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 microsuspension 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/Suppl6/1435-1440brooks/abstract.html

Key words: fractionation, bioremediation, polycyclic aromatic hydrocarbons, Salmonella, mutagenicity

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 × 106 revertants [rev]/kg dry soil) and BP (3.0 × 106 rev/kg dry soil) treatments than in the UTS (0.008 × 106 rev/kg dry soil) (4). A significant increase in mutagenicity in the CMP and LT extracts

This paper is based on a presentation at the Conference on Current Issues on Chemical Mixtures held 11–13 August 1997 in Fort Collins, Colorado. Manuscript received at EHP 17 February 1998; accepted 30 June 1998.

The authors thank L. Stephens, A. Warren, P. Matthews, H. Carlsen, and B. Eischen for their valuable assistance. The Reilly Tar Site samples were prepared by the following people at the U.S. Environmental Protection Agency (U.S. EPA) in Cincinnati, Ohio: untreated soil (P. McCauley), bioslurry U. Glaeser, biosolids and compost (C. Potter), and land treatment (G. Sayles). The authors thank them for supplying soil samples and the chemical analysis (percent reduction in PAH) for this study. The authors thank J. Ryan and E. George for their critical technical review of the manuscript.

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) 541-1385. Fax: (919) 541-3066. 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 arachidonic-induced rat liver; 2-S9, without arachidonic-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 with sodium sulfate and filtered through a 0.45-μm 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 μg 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 quantitate 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 x 0.25 mm, 0.50-μm 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 μm, 250 x 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 μl 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, and LT) were bioassayed in the plate incorporation assay using strains YG1041 and YG1042 with and without exogenous mammalian metabolic activation (59) (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 μl of 10-fold concentrated cells in buffer (from 16-hr cultures) and 100 μl of 0.015 M phosphate buffer or 100 μl S9 mix (6%), pH 7.2, were added to each fraction that had been solvent-exchanged into 5 μl DMSO. Contents of the tubes were mixed, incubated at 37°C for 90 min, then poured onto minimal medium plates. 2-Nitrofluorenone (0.3 μg/plate) served as the positive control for YG1041 (−S9) and yielded a mean of 204 rev. 2-Aminoantrachene was the positive control for YG1041 (+S9) with 0.25 μg 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 μl 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 x 0.25 mm, 0.25-μm 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 ± 5 rev +S9 and 131 ± 41 rev −S9. A treatment fraction was not considered mutagenic unless it was ≥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
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 acenaphthropyridine, acridine, 1-azapyrene, benzo(c)carbazole, benzoquinoine, benzothiazolylphenol, carbazoles, diaminotriazoles, diphenylpyrazole, indenopyridine, methylacridine, methylazaphenanthrene, methylbenzacridine, and methylisothiazole. Some fractions also contained S- and O-heterocyclics such as benzothiazolylphenol, benzoquinoine, benzoquinone, fluoroscein, methylisothiazole, and phenanthrofuran (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

| Soil treatment | Average initial concentration, mg/kg soil | Average final concentration, mg/kg soil | Percent reduction |
|----------------|-----------------------------------------|-----------------------------------------|------------------|
| Bioslurry      |                                         |                                         |                  |
| Two- and three-ring PAHs
d | 443 ± 62                                | 114 ± 75                                | 74               |
| Four-ring PAHs  | 874 ± 219                               | 217 ± 81                                | 75               |
| Five- and six-ring PAHs | 388 ± 90                              | 309 ± 17                                | 20               |
| Total PAHs      | 1705 ± 361                              | 640 ± 161                               | 62               |
| Biopile         |                                         |                                         |                  |
| Two- and three-ring PAHs | 1498 ± 183                      | 458 ± 159                                | 69               |
| Four-ring PAHs  | 1084 ± 65                               | 651 ± 253                                | 40               |
| Five- and six-ring PAHs | 408 ± 75                           | 450 ± 49                                | 10%              |
| Total PAHs      | 2992 ± 326                              | 1559 ± 726                               | 48               |
| Compost         |                                         |                                         |                  |
| Two- and three-ring PAHs | 2628 ± 57                        | 217 ± 23                                 | 92               |
| Four-ring PAHs  | 1245 ± 35                               | 488 ± 9                                 | 62               |
| Five- and six-ring PAHs | 444 ± 40                        | 457 ± 3                                 | 3%               |
| 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      | 2785 ± 292                              | 1695 ± 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               |

*As determined by U.S. EPA method 8100 (6).* Two- and three-ring PAHs include naphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, dibenzofuran, fluorene, phenanthrene, and anthracene. Four-ring PAHs include fluoranthenes, pyrene, benzo[a]anthracene, and chrysene. Five- and six-ring PAHs include benzofluoranthene, benzo[ghi]perylene, benzo[ghi]perylene, benzo[ghi]pyrene, benzo[ghi]pyrene, indeno[123-cd]pyrene, dibenz[a]anthracene, and dibenzog,h,pdiphenylene. *Denotes an increase in concentration.

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.4

| Fraction no. | Untreated soil | Bioslurry | Biopile |
|--------------|----------------|-----------|---------|
| 14           | Not analyzed   | Methylated and oligomethylated carboxylates, naphazulenes, indenoantraerencene | Methylated and oligomethylated carboxylates, benzoxanthene, phenylquinoline |
| 15           | 9H-carbazole, benzof[carbazole | 9H-carbazole, acenaphthylene, methylbenzacridine | 9H-carbazole |
| 16           | Acenaphthene, benzof[carbazole, anthracencarbonitrile | Acenaphthene, benzof[carbazole, anthracencarbonitrile, benzanthracene, fluorencarbonitrile, 1-azaprylene | Acenaphthene, benzof[carbazole, anthracencarbonitrile, benzanthracene, fluorencarbonitrile, phenetrol, indenoisouquinoline, hydroxy-thienyl-quinoline |
| 18           | Benzoquinoline, antracenedione, cyclopenta[d]phenanthrene, fluorencarbonitrile | Tetramethylphenol, antrosoine, 9,10-anthrachinone, indacenodione, benzanthracene | Anthracencarbonitrile, aromatic ketones |
| 20           | 9H-fluoren-9-one, antracenone | Benzoquinoline, cyclopentaphenanthrene, hydroxypirenne, anthracene dione, cyanopyrene, benzothiaxoylphenol | Aromatic ketones |
| 23           | Dihydrocyclobuta[b]naphthalene | Benzantracene, diphenylpyrazole | Benzantracene |
| 24           | Benzantracene, dihydrocyclobuta[b]naphthalene, naphthoyrlandine | 2-Hexanol, methylcycloptanone, tetrachloroethane, 3-methylpentanone, diaminotrazole | 2-Hexanol, benzantracene, tetrahydrophenanthrene, styrylquinoline, therahydrobenzazacene, phenanthrofuran |
| 25           | No identified compounds | Dihydrocyclobuta[b]naphthalene, phenanthrofuran | Phenanthrofuran, aromatic ketones |
| 27           | No identified compounds | No identified compounds | Phenanthrofuran, benzofuranol, benzoceammin |
| 30           | Not analyzed | Acenaphthylene, anthracene, dihydroindene, pyrene, chrysene, hexanois, heptanois, hexones, heptanones, methylthiothropyrene, acenaphthernedione, anthracendiamine, benzanathrothiophene | Acenaphthylene anthracene, dihydroindene, methyldiene, aliphatic ketones and alcohols |
| 31           | Acenaphthylene, benz[a]anthracene, benzof[jacridine, hexanediol, dihydroindene, acenaphthyryidine, methylindane, demethylpyridine, naphthopyran, indenquinoline, phenylcarbazole | Acenaphthylene, benz[a]anthracene, anthracene, chrysene, hexanediol, dihydroindene, acenaphthyryidine, 2-pentane, indenopyridine, acridine, methylazacridine, methylazaphrentherene, naphthopyrlandine, anthracencarbonitrile, palmitic acid | Acenaphthylene, benz[a]anthracene, benzof[jacridine, anthracene, chrysene |

*Compounds in italics are azaraenes.

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 azaraenes (i.e., PAHs where a nitrogen replaces a carbon in the ring structure). Several of these azaraenes (acridine, 1-azaprylene, diaminotrazole, and methylazaphrentherene) 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 Rosenkrantze and Mermelstein (17). Nitroarenes are metabolized by the bacterial enzymes nitroreductase and
Table 3. Identified compounds tested in the Salmonella mutagenicity assay.

| Fraction no. | Compound                  | Bioslurry Result | Reference |
|--------------|---------------------------|------------------|-----------|
| 14           | No identified mutagens in the literature |                  |           |
| 15           | 3H-carbazole              | Negative         | (12)      |
| 16           | Acenaphthene              | Negative         | (13)      |
|              | Benzanthrone              | Positive, −S9    | (12)      |
|              | 1-Azapyrene               | Positive, −S9, +S9 | (12,14) |
| 18           | Fluorescein               | Negative         | (12,15)  |
| 20           | Anthracene dione          | Positive, −S9, +S9 | (12)      |
| 23           | No identified mutagens in the literature |                  |           |
| 24           | Diaminotriazole           | Positive, −S9, +S9 | (12)      |
| 25           | No identified mutagens in the literature |                  |           |
| 27           | No identified mutagens in the literature |                  |           |
| 30           | Pyrene                    | Negative         | (12,15)  |
|              | Chrysene                  | Positive, +S9    | (12,16)  |
|              | Hexone                    | Negative         | (13)      |
| 31           | Acenaphthylene            | Positive, +S9    | (12)      |
|              | Benz(a)anthracene         | Positive, +S9    | (15)      |
|              | Anthracene                | Positive, +S9    | (12,15)  |
|              | Chrysene                  | Positive, +S9    | (12,16)  |
|              | Acidine                   | Positive, −S9    | (12)      |
|              | Methylazaphenanthrene     | Positive, −S9, +S9 | (12,16) |

| Compound                  | Biopile Result | Reference |
|---------------------------|----------------|-----------|
| No identified mutagens in the literature |                  |           |
| 3H-carbazole              | Negative         | (12)      |
| Acenaphthene              | Negative         | (13)      |
| Benzanthrone              | Positive, −S9   | (12)      |
| 1-Azapyrene               | Positive, −S9, +S9 | (12,14) |
| Fluorescein               | Negative         | (12,15)  |
| Anthracene dione          | Positive, −S9, +S9 | (12)      |
| Diaminotriazole           | Positive, −S9, +S9 | (12)      |
| No identified mutagens in the literature |                  |           |
| No identified mutagens in the literature |                  |           |
| No identified mutagens in the literature |                  |           |
| No identified mutagens in the literature |                  |           |
| No identified mutagens in the literature |                  |           |
| Acenaphthylene            | Positive, +S9   | (12)      |
| Anthracene                | Positive, +S9   | (13,15)  |
| Benz(a)anthracene         | Positive, +S9   | (15)      |
| Anthracene                | Positive, +S9   | (13,15)  |
| Chrysene                  | Positive, +S9   | (12,16)  |

*Results are shown only for the mutagenic fractions. *Compounds 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[a]carbazole, benzoquinoline, benzothiazolylphenol, carbazoles, diamino- triazole, 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 tetrachloroethene (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.

Disclaimer: This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, and the mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES AND NOTES

1. U.S. EPA. Treatment Technology Performance and Cost Data for Remediation of Wood Preserving Sites. EPA/625/R-97/005. Washington:U.S. Environmental Protection Agency, 1997.
2. U.S. EPA. Development Document for Effluent Limitation Guidelines and Standards for the Timber Products Source Category. EPA 440/1–81/023. Washington:U.S. Environmental Protection Agency, 1981.
3. U.S. EPA. Bioremediation Field Initiative Site Profile: Reilly Tar and Chemical Corporation Superfund Site. EPA 540-F-95–506H. Washington:U.S. Environmental Protection Agency, 1995.
4. Hughes TJ, Claxton LD, Brooks L, Warren S, Brenner R, Kremer F. Genotoxicity of bioremediated soils from the Reilly Tar Site, St. Louis Park, Minnesota. Environ Health Perspect 106(Suppl 6):1427–1433 (1998).
5. Maron D, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res 113:173–215 (1983).
6. U.S. EPA. Test Methods for Evaluating Solid Waste. PB-88-239223. Washington:U.S. Environmental Protection Agency, 1986.
7. Hagiwara Y, Watanabe M, Oda Y, Sofuni T, Nohmi T. Specificity and sensitivity of Salmonella typhimurium YG1041 and YG1042 strains possessing elevated levels of both nitroreductase and acetyltransferase activity. Mutat Res 291:171–180 (1993).
8. DeMarini D, Dallas MM, Lewtas J. Cytotoxicity and effect on mutagenicity of buffers in a microsuspension assay. Teratog Carcinog Mutagen 9:287–295 (1989).
9. Wiley Registry of Mass Spectral Data. 6th ed. Newfield, NY: Palisade Corporation, 1998.
10. Barr DP, Aust SD. Pollutant degradation by white rot fungi. Rev Environ Contam Toxicol 138:49–72 (1994).
11. Lewtas J, King LC, Williams K, Ball LM, DeMarini DM. Bioassay-directed fractionation of 1-nitropyrene metabolites: generation of mutagens by coupling reverse-phase HPLC with microsuspension mutagenicity assays. Mutagenesis 5:481–489 (1990).
12. RTECS. Registry of Toxic Effects of Chemical Substances. Cincinnati, OH:National Institute for Occupational Safety and Health, 1997.
13. NLM. Hazardous Substance Data Bank. Bethesda, MD:National Library of Medicine, 1997.
14. Tanga MJ, Miao RM, Reist EJ. Bacterial mutagenicity and carcinogetic potential of some azapyrene derivatives. Mutat Res 172:11–17 (1986).
15. Gold LS, Zeiger E, ed. Carcinogenic Potency and Genotoxicity Databases. New York:CRC Press, 1997.
16. Graedel TE, Hawkins DT, Claxton LD. Atmospheric Chemical Compounds: Sources, Occurrence, and Bioassay. New York:Academic Press, 1986.
17. Rosenkranz HS, Mermelstein R. Mutagenicity and genotoxicity of nitroarones: all nitro-containing chemicals were not created equal. Mutat Res 114:217–267 (1983).
18. Nylund L, Heikkila P, Hameila M, Pyy L, Linnainmaa K, Sorsa M. Genotoxic effects and chemical compositions of four creosotes. Mutat Res 265:223–226 (1992).
19. Brumley WC, Brownrigg CM, Brilis GM. Characterization of nitrogen-containing compounds in soil and sediment by capillary gas chromatograph-mass spectrometry after fractionation. J Chromatogr 558:223–233 (1991).
20. Pereira WE, Rostad CE, Garbarino JR, Hult MF. Groundwater contamination by organic bases derived from coal-tar wastes. Environ Toxicol Chem 2:283–294 (1983).
21. Bodzek D, Janoszka J, Dobosz C, Warzecha L, Bodzek M. Determination of polycyclic aromatic compounds and heavy metals in sludges from biological sewage treatment plants. J Chromatogr A 774:177–192 (1997).
22. Gruffol M, Solanas A, Bayona J. Characterization of genotoxic components in sediments by mass spectrometric techniques combined with Salmonella/microsomal test. Arch Environ Contam Toxicol 19:175–184 (1990).
23. Sayato Y, Nakamuro K, Ueno H, Goto R. Identification of polycyclic aromatic hydrocarbons in mutagenic adsorbates to a copper-phthalocyanine derivative recovered from municipal river water. Mutat Res 300:207–213 (1993).