

Bioassay-Directed Fractionation and Chemical Identification of Mutagens in Bioremediated Soils

Lance R. Brooks,¹ Thomas J. Hughes,¹ Larry D. Claxton,¹ Barry Austern,² Richard Brenner,² and Fran Kremer²

¹Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina; ²National Risk Management Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio

Soil from a Superfund site (Reilly Tar Site, St. Louis Park, Minnesota) contaminated with polycyclic aromatic hydrocarbons (PAHs) from creosote was treated with several bioremediation technologies including bioslurry (BS), biopile (BP), compost (CMP), and land treatment (LT). These treatment technologies are being evaluated in pilot scale laboratory systems by the U.S. Environmental Protection Agency's National Risk Management Research Laboratory in Cincinnati, Ohio. To evaluate the genotoxicity and identify the mutagens in the soil before and after the various treatments, fractionated extracts of five soils were bioassayed for mutagenic activity with a micro-suspension modification of the *Salmonella* histidine reversion assay. Soils were extracted by sonication using dichloromethane (DCM). The five extracts were fractionated in triplicate (two for bioassay and one for chemical analysis) by reverse-phase high-performance liquid chromatography (HPLC) using hexane/DCM/methanol, and the fractions for bioassay were solvent-exchanged into dimethyl sulfoxide by nitrogen evaporation. Forty HPLC fractions for each sample were bioassayed in strain YG1041 with and without exogenous liver metabolic activation. As shown in a companion paper, the mutagenicity of two treatments (BS and BP) was significantly greater than the mutagenicity of the untreated soil. Mutagenic fractions (> 500 revertants) were analyzed by gas chromatography/mass spectrometry (GC/MS). PAH analysis of the soils indicated that all treatments were effective in reducing the total PAH concentration (48–74%). Qualitative GC/MS analysis of the mutagenic fractions from the BS and BP treatments indicated that they contained azarenes, which are mutagens. The CMP and LT processes were the most effective and least toxic bioremediation procedures based on mutagenic potency and chemical analysis. This research demonstrated that the combination of bioassays and chemical analysis provided a more accurate determination of toxicity in these complex environmental mixtures. — *Environ Health Perspect* 106(Suppl 6):1435–1440 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1435-1440brooks/abstract.html>

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There are over 700 wood-preserving facilities documented in the United States (1,2). Many designated Superfund sites are a result of wood treatment activities involving creosote (1,2). During the pressure

treatment of wood products, excess creosote is released from the treated materials, and the leaching of creosote wastes from treated materials contaminating the soil and groundwater has been common (1,2). One

such contaminated site is in St. Louis Park, Minnesota, the former site of the Reilly Tar and Chemical Corporation's coal tar distillation and wood preserving plant. From 1917 to 1972, dumping from this plant contaminated about 80 acres of soil and the underlying groundwater with creosote wood-preserving waste (3). In 1978, the Minnesota Department of Health discovered significant concentrations of polycyclic aromatic hydrocarbons (PAHs) in six municipal drinking water wells near the Reilly Tar Site plant (3). Currently, St. Louis Park is pumping and treating the contaminated groundwater plume leaching from the creosote-contaminated soil and has placed a soil cover with grass over the contaminated soil.

To reduce the length of time required for pump and treat operations, the U.S. Environmental Protection Agency (U.S. EPA) National Risk Management Research Laboratory (Cincinnati, Ohio) is evaluating various bioremediation technologies for their efficiency in removing PAHs and reducing toxicity of soil collected from St. Louis Park. The bioremediation technologies that are being examined in pilot-scale laboratory studies are bioslurry (BS), biopile (BP), compost (CMP), and land treatment (LT). These technologies are presented and described by Hughes et al. (4). The goal of these bioremediation technologies is to reduce the PAH concentrations and toxicity of contaminated soils. The PAH concentrations were chemically monitored throughout this study. The mutagenic activity of the soil extracts was measured in the untreated soil (UTS) and in the four treatment soils at end of the study. Mutagenic activity was measured using the *Salmonella* mutagenicity assay developed by Maron and Ames (5). The bioassay indicated higher mutagenicity in the BS (163.3×10^6 revertants [rev]/kg dry soil) and BP (3.0×10^6 rev/kg dry soil) treatments than in the UTS (0.008×10^6 rev/kg dry soil) (4). A significant increase in mutagenicity in the CMP and LT extracts

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Address correspondence to L.R. Brooks, Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, MD-68, Research Triangle Park, NC 27711. Telephone: (919) 541-1365. Fax: (919) 541-3966. E-mail: brooks.lance@epamail.epa.gov

Abbreviations used: BP, biopile; BS, bioslurry; CMP, compost; DCM, dichloromethane; DMSO, dimethyl sulfoxide; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LT, land treatment extract; rev, revertants; +S9, with aroclor-induced rat liver; -S9, without aroclor-induced rat liver; PAH, polycyclic aromatic hydrocarbon; U.S. EPA, U.S. Environmental Protection Agency; UTS, untreated creosote-contaminated soil.

compared to the UTS was not detected (4). In this study the mutagenic BS and BP extracts, along with the UTS extracts, were fractionated by high-performance liquid chromatography (HPLC) and the resulting mutagenic fractions were analyzed by gas chromatography/mass spectrometry (GC/MS) to identify the mutagens present in the BS and BP.

Materials and Methods

Soil Extraction

Five soil samples from the end of the study (UTS, BS, BP, CMP, and LT) were placed individually in glass jars, as described by Hughes et al. (4). Twice the volume of dichloromethane (DCM) (GC grade, Burdick & Jackson, Muskegon, Michigan) was added to each jar. Each sample was sonicated for 15 min and the extract was decanted into a glass collection vessel. This procedure was repeated twice more. Each organic extract was then dried of water with sodium sulfate and filtered through a 0.45- μ m Teflon filter (Millipore, Marlborough, Massachusetts). Sample volume was reduced by rotoevaporation and normalized to 25 ml. The concentration of each extract was then determined by gravimetric analysis (milligrams of organics per milliliter DCM).

Polycyclic Aromatic Hydrocarbon Analysis

A 40-ml volatile organic analysis vial served as the extraction vessel. Four grams of the soil was mixed with 10 g sodium sulfate and 1000 μ g surrogate (2-fluorobiphenyl) (two to six replicates of each treatment were used for analysis). After mixing the soil and sodium sulfate, 20 ml of a DCM/acetone extraction solvent mixture was added to the vial. The vial was then placed on a reciprocating shaker for 18 hr. After the vial was centrifuged at 1500 rpm for 30 min, a 1-ml aliquot was removed for GC analysis. U.S. EPA method 8100 (6) was used to quantify 19 PAHs in the sample extracts. A Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard, Wilmington, Delaware), a Supelco SPB-5 column (Supelco, Bellefonte, Pennsylvania) (30 m \times 0.53 mm, 0.50- μ m film thickness), and a flame ionization detector were used for this analysis.

High-Performance Liquid Chromatography Fractionation

For fractionation, 5 mg of each of the five soil extracts was injected onto a silica column (Econsil Silica, 10 μ m, 250 \times 10

mm, Alltech, Deerfield, Illinois). The extracts were eluted at 7 ml/min using a Waters 600E HPLC (Millipore) equipped with a 717 autosampler, a M996 photodiode array detector, and an Isco Foxy Jr. (Isco, Lincoln, Nebraska) fraction collector. The gradient initially was 99% hexane (HPLC grade, Burdick & Jackson) and 1% DCM, which was held for 5 min. This was followed by a linear gradient to 100% DCM in 20 min and then held for 1 min. This was followed by a linear gradient to 100% methanol (HPLC grade, Burdick & Jackson) in 5 min and then held for 10 min. Fractions were collected at the rate of 1/min during the 40-min run in 8-ml vials containing 5 μ l dimethyl sulfoxide (DMSO) for bioassay. The vials contained no DMSO when collected for chemical analysis. The resulting fractions were then concentrated by nitrogen evaporation.

Mutagenicity Assay

Whole extracts of the five soils (UTS, BS, BP, CMP, LT) were bioassayed in the plate incorporation assay using strains YG1041 and YG1042 with and without exogenous mammalian metabolic activation (S9) (4). The HPLC fractions were bioassayed in a microsuspension modification of the *Salmonella* mutagenicity assay (4,7) using strain YG1041. Hughes et al. (4) showed that YG1041 was the most sensitive strain for detecting mutagenicity of the whole extracts from the Reilly Tar Site. The extracts and fractions were bioassayed with arochlor-induced rat liver (+S9) and without arochlor-induced rat liver (–S9) metabolic activation according to the method of DeMarini (8). Briefly, 100 μ l of 10-fold concentrated cells in buffer (from 16-hr cultures) and 100 μ l of 0.015 M phosphate buffer or 100 μ l S9 mix (6%), pH 7.2, were added to each fraction that had been solvent-exchanged into 5 μ l DMSO. Contents of the tubes were mixed, incubated at 37°C for 90 min, then poured onto minimal medium plates. 2-Nitrofluorene (0.3 μ g/plate) served as the positive control for YG1041 (–S9) and yielded a mean of 204 rev. 2-Aminoanthracene was the positive control for YG1041 (+S9) with 0.25 μ g yielding 738 rev. Solvent (DMSO) control values for YG1041 were 109 (–S9) and 106 (+S9). A laboratory blank (DCM) was also tested as a control for the HPLC system.

Gas Chromatography/Mass Spectrometry Analysis

For qualitative GC/MS analysis, a total of 25 mg for each extract (UTS, BS, BP) was

fractionated. The HPLC fractions were evaporated to dryness and then dissolved into 100 μ l DCM. The fractions were then analyzed using a Hewlett-Packard 5890 gas chromatograph with a 5973 mass spectrometer interfaced to a dedicated data system. The gas chromatograph oven contained an HPB-5ms column (30 m \times 0.25 mm, 0.25- μ m film thickness) (Hewlett Packard), which was at 40°C initially and then increased to 300°C at a rate of 5°C/min. GC/MS data were acquired from 35 to 500 atomic mass units. The results interpretation was done on the basis of data collected in a computer library containing 275,821 mass spectrometry spectra (9). All identifications are tentative, as no authentic standards were used for comparison.

Results

Polycyclic aromatic hydrocarbon analysis of the soils before and after each treatment demonstrated observable decreases (from 48 to 74%) in the concentrations of priority pollutant PAHs, as seen in Table 1. The greatest reduction (59 to 92%) was in the two- and three-ring PAHs for all treatments. The next greatest reduction (40 to 75%) was in the four-ring PAHs. The percent reduction (–10 to 20%) in the five- and six-ring PAHs indicated no real change in these concentrations. Higher-ring PAHs take longer to be degraded (10) because of the induction of complex biodegradation enzymes in microorganisms, low solubility, and sorption to the soil. The largest average reduction in total PAHs for the four extracts tested in the mutagenicity assay was in the CMP (74%), followed by the BS (62%), LT (62%), and BP treatments (48%).

After the bioassay of each fraction was completed, the resulting mutagenicity profile of the HPLC fractions constituted a mutagram (11). Mutagrams from all four bioremediation treatments, UTS, and a solvent (DCM) method blank, bioassayed in the *Salmonella* mutagenicity assay in YG1041 +S9 and –S9, are shown in Figures 1 and 2. The average mutagenicity of the solvent blank fractions was 138 \pm 55 rev +S9 and 131 \pm 41 rev –S9. A treatment fraction was not considered mutagenic unless it was \geq 500 rev (greater than three times the solvent blank) per fraction. The BS treatment had the most mutagenic fractions (both revertants per fraction and the number of fractions) both +S9 and –S9. The BP treatment showed less mutagenicity (both revertants per fraction and the

Table 1. Polycyclic aromatic hydrocarbon concentration of the four treatment soils.^a

Soil treatment	Average initial concentration, mg/kg soil	Average final concentration, mg/kg soil	Percent reduction
Bioslurry			
Two- and three-ring PAHs ^b	443 ± 62	114 ± 75	74
Four-ring PAHs ^c	874 ± 219	217 ± 81	75
Five- and six-ring PAHs ^d	388 ± 90	309 ± 71	20
Total PAHs	1705 ± 361	640 ± 161	62
Biopile			
Two- and three-ring PAHs	1490 ± 183	458 ± 159	69
Four-ring PAHs	1094 ± 85	651 ± 253	40
Five- and six-ring PAHs	408 ± 75	450 ± 49	[10] ^e
Total PAHs	2992 ± 326	1559 ± 726	48
Compost			
Two- and three-ring PAHs	2628 ± 57	217 ± 23	92
Four-ring PAHs	1245 ± 35	468 ± 9	62
Five- and six-ring PAHs	444 ± 40	457 ± 3	[3] ^e
Total PAHs	4317 ± 132	1142 ± 11	74
Land treatment			
Two- and three-ring PAHs	1232 ± 111	295 ± 102	76
Four-ring PAHs	1126 ± 73	361 ± 243	68
Five- and six-ring PAHs	438 ± 116	399 ± 159	68
Total PAHs	2796 ± 292	1055 ± 719	62
Untreated soil			
Two- and three-ring PAHs	1580 ± 75	NA	NA
Four-ring PAHs	880 ± 44	NA	NA
Five- and six-ring PAHs	325 ± 134	NA	NA
Total PAHs	2785 ± 230	NA	NA

^aAs determined by U.S. EPA method 8100 (6). ^bTwo- and three-ring PAHs include naphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, dibenzofuran, fluorene, phenanthrene, and anthracene. ^cFour-ring PAHs include fluoranthene, pyrene, benzo[a]anthracene, and chrysene. ^dFive- and six-ring PAHs include benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, indeno[123-cd]pyrene, dibenzo[a,h]anthracene, and dibenzo[g,h,i]perylene. ^eDenotes an increase in concentration.

number of fractions) than the BS treatment but was more mutagenic than the other treatments (CMP, LT) and the UTS. The mutagenicity of the CMP and LT fractions was not qualitatively different from that in the UTS fractions both in our study and in Hughes et al. (4). Therefore, extracts from these two treatments were not chemically analyzed.

GC/MS identification of compounds in the mutagenic fractions from the BS and BP HPLC fractionations is summarized in Table 2. The HPLC fractionations from the UTS also were analyzed by GC/MS for comparison to the BS and BP fractions and are included in Table 2. The priority pollutant PAHs (listed in Table 1) only were detected in three fractions for both the BS

and BP treatments. In both the BS and the BP treatments, fraction 16 contained acenaphthene and fractions 30 and 31 contained acenaphthylene, anthracene, benzo[a]anthracene, and chrysene. Fraction 16 of the UTS contained acenaphthene, and fraction 31 contained acenaphthylene and benzo[a]anthracene. The other compounds seen in the remaining BS and BP fractions were mainly *N*-heterocyclics (italicized in Table 2) such as acenaphthopyridine, acridine, 1-azapyrene, benzo(c)carbazole, benzoquinoline, benzothiazolylphenol, carbazoles, diaminotriazole, diphenylpyrazole, indenopyridine, methylacridine, methylazaphenanthrene, methylbenzacridine, and methylisothiazole. Some fractions also contained *S*- and *O*-heterocyclics such as benzothiazolylphenol, benzocoumarin, benzofuranol, fluoroscein, methylisothiazole, and phenanthrofurane (Table 2).

Discussion

It has been estimated that creosote consists of 85% PAHs, 10% phenolics, and 5% other *N*-, *S*-, and *O*-heterocyclics (1). In this study, the bioremediation treatments (BP, BS, CMP, and LT) were successful in reducing the priority pollutant PAHs by 48% or more (Table 1). However, when the soil extracts from the bioremediation treatments and the UTS (extracted at the same time as the treatment soils) were tested in the *Salmonella* mutagenicity assay in strain YG1041, two treatments (BS and BP) had increased mutagenic activity, both with and without S9 addition, when compared to UTS (4). All four bioremediation treatments and the UTS were fractionated by HPLC and tested in a microsuspension modification of the *Salmonella* mutagenicity assay using

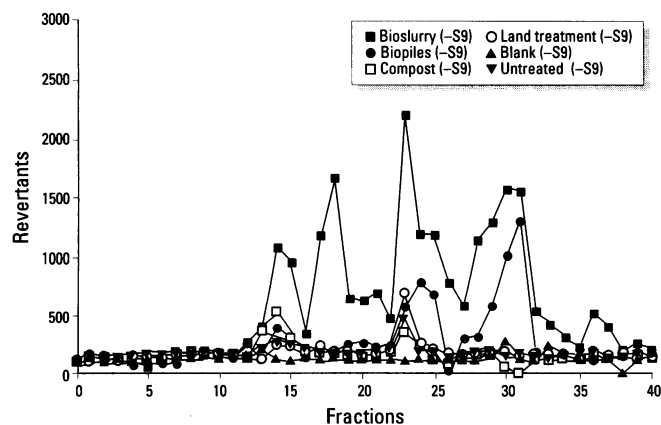


Figure 1. Mutagram of high-performance liquid chromatography fractions tested in the *Salmonella* mutagenicity assay without metabolic activation (-S9).

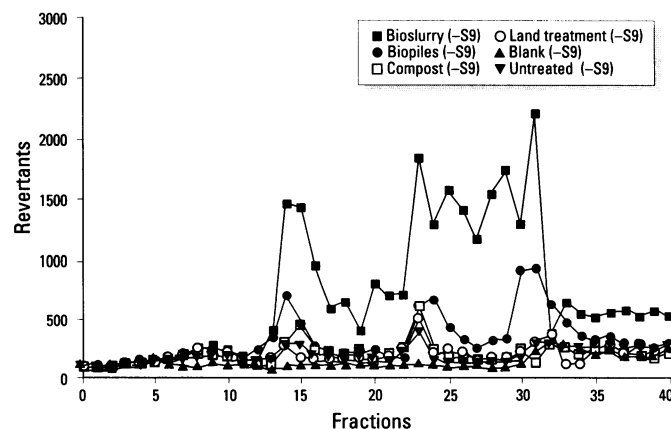


Figure 2. Mutagram of high-performance liquid chromatography fractions tested in the *Salmonella* mutagenicity assay with metabolic activation (+S9).

Table 2. Results of the gas chromatography/mass spectrometry analysis of the mutagenic high-performance liquid chromatography fractions.^a

Fraction no.	Untreated soil	Bioslurry	Biopile
14	Not analyzed	<i>Methylated and oligomethylated carbazoles</i> , naphazulenone, indenoanthracenone	<i>Methylated and oligomethylated carbazoles</i> , benzonitrile, phenylquinoline
15	⁹ H-carbazole, <i>benzo[c]carbazole</i>	⁹ H-carbazole, acenaphthalene, <i>methylbenzacridine</i>	⁹ H-carbazole
16	Acenaphthene, <i>benzo[c]carbazole</i> , anthracenecarbonitrile	Acenaphthene, <i>benzo[c]carbazole</i> , anthracenecarbonitrile, benzanthracenone, fluorene carbonitrile, <i>1-azapyrene</i>	Acenaphthene, <i>benzo[c]carbazole</i> , anthracenecarbonitrile, benzanthracenone, fluorene carbonitrile, phenethrol, <i>indenoisoquinoline</i> , <i>hydroxy-thienyl-quinoline</i>
18	<i>Benzoquinoline</i> , anthracenedione, cyclopenta(def)phenanthrenone, fluorene carbonitrile	Tetramethylphenol, fluoroscein, 9,10-anthraquinone, indacenedione, benzanthracenone	Anthracenecarbonitrile, aromatic ketones
20	⁹ H-fluorene-9-one, anthracenone	<i>Benzoquinoline</i> , cyclopentaphenanthrenone, hydroxypyrene, anthracene dione, cyanopyrene, benzothiazolylphenol	Aromatic ketones
23	Dihydrocyclobuta[<i>b</i>]naphthalene	Benzanthracenone, <i>diphenylpyrazole</i>	Benzanthracenone
24	Benzanthracenone, dihydrocyclobuta[<i>b</i>]naphthalene, naphthopyrandione	2-Hexanol, methylcyclopentanone, tetrachloroethane, 3-methylpentanone, <i>diaminotriazole</i>	2-Hexanol, benzanthracenone, tetrahydrophenanthrenone, <i>styrylquinoline</i> , <i>therahydrobenzanthracene</i> , phenanthrofurane
25	No identified compounds	Dihydrocyclobuta[<i>b</i>]naphthalene, phenanthrofurane	Phenanthrofurane, aromatic ketones
27	No identified compounds	No identified compounds	Phenanthrofurane, benzofuranol, benzocoumarin
30	Not analyzed	Acenaphthylene, anthracene, dihydroindenone, pyrene, chrysene, hexanols, heptanols, hexones, heptanones, <i>methylisothiazole</i> , acenaphthylenedione, anthracendiamine, benzoanthrothiophene	Acenaphthylene anthracene, dihydroindenone, methylindene, aliphatic ketones and alcohols
31	Acenaphthylene, benzo[<i>a</i>]anthracene, <i>benzo[c]acridine</i> , hexanediol, dihydroindenone, <i>acenaphthopyridine</i> , methylindanone, demethylfurylpyridine, naphthopyran, <i>indenquinoline</i> , <i>phenylcarbazole</i>	Acenaphthylene, benzo[<i>a</i>]anthracene, anthracene, chrysene, hexanediol, dihydroindenone, <i>acenaphthopyridine</i> , 2-pentanone, <i>indenopyridine</i> , <i>acridine</i> , <i>methylacridine</i> , <i>methylazaphenanthrene</i> , naphthopyrandione, anthracenecarbonitrile, palmitic acid	Acenaphthylene, benzo[<i>a</i>]anthracene, <i>benzo[c]acridine</i> , anthracene, chrysene

^aCompounds in italics are azaarenes.

strain YG1041, which yielded mutagrams (Figures 1 and 2). Again, the two treatments (BS and BP) had increased mutagenicity as compared to the UTS, especially in 11 of 40 fractions of the BS (+S9, -S9) and in 6 fractions of the BP (+S9, -S9) (Figures 1 and 2).

To determine the cause of the increased mutagenicity in these two treatments, the mutagenic HPLC fractions, along with corresponding fractions from the UTS, were analyzed by GC/MS. Of the 11 mutagenic fractions of the BS and BP treatments

analyzed by GC/MS, only 3 fractions contained any of the priority pollutant PAHs (Table 1). The mutagenicity (+S9) seen in fractions 30 and 31 of the BS and BP treatments is likely due to PAHs that are positive in the *Salmonella* mutagenicity assay (Table 3). The mutagenicity in other fractions, therefore, must be attributed to compounds other than the 19 priority PAHs. Many of the other compounds identified by GC/MS in the fractions are azaarenes (i.e., PAHs where a nitrogen replaces a carbon in the ring

structure). Several of these azaarenes (acridine, 1-azapyrene, diaminotriazole, and methylazaphenanthrene) are mutagenic -S9 (Table 3).

Nitroarenes are another class of chemicals that were of concern in these samples. Many nitroarenes (i.e., a nitro group attached to a PAH) are direct-acting mutagens (i.e., they do not need S9 to be mutagenic). For a review of their mutagenicity see Rosenkranz and Mermelstein (17). Nitroarenes are metabolized by the bacterial enzymes nitroreductase and

Table 3. Identified compounds tested in the *Salmonella* mutagenicity assay.^{a,b}

Fraction no.	Compound	Bioslurry Result	Reference	Compound	Biopile Result	Reference
14	No identified mutagens in the literature			No identified mutagens in the literature		
15	<i>9H-carbazole</i>	Negative	(12)	<i>9H-carbazole</i>	Negative	(12)
16	Acenaphthene	Negative	(13)	Acenaphthene	Negative	(13)
	Benzanthrone	Positive, -S9	(12)	Benzanthrone	Positive, -S9	(12)
	<i>1-Azapyrene</i>	Positive, -S9, +S9	(12,14)			
18	Fluorescein	Negative	(12,15)	No identified mutagens in the literature		
20	Anthracene dione	Positive, -S9, +S9	(12)	No identified mutagens in the literature		
23	No identified mutagens in the literature			No identified mutagens in the literature		
24	<i>Diaminotriazole</i>	Positive, -S9, +S9	(12)	No identified mutagens in the literature		
25	No identified mutagens in the literature			No identified mutagens in the literature		
27	No identified mutagens in the literature			No identified mutagens in the literature		
30	Pyrene	Negative	(12,15)	Acenaphthylene	Positive, +S9	(12)
	Chrysene	Positive, +S9	(12,16)	Anthracene	Positive, +S9	(13,15)
	Hexone	Negative	(13)			
31	Acenaphthylene	Positive, +S9	(12)	Acenaphthylene	Positive, +S9	(12)
	Benzo[a]anthracene	Positive, +S9	(15)	Benzo[a]anthracene	Positive, +S9	(15)
	Anthracene	Positive, +S9	(13,15)	Anthracene	Positive, +S9	(13,15)
	Chrysene	Positive, +S9	(12,16)	Chrysene	Positive, +S9	(12,16)
	Acridine	Positive, -S9	(12)			
	<i>Methylazaphenanthrene</i>	Positive, -S9, +S9	(12,16)			

^aResults are shown only for the mutagenic fractions. ^bCompounds in italics are azaarenes.

acetyltransferase. *Salmonella* strains YG1041 and YG1042 were developed from the standard *Salmonella* tester strains TA98 and TA100 (5), respectively, and they contain elevated levels (50×) of both nitroreductase and acetyltransferase activities (7). If higher mutagenic activity is detected in these strains than in TA98 and TA100, especially without S9 addition, the presence of nitroarenes could account for the mutagenicity in the test sample. This condition was true for the BS and BP extracts in this study. The BS had mutagenic slopes (revertants per microgram; calculated from the linear regression of the data) of 38.6 (-S9) and 31.4 (+S9), and the BP had mutagenic slopes of 3.0 (-S9) and 5.0 (+S9) (4). In addition, when TA98NR (which lacks nitroreductase and has limited ability to metabolize nitroarenes into active mutagens) was used, the mutagenic activity of the BS extract was reduced by 50% when mutagenic results were compared to TA98, which contains normal *Salmonella* nitroreductase (4). Therefore it was hypothesized from these data that the mutagenic activity in the BS extract may be due to nitroarenes. This hypothesis, however, was not upheld by chemical analysis. The mutagenic HPLC fractions in the BS and BP extracts were analyzed by GC/MS and nitroarenes were not detected; however, azaarenes were detected (Table 2). Nitroarenes can be detected as mutagens at nanogram levels in

strains YG1041 and YG1042 (7). The detection limits of the GC/MS were not low enough to detect any nitroarenes that may have been present in the BS. Examples of the azaarenes found in these fractions were acenaphthopyridine, acridine, 1-azapyrene, benzo[d]carbazole, benzoquinoline, benzothiazolylphenol, carbazoles, diaminotriazole, diphenylpyrazole, indenopyridine, methylacridine, methylazaphenanthrene, methylbenzacridine, and methylisothiazole. A large number of azaarenes have been reported in various creosotes (18) and in creosote-contaminated soil (19). The azaarenes are of concern because of their mutagenic activity and because the nitrogen atom in the ring system causes these compounds to be weakly polar and considerably more water soluble than related PAHs. Azaarenes have been reported in groundwater near creosote-contaminated sites including the Reilly Tar Site (1,2,10).

One reason the BS treatment was much more mutagenic than the other treatments could be that it was amended with 1% activated sludge (primary solid waste aerated and stirred to encourage bacterial growth) from a municipal wastewater-treatment facility that processed both industrial and municipal waste (4). The sludge was added in an effort to increase the diversity of the bacterial population in the BS. Municipal wastewater and sludge from municipal wastewater-treatment

facilities contain azaarenes, oxygenated PAH derivatives, and nitroarenes (21-23). Azaarenes were also detected in BS fractions 20 and 24 (Table 2) and may have been increased in the BS by the addition of the activated sludge. For example, acridine, 1-azapyrene, diaminotriazole, and methylazaphenanthrene were uniquely identified in the BS. All have been cited as direct-acting mutagens (Table 3). The mutagenic fractions for the BS in Figure 2 may be due to these compounds and the higher number of PAHs in the BS. In addition, acridine, fluorescein (dyes), and tetrachloroethane (an industrial product) were identified in BS fractions 31, 18, and 24, respectively. The presence of these three compounds may be evidence that the activated sludge added to the BS contained industrial chemicals.

Another possible reason the BS was more mutagenic than the other treatments was that the BS was the only treatment that was constantly aerated and mixed. The BS had air bubbled to the bottom of the reaction vessels at the rate of 1.5 ml/min and was stirred at 500 rpm (4). BS had 1% activated sludge added and BP had 1% cow manure added in an effort to increase the diversity of the bacterial population. The constant aeration in the BS caused maximum mixing of all components, including the azaarenes. The BP contained azaarenes but mixing did not

occur. CMP had 1% cow manure added, but the treatment vessel was not aerated, and was mixed once a day by rolling the vessel for 30 min. LT had nothing added and was tilled once per week. Each of these processes, therefore, had different levels of anaerobic and aerobic metabolism occurring. The differences in aeration, mixing, and addition of sludge/manure between the mutagenic treatments (BS, BP) and the other nonmutagenic treatments (CMP, LT) may account for the mutagenicity. Weak mutagenic activity was also detected in all five extracts in TA102 (4). This strain detects mutagenic aldehydes and ketones, which were detected in the BS and BP (Table 2).

Conclusion

The pilot scale laboratory experiments of the creosote-contaminated field soil were conducted to determine which treatment

was most successful not only in reducing the total PAH concentration but also the toxic potential of this soil. The PAH analysis demonstrated that each treatment was successful in reducing the total PAH concentration. However, the mutagenic activity demonstrated that some treatments (BP and BS) actually increased the toxic potential of these soils. Also, chemical analysis along with the bioassay identified several problems. The initial PAH concentrations (Table 1) varied among the treatments and were due to the different sieving sizes (1/4 to 1 in) used for each of them. In the future, uniform particle sizes should be used to more accurately compare the treatments. Also, the increased mutagenicity in the treatments (BP and BS) could not be directly associated with the amendments (i.e., activated sludge) added to these treatments because they were not analyzed directly in the chemical analysis and the

mutagenicity assay. Amendments should be evaluated for toxicity before addition to a bioremediation process to ensure that they are nontoxic. Complex environmental mixtures such as these soil extracts can contain hundreds of chemicals. Bioassay-directed fractionation is an efficient method to identify signature mutagenic chemicals in such mixtures. The combination of bioassays and chemical analysis provided a more complete and accurate evaluation of the four bioremediation treatments in this pilot study.

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