of the incubation period in contaminated and uncontaminated soils (7, 10). Specific waste characteristics of the Libby LTU 2 soil may have inhibited binding with the soil matrix, or the hexane:acetone solvent used in the extraction process may have been more efficient in removal of ^{14}C compounds than dichloromethane used in other studies (7, 8, 10).

Results of this study suggest that the bound lipid component of the soil humin was the primary sink of bound ^{14}C under biologically inhibited conditions in the Libby LTU 2 soil. The amount of radiolabeled carbon associated with the bound lipid fraction remained constant over time (Figure 3) and was only slightly higher under biologically active conditions (Figure 2) possibly due to the added association of ^{14}C metabolites. Kohl and Rice (14) found that the bound lipid fraction of humin had a high affinity for benzo[a]pyrene, phenanthrene, and naphthalene in laboratory-contaminated soils in which microbial activity may have been inhibited by contact with dichloromethane used as a carrier for the contaminant. The bound lipid fraction isolated from the contaminated Libby soil did not appear to have a high affinity for pyrene over other humic fractions (Table 1) but was most likely the primary accumulator of ^{14}C in poisoned and unincubated treatments because it contained the highest fraction of soil organic carbon. Association of ^{14}C with the bound lipid fraction may account for the high percentage of nonextractable radiolabeled carbon observed by others (7, 8, 10) under biotic and poisoned conditions at different incubation times.

The percentage of total soil organic carbon isolated as the bound lipid fraction of the contaminated Libby soil was $34.5 \pm 7.3\%$, which is significantly higher than that of uncontaminated soils (4−6%) that have been fractionated by the MIBK method (14). This difference in organic carbon distribution may be caused by the presence of the residual nonaqueous-phase liquids (NAPL) in the contaminated soil or by inherent differences in soil type or differences in extraction procedures. It is known that sorption of organic compounds varies among different humic fractions (25, 26). Variations in the capacity of humic fractions to retain bound residues of hydrophobic contaminants could explain the observed lack of correlation between total soil organic carbon content and measures of bioavailability (24). Quantification

of the bound lipid organic carbon content of a soil could be an indicator of the soil capacity to bind PAH residues through abiotic mechanisms.

Biological Bound Residue Formation. The humic acid fraction of soil organic carbon was the primary accumulator of ^{14}C activity in biologically active microcosms, although an increase was observed in all organic carbon fractions over time. Pyrene was shown to be biologically transformed and mineralized through (1) active ^{14}CO$_2$ production in biotic microcosms, (2) an increase in water-extractable ^{14}C from $0.5 \pm 0.03\%$ in poisoned treatments to $3.1 \pm 0.5\%$ in biotic treatments indicating production of polar metabolites, and (3) a decrease in solvent-extractable nonradiolabeled pyrene from 6.57 ± 0.2 mg/kg in poisoned microcosms to 2.14 ± 0.1 mg/kg in biotic microcosms. Radiolabeled carbon associated with the humic acid and other organic carbon fractions are most likely transformation products of the added [^{14}C]pyrene rather than the parent compound, since similar increases in organic carbon-associated ^{14}C were not observed in poisoned microcosms. These bound products may range from simple transformation products, which could be released in forms similar to pyrene, to more extensively degraded metabolites.

The data of Guthrie and Pfaender (10) showed a similar increase in ^{14}C activity in combined humic/fulvic acid extracts and soil humin after 270 days of incubation in biologically active microcosms. The humin fraction of the soil studied by Guthrie and Pfaender was the primary sink for solvent-nonextractable ^{14}C while the humin fraction of the Libby soil played a less significant role in bound residue formation. Pyrene−NAPL interactions (5) in the contaminated Libby soil may have significantly affected the formation and distribution of bound residues in comparison to residues formed in uncontaminated or less contaminated soils.

Association of pyrene metabolites with humic acid and other organic carbon fractions due to biological activity may be due to covalent and noncovalent bonding (27). Covalent bonding through oxidative coupling would result in stable metabolite−organic matter complexes that would likely be low in bioavailability and toxicity (2, 3, 23). Noncovalent metabolite−organic matter interactions may allow the release of metabolic products at very slow rates, allowing them to become bioavailable and be mineralized by the microbial community. Resistance to sequential water and solvent

extractions indicate that pyrene metabolite−organic matter associations that increased under biologically active conditions may be covalent in nature.

The risk associated with bound residues of PAHs are related to their chemical form and potential for release into the environment. Guthrie and Pfaender showed that ^{14}C residues of pyrene associated with humic/fulvic acid extracts and residual soil after acid digestion were not readily bioavailable to a pyrene-mineralizing microbial community (10). Eschenbach et al. (28) also recently showed that bound PAH residues, including those from pyrene, were degraded at limited rates similar to the natural humus turnover rate and that the residue extractability did not increase after exposure to extreme environmental changes such as freezing/thawing or wetting/drying cycles. Chemical treatment with EDTA did however release ^{14}C activity that was concluded to be attached to colloidal or dissolved organic matter (28).

Because of differences in extraction techniques, soil/waste characteristics, and microbial communities, comparison of study results involving bound residues from PAHs is difficult. It can be concluded however that the biologically mediated formation of PAH residues can play an important role in the reduction of PAH concentrations in contaminated soils as determined by standard extraction techniques. Further research is needed to assess differences in bound residue formation in different soil types, as assessed by the MIBK fractionation method, and to determine the form and toxicity of these bound residues.

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