Genotoxicity of Bioremediated Soils from the Reilly Tar Site, St. Louis Park, Minnesota

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An in vitro approach was used to measure the genotoxicity of creosote-contaminated soil before and after four bioremediation processes. The soil was taken from the Reilly Tar site, a closed Superfund site in Saint Louis Park, Minnesota. The creosote soil was bioremediated in bioslurry, biopile, compost, and land treatment, which were optimized for effective treatment. Mutagenicity profiles of dichloromethane extracts of the five soils were determined in the Spirochilus stain of the Salmonella assay with seven tester strains. Quantitative mutagenic responses in the plate incorporation technique were then determined in the most sensitive strains, YG1041 and YG1042. Mutagenic potency (revertants per microgram extract) in YG1041 suggested that compost, land treatment, and untreated creosote soil extracts were moderately mutagenic with Aroclor-induced rat liver (S9) but were nonmutagenic without S9. However, the bioslurry extract was strongly mutagenic and the biopile extract was moderately mutagenic either with or without S9. A similar trend was obtained in strain YG1042. The strong mutagenic activity in the bioslurry extract was reduced by 50% in TA98NR, which suggested the presence of mutagenic nitroaromatics. Variation in reproducibility was 15% or less for the bioassay and extraction procedures. Bioavailability of mutagens in the biopile soil was determined with six solvents; water-soluble mutagens accounted for 40% of the total mutagenic activity and they were stable at room temperature. The mutagenic activity in the bioslurry and biopile samples was due to either the processes themselves or to the added sludge/manure amendments. The in vitro approach was effective in monitoring bioremediated soils for genotoxicity and will be useful in future laboratory and in situ studies. — Environ Health Perspect 106(Suppl 6):1427–1433 (1999). http://ehpnet1.nih.gov/docs/19996/S61427-1433hughes/abstract.html

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Coal tar creosote consists mainly (80–85%) of polycyclic aromatic hydrocarbons (PAHs) (1); therefore, creosote generally requires metabolic activation to show mutagenic activity (2–6). Creosote contains carcinogens (7,8) and causes DNA adducts in mouse skin (9) and tumors in fish (10,11). Creosote is an animal carcinogen and there is limited evidence for carcinogenicity in humans (12). Because it has been used as a wood preservative since the early 1900s, approximately 415 Superfund sites have soil and groundwater contaminated with creosote (1,13–17). There are many methods available to remediate a hazardous waste site soil (18–22). The presumptive treatments for soils, sediments, and sludge contaminated with organics at wood treatment sites are bioremediation, thermal desorption, and incineration (19).

Bioremediation is a cost-effective process to degrade wastes, and techniques are reviewed elsewhere (23–26). Bioremediation takes advantage of the ability of bacteria (27–30), fungi (31,32), and plants (33) to degrade many toxics. Indigenous bacteria and fungi often utilize organic toxics as sources of carbon and energy (34,35) and can be augmented with nutrients in situ or isolated and propagated outside the laboratory for use with specific hazardous soils (22,36,37). Biosurfactants increase the solubility of the toxics, which releases them from soil particles so that bacteria and fungi can more easily metabolize them (38–40), but increased water solubility can lead to water contamination.

This research investigated the genotoxicity of creosote-contaminated soil from a closed Superfund site, the Reilly Tar site (RTS) in St. Louis Park, Minnesota, before and after bioremediation in four laboratory-scale processes: bioslurry (BS), biopile (BP), compost (CMP), and land treatment (LT). Mutagenic activity was measured in two Salmonella assay techniques (41,42). Reproducibility of the mutagenicity assay and the extraction scheme was determined, and the bioavailability of the mutagens in the BS soil was investigated. Chemical fractionation and identification of signature mutagens in the RTS soils are presented in a companion paper (43).

Materials and Methods

Chemicals

The dichloromethane (DCM) used for the extraction of organics from the RTS soils and as a solvent for the Spiral mutagenicity assay (42) was gas chromatography grade, and the dimethyloxide (DMO) used as a solvent for the plate incorporation mutagenicity bioassay (41) was a spectrophotometric grade. Both DCM and DMO were obtained from Burdick and Jackson (Muskegon, Michigan). The water used for the bioavailability experiment was deionized-distilled, sterilized municipal...
water from Durham, North Carolina, prepared at the U.S. Environmental Protection Agency (Research Triangle Park, North Carolina). The artificial seawater (saltwater) was obtained from Aquarium Systems ( Mentor, Ohio). The Corexit 9527 is a commercial oil dispersant and was obtained from Shell Oil Company (Dallas, Texas). Toluene used for the bioavailability experiment was a spectrophotometric grade from Burdick and Jackson. The positive controls for the direct-acting (-S9) conditions were 2-nitrofluorene (TA98, YG1041), sodium azide (TA100, YG1042), methylmethane sulfonate (TA104), mitomycin C (TA102), and 9-aminoacridine (TA97). The positive controls for the mammalian liver activation (+S9) conditions were: 2-aminoanthracene (TA97, TA98, TA100, YG1041, YG1042, TA104) and dantheron (TA102). All control chemicals were obtained from the Sigma Chemical Company (St. Louis, Missouri).

Reilly Tar Site Soils
Un-treated Creosote-Contaminated Soil from the Reilly Tar Site. The untreated creosote-contaminated soil (UTS) was obtained from a former Superfund site that was a creosote (coal tar) wood treatment facility in operation from 1917 to 1972. The UTS, which had a maximum concentration of 3000 mg PAH/kg dry soil, extended to a depth of 80 ft and was a sandy loam soil. A composite UTS sample was created by mixing twenty-two 55-gal drums of soil and passing the soil through a vibrating screen to remove large rocks and debris. Each process used a different size of screened soil, from less than 1 inch to less than 1/4 inch. The UTS was collected in the spring of 1996 and was kept at 4°C until it was extracted for mutagenicity testing in January 1997. The UTS was the starting soil for all four bioremediation processes.

Bioslurry. The BS mixture used UTS of less than 1/4-inch particle size. Treatment was performed in stirred (500 rpm) glass laboratory reactors (8-liter capacities) and air was bubbled to the bottom of the reactors. Ports were provided at the top of the reactor for periodic addition of nutrients, slurry, or air. The soil slurry was amended to keep the carbon:nitrogen:phosphorus ratio at 100:10:1, and an Organisation for Economic Co-operation and Development (OECD) nutrient mixture was added that contained trace minerals and vitamins (44). The BS soil samples for the mutagenicity assays were a composite sample from three separate BS treatments. A 1% activated sludge from a municipal wastewater treatment facility was used for one of the treatments to increase the bacterial diversity. Detailed descriptions of the bioslurry treatments are available (45). BS soil samples for mutagenicity assays were taken after 41 days of operation.

Biopile. The BP process used UTS screened to less than 1-inch particle size. The BP reactor was a rectangular polyethylene tank that contained 2 cubic yards of soil with two humidified air ports (0.5 liter/min, equal to one complete air change/24 hr). Cow manure was added to the reactor at 1%, and nutrients were added to keep a constant ratio of 100:10:1 for carbon: nitrogen:phosphorous. The reactor was sealed during the course of this study. The BP sample for the mutagenicity studies was taken at 20 weeks from the middle of the BP reactor, which contained 5% fungi by weight from oyster mushrooms (Pleurotus ostreatus).

Compost. The CMP process used UTS screened to less than 1/2-inch particle size. The CMP reactors consisted of 55-gal stainless steel inner drums that were welded, sealed, and insulated within 95-gal outer drums that prevented heat loss during the composting process. Details for the CMP treatment are available (46). The composters were placed on a conveyor belt every day and rolled for 30 min. Samples of CMP for mutagenicity testing were taken at 12 weeks of operation.

Land Treatment. The LT process used UTS screened to less than 1/2-inch particle size. The LT reactors are described in detail elsewhere (47,48). In brief, an LT reactor consisted of a stainless steel pan containing about 30 kg of dry soil (1.5 cubic feet of soil). Soil was placed in the pan to a depth of 8 inches, which was characteristic of field-scale LT. Four of these pans were suspended in a water bath and housed in a custom-built Plexiglas and steel glove box (47). Air entered the box through a high-efficiency particulate air filter and was vented through a ventilation duct. Six glove boxes held the 24 pans used in this study. The soil was aerated through weekly tilling by hand with a pitchfork. The temperature was controlled at 20°C (± 3°C). Soil moisture was maintained in the range of 12 to 14% (moisture mass relative to wet soil) by weekly moisture measurements and corresponding weekly additions of a calculated amount of distilled water. Soil pH was monitored at 0, 8, 16, and 25 weeks, but no effort was made to control the soil pH. Soil pH varied little, ranging from 8.04 to 8.41. The LT sample for this study was a composite sample from six of these reactors to which no amendments were added. Samples for mutagenicity testing were taken after 25 weeks of operation.

Dichloromethane Extraction Procedure to Remove the Organic Fraction from the Reilly Tar Site Soils for Mutagenicity Testing. Dichloromethane (200 ml) was added to 100 g of each of the five soil types (UTS, BS, BP, CMP, and LT) taken at the end of each process. The UTS was used as the starting soil for each process and therefore was the control soil at time zero. The soil/DCM mixture was sonicated for 15 min; this procedure was performed three times. The DCM was siphoned from the extracted soil, combined, dried with sodium sulfate and filtered through a 0.45 μ Teflon filter (Millipore Corporation, Marlborough, Massachusetts). The DCM extract was concentrated under a stream of gaseous nitrogen to 25 ml. Soils were initially concentrated to 10 mg organics per milliliter DCM for Spiral technique testing. The DCM extract was solvent-exchanged into DMSO and filter-sterilized (0.45 μ Millipore filters) before it was tested for mutagenic activity in the plate incorporation assay. The DCM was taken through these same procedures and was used as a procedural control (i.e., lab solvent blank in DCM [LRB]).

Mutagenicity Procedures
Spiral Technique. The Spiral technique (42) was used on the DCM soil extracts (10 mg organics per milliliter DCM) to determine which samples were mutagenic and which specific strain and activation conditions were most appropriate for subsequent studies. The Salmonella strains used in the Spiral testing were TA97, TA98, TA100, TA102, TA104, YG1041, and YG1042 (41,49). The TA strains were obtained from B. Ames (University of California, Berkeley, California); the YG strains were obtained from T. Nohmi (National Institute of Hygienic Sciences, Tokyo, Japan). The Aroclor-induced (500 mg Aroclor 1254 per kilogram rat body weight) rat liver 9000×g supernatant (S9) was obtained from Organon Teknika Corporation (Durham, North Carolina) and was stored at −80°C. The protein concentration of the S9 was 45.3 mg protein per milliliter. The colonies were counted on a Spiral laser counter using the SALS program (42), which transformed the Spiral data to an equivalent number of
revertants at each specific dose level for a 100-mm petri dish. The Spiral plater and counter were obtained from Spiral System Instruments, Inc. (Cincinnati, Ohio).

**Plate Incorporation Technique.** The plate incorporation technique was conducted as described by Maron and Ames (41), except that the base agar layer contained the trace amounts of histidine and biotin necessary for initial replication, and the plates were incubated for 72 hr (50). Based on the Spiral results, soil samples were tested with strains YG1041 and YG1042 with and without S9 at 10 doses, triplicate plates per dose. The exogenous activation system was from Aroclor-induced rat liver S9, and 500 μl of a 6% S9 mixture was added to each plate. Mutagenic potencies (revertants per microgram) were determined by the Bernstein linear regression rejection model, which rejects nonlinear data from the upper portion of the dose–response curve. The Bernstein model does not adjust for toxicity, requires three acceptable dose levels, and therefore provides very conservative mutagenic potencies (51,52). Statistical models that adjust for toxicity and include all data in their calculations [e.g., Sted model (53)] have higher mutagenic potencies. The GeneTox Manager statistical analysis program (54) was used to record the data and generate the Bernstein mutagenic potencies (55).

**Nitroreductase Study.** Nitroreductase-deficient strain TA98NR was obtained from H. Rosenkranz (University of Pittsburgh, Pittsburgh, Pennsylvania). The methods for the nitroreductase strains are contained in Rosenkranz and Poirier (56).

**Reproducibility Studies.** Experiments were performed to evaluate the reproducibility of the extraction and bioassay techniques over time. Reproducibility was measured as percent coefficient of variation (CV) in the mutagenic activity of BP extracts over time. Variation below 25% was considered acceptable. Three separate BP samples were extracted by DCM on the same day and solvent-exchanged in DMSO for mutagenicity testing. Bioassay parameters were as follows: strain YG1041, without S9, and 10 dose levels (10–100 μg/plate in intervals of 10 μg), with triplicate plates per dose. Three experiments were conducted on these three BP extracts on three separate days; experiments were spaced one week apart.

**Bioavailability Study.** The following six solvents (200 ml each) were added to 100 g UTS and BP soil to measure the mutagenic activity that could be extracted (i.e., the bioavailability of mutagens) by each solvent: Corexit 9527 (a widely used dispersant) at a dilution of 1:100 in water; sterile water; artificial seawater; a mixture of 50% water and 50% artificial seawater; toluene; and DCM. The soil/extract mixtures were shaken in glass jars for 2 min then centrifuged for 20 min at 6000 rpm. The supernatants were decanted and 200 ml DCM was added to each of the supernatants. This mixture was then shaken for 2 min and the organic phase was removed. This procedure was repeated a total of three times. The three organic phases were combined, filtered through a Millipore filter (0.45 μm) overlaid with 1 to 2 cm sodium sulfate to dry the extract, and concentrated to 25 ml in a rotovapornicator apparatus. A mutagenicity assay was performed on each extract with YG1041, no S9 addition, five dose levels (100, 75, 50, 25, and 10 μg/plate), triplicate plates per dose. The three waters were used to simulate various aquatic environments contaminated by various PAH-containing pollutants, the Corexit 9527 was utilized as a positive control dispersant, the toluene was used to simulate a potential solvent front commonly seen moving away from a creosote point source, and DCM was tested as the standard extraction solvent. The DMSO was the bioassay solvent control.

**Results**

**Mutagenic Activity of the Untreated and Bioremediated Reilly Tar Site Soils.**

Preliminary mutagenic evaluation of the five RT's soils in the Spiral technique with seven Salmonella strains clearly identified strains YG1041 and YG1042, both with and without S9, as the most responsive test conditions (Table 1). Subsequently, these two strains were used in the Salmonella plate incorporation assay and the mutagenic potencies (revertants per microgram) from the Bernstein linear regression model are shown in Table 2. A summary of the RTS soil data for YG1041 and YG1042 in the plate incorporation technique is available (57). The mutagenic potencies for the BS in YG1041 were >25-fold more mutagenic than those of the UTS; the BP mutagenic potencies were >3-fold more mutagenic than the UTS in YG1041. The mutagenic activity for the BS and BP extracts in YG1042 was reduced when compared to YG1041 but their mutagenic potencies were considerably above those for the UTS. The CMP, LT, and UTS mutagenic potencies were moderate with YG1041, +S9, but nonmutagenic without S9 in both strains. There was not a doubling of mutagenic potencies (in YG1041, +S9) for the compost and land treatment extracts compared to the UTS extract; therefore, their mutagenic potencies were not appreciably different from the UTS mutagenic potencies (58). LRB, the procedural solvent control, was not mutagenic, as expected.

**Nitroreductase Study**

The strong mutagenic activity present in the YG strains, especially without S9 addition, suggested that the BS extract contained nitroarenes. To further investigate this hypothesis, the BS extract was tested for mutagenic activity in TA98 and TA98NR (nitroreductase deficient) at five

### Table 1. Mutagenic potencies (revertants per microgram) of Reilly Tar site soil extracts in the preliminary spiral mutagenicity assay.

| Strain  | S9 | BS | BP | CMP | LT | UTS | LRB |
|---------|----|----|----|-----|----|-----|-----|
| YG1041  |    |    |    |     |    |    |     |
| –       | 16.0 | 3.6 | 1.4 |     | NM | NM | NM  |
| +      | 16.4 | 3.8 | NM | NM | NM | NM | NM  |
| YG1042  |    |    |    |     |    |    |     |
| –       | 6.7 | NM | 1.4 |     | NM | NM | NM  |
| +      | 4.3 | 2.2 | NM | NM | NM | NM | NM  |
| TA98    |    |    |    |     |    |    |     |
| –       | NM | NM | 0.7 |     | NM | NM | NM  |
| +      | 2.3 | NM | NM | NM | NM | NM | NM  |
| TA100   |    |    |    |     |    |    |     |
| –       | NM | 1.3 | 1.1 |     | NM | 0.7 | NM  |
| +      | NM | 1.9 | NM | NM | NM | NM | NM  |
| TA97    |    |    |    |     |    |    |     |
| –       | 0.6 | NM | NM |     | NM | NM | NM  |
| +      | 0.8 | NM | NM | NM | NM | NM | NM  |
| TA102   |    |    |    |     |    |    |     |
| –       | 0.6 | NM | NM |     | 1.0 | NM | NM  |
| +      | NM | 1.4 | 0.6 | 0.6 | NM | NM | NM  |
| TA104   |    |    |    |     |    |    |     |
| –       | NM | NM | NM |     | NM | NM | NM  |
| +      | NM | NM | NM | NM | NM | NM | NM  |

NM, not mutagenic. *Mutagenic potencies from the Bernstein linear regression model (57). Mutagenic potencies less than 0.5 and those that were nonsignificant are noted as NM.
Table 2. Mutagenic potencies of the strains from the Reilly Tar site in the plate incorporation mutagenicity assay.

| Strain | -S9 | +S9 | -S9 | +S9 |
|--------|-----|-----|-----|-----|
| UTS    | NM  | NM  | NM  | NM  |
| LRB    | 38.6b | 31.4b | 18.0b | 8.9b |
| BS     | 3.0b | 5.0b | 1.3b | 1.7b |
| CMP    | NM  | 2.3b | NM  | 0.2 |
| LT     | 0.16 | 1.8b | NM  | 0.2 |

*Mutagenic potencies were calculated from the linear regression model of Bernstein (51). Values greater than 0.1 were mutagenic. The rankings were as follows: values ≤0.1 were NM; values >0.1 to 1.0 were weakly mutagenic; values 1.0 to 10.0 were moderately mutagenic; values ≥10.0 were strongly mutagenic.

Table 3. Reproducibility of the Salmonella assay and the extraction scheme by analyses of three separate extractions of the biopile soil from the Reilly Tar site.

| Sample          | Assay 1 | Assay 2 | Assay 3 | Assay CV%
|-----------------|---------|---------|---------|-------------|
| Biopile 1       | 3.6     | 3.5     | 2.9     | 11.4        |
| Biopile 2       | 3.0     | 2.7     | 2.2     | 15.2        |
| Biopile 3       | 3.3     | 2.7     | 2.6     | 13.2        |
| Extraction:     | 9.1     | 15.5    | 13.6    |             |

*Mutagenic potencies from the linear regression Bernstein model, which rejects nonlinear points from the upper portion of the dose–response curve (51). Three separate BP soil samples (1, 2, and 3) were extracted on the same day. Assay parameters were as follows: Salmonella strain YG1041, 10 dose levels in the linear portion of the dose–response curve (10–100 μg in 10 μg intervals), three plates/dose, no S9 addition, plate incorporation assay; assays were conducted 1 week apart from each other. *SD/mean × 100%.

Dose levels, triplicate plates per dose. If mutagenic activity were reduced in TA98NR, the presence of nitroarenes would be further implicated. Mutagenic activity was reduced by 50% in the BS extract with strain TA98NR (i.e., at 1000 μg/plate, mutagenicity was reduced to 135 revertants in TA98NR from 267 revertants in TA98 (data not shown)). These data supported the hypothesis that the mutagenic activity in the BS was due to nitroarenes.

Reproducibility Studies

Table 3 contains the data for the reproducibility evaluation of both the bioassay and the extraction scheme. The mean variation in the bioassay for each BP extract over a 3-week period (horizontal columns in Table 3) was 13.3%. The mean variation in the extraction scheme for the three BP extracts over a 3-week period (vertical columns in Table 3) was 12.7%. These low variations confirmed our confidence in the mutagenic potencies and suggested that the mutagens in the BP extract were stable at room temperature for at least 3 weeks.

Bioavailability Study

The bioavailability of the mutagens in the UTS and the BP extracts was investigated by extracting these two soils with six different solvents and then testing these extracts in YG1041 with and without S9 addition. Samples were tested at five dose levels, and the 100 μg/plate data are depicted graphically in Figure 1 (−S9) and Figure 2 (+S9). The dotted lines on the two graphs denote DMSO solvent control values (100 revertants). An unambiguous mutagenic response was considered to be 300 revertants or three times the solvent control. Figure 1 demonstrated that for the direct-acting conditions, the BP extract had considerably more mutagenic activity than the UTS, especially with the water extract, which accounted for approximately 40% of the total mutagenicity in the BP extract. The toluene and DCM extracts of the BP soil without S9 also demonstrated appreciable mutagenic activity (Figure 1). When tested with S9 (Figure 2), the mutagenicity in the water extract of the BP soil was equal to the mutagenicity seen with DCM extraction for the UTS and BP. These data demonstrated that the mutagens in the BP soil were soluble in water, DCM, and toluene. Neither the saltwater (artificial seawater) nor the 50:50 mix nor the Corexit 9527 extracted significant mutagenic activity from the BP soil (Figures 1 and 2).

Discussion

Mutagenic Activity in the Reilly Tar Site Soil Extracts

The in vitro approach used for the genotoxic analyses of bioremediated soils in this study contained three main parts: initial mutagenicity screening of soil extracts in the Spiral technique; subsequent plate incorporation assays to accurately determine mutagenic potencies (revertants per microgram); and a bioavailability study with a multiple solvent testing scheme. The value of this approach was the Spiral screening provided a rapid inexpensive
method to determine mutagenic activity; the plate incorporation assay permitted the determination of mutagenic potencies that could be ranked; and the bioavailability study identified the solubility of the mutagens. Obviously, the test strains and solvents could be modified to account for varying environmental conditions surrounding different types of hazardous waste sites.

Ranking mutagenic activity is important in environmental testing to identify the most mutagenic samples and to prioritize multiple samples from the same site for future testing. Bioavailability studies are important for Superfund sites with both marine and freshwater boundaries (e.g., a marsh or a river that flows into the sea) because mutagens in such a site may be more soluble in either fresh water or seawater. Bioavailability could change depending on the soil type, the specific environmental conditions at the site, and the chemistry of the toxicant. In addition, on-site bioremediation could metabolize the mutagens into more water-soluble chemicals, which then could more easily contaminate groundwater (Figures 1 and 2).

Fractionation and chemical analysis of the RTS soils were conducted to identify signature mutagens and are more fully discussed in Brooks et al. (43). When conducting toxicologic evaluation of hazardous waste sites, chemistry alone is not adequate to fully measure decreases or increases in toxicity of the soil or water. Only by the coupling of chemical and biologic analyses can a complete toxicologic evaluation be made. For example, the mutagenic activity of the BS sample in this research increased significantly even though the priority PAH concentrations were reduced 62% by bioremediation (43).

The initial use of the Spiral technique (42) permitted a large amount of mutagenicity testing to be completed with a minimal amount of supplies and effort (Table 1). Each Spiral plate contained 11 dose levels over more than an order of magnitude in range (e.g., 138–1760 µg/plate equivalents). In addition, the S9 plates provided an S9 concentration range from 100 to 8% of the S9 mix, which gave a dose response for both S9 and sample concentrations. The major disadvantage of the Spiral technique was that the variation in this technique was large (20–100% CV); therefore, the minimal mutagenic potency for a positive mutagenic result in the Spiral technique was raised to 0.5 from 0.1 in the plate incorporation assay (Tables 1 and 2). The strategy in this study was to test seven strains initially: TA98 and TA100 (standard Salmonella tester strains), YG1041 and YG1042 (derived from TA98 and TA100, respectively, that contain plasmids with the metabolic activation enzymes nitroreductase and acetyltransferase); TA102 and TA104 (that detect oxidative aldehydes and ketones such as formaldehyde); and TA97 (that detects unusual mutagens such as 9-aminopurine, which distort DNA but do not directly bind to DNA).

After the Spiral assay defined YG1041 and YG1042 as the most responsive strains, the plate incorporation technique permitted the generation of mutagenic potencies to rank mutagenic activity (Table 2). The protocol for the plate assay (10 dose levels in the linear portion of the dose–response curve, triplicate plates per dose) reduced variation in the Salmonella assay to 15% or less (Table 3). When the CV remain below 25%, a doubling of mutagenic potency becomes significant (58) and accurate ranking of mutagenic activity can be accomplished (59,60). When other Salmonella testing protocols are used (i.e., five dose levels over several orders of magnitude for the test sample), toxicity or saturation of the S9 metabolic activation system can cause the variation in mutagenic potencies to exceed 100% (58).

In this research, the ability to accurately measure and quantify mutagenic activity of the four bioremediation processes allowed their effectiveness to be determined (Table 2). Elevated mutagenic activity was detected in the BS and BP extracts that was not present in the UTS (Table 2). The high direct-acting mutagenic activity in the BS extract was surprising because creosote had been mutagenic only with S9 addition (5). One possible reason for this direct-acting mutagenic activity may be that the BS process contained activated sludge from a municipal wastewater treatment facility that had a high input from industrial sources. The sludge may have contained nitroarenes, which are direct-acting mutagens, are highly mutagenic (61), and have been detected in sludge from wastewater treatment plants from an industrial area (62). Although unlikely, the BS process may have allowed both anaerobic and/or aerobic processes to generate nitrogen-containing hydrocarbons. Nitroarenes were indicated in the BS extract by mutagenic activity in the YG strains without S9 and by a 50% reduction of mutagenic activity in TA98NR. However, because the BS was not tested without sludge, we cannot state if the increased mutagenic activity was due to the added sludge or the process itself. This will require further testing. The BP sample did not contain activated sludge; it contained 1% cow manure, which could have contained trace amounts of nitroarenes from the cows'
diet. The BP sample also contained fungi in addition to bacteria, which may have changed the metabolism in the BP reactor. The CMP also contained cow manure but the corn cobs may have eliminated the relatively small amount of direct-acting mutagenic activity by absorption. The LT did not contain any added manure or fungi and had little mutagenic activity. All possible modifications to any one procedure were not investigated in this research. However, the four bioremediation procedures were calibrated by the engineers to perform in an optimal manner.

The reductions in PAH concentrations (48 to 74%) in this study (43) are in agreement with a 50% reduction in creosote (by weight) conducted by Chapman and co-workers (63). The PAH reductions indicated that the four bioremediation processes were closely equivalent. When the mutagenicity data were evaluated along with the PAH data, CMP and LT processes were the most efficient and least toxic bioremediation procedures.

**Reproducibility and Bioavailability Studies**

The reproducibility and low variation of the extraction scheme and the bioassay results were excellent (Table 3), which then allowed a meaningful comparative investigation into the bioavailability of the mutations in the UTS and BP soils (Figures 1 and 2). The bioavailability results (Figures 1 and 2) agreed with the mutagenicity results in Table 2. Mutagenicity was detected in the UTS with S9 and DCM; however, water was able to extract both direct-acting and indirect-acting mutagens from the BP soil. The fact that the BP process made the mutagens soluble in water may be significant for on-site bioremediation, where water-soluble mutagens could contaminate groundwater supplies. Neither the saltwater extraction nor the 50:50 mixture removed any mutagens from the BP sample. The addition of a salt will generally change the characteristics of a solvent, including water. Because oil spills and creosote waste sites may have solvent fronts moving away from the point source of the contamination that may contain contaminants, the toluene extract data in Figure 1 showed that such a solvent front could contain solubilized mutagenic components.

Bioremediation is a powerful tool that can be used to lessen the mutagenic and carcinogenic hazards present at Superfund sites. However, more research is needed on the techniques of bioremediation to increase its efficiency. The coupling of chemical and in vivo biologic analyses can play a key role in the evaluation and monitoring of bioremediation processes, as demonstrated by this research (43).

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**REFERENCES AND NOTES**

1. Barbee GC, Brown KW, Thomas JC, Donnelly KC, Murray HE. Mutagenic activity (Ames Test) of wood-preserving waste sludge applied to soil. Bull Environ Contam Toxicol 57:54–62 (1996).

2. Bos RP, Prinsen WJC, van Rooy JGM, Jongeneelen FJ, Theeuws JLG, Henderson PTh. Fluoranthene, a volatile mutagenic compound, present in creosote and coal tar. Mutat Res 187:119–125 (1987).

3. Bos RP, Jongeneelen FJ, Theeuws JLG, Henderson PTh. Detection of volatile mutagens in creosote tar and oil. Mutat Res 156:195–198 (1985).

4. Bos RP, Theeuws JLG, Leijdekkers Ch-M, Henderson PTh. The presence of mutagenic polycyclic aromatic hydrocarbons benz[a]pyrene and benz[a]anthracene in creosote P1. Mutat Res 130:153–158 (1984).

5. Bos RP, Huishof CTJ, Theeuws JLG, Henderson PTh. Mutagenicity of creosote in the Salmonella/microsome assay. Mutat Res 119:21–25 (1983).

6. Nylund L, Heikkila P, Hameila M, Pyy I, Linnainmaa K, Sorsa M. Genotoxic effects and chemical compositions of four creosotes. Mutat Res 265:223–236 (1992).

7. Donnelly KC, Davol P, Brown KW, Estirl M, Thomas JC. Mutagenic activity of two soils amended with a wood-preserving waste. Environ Sci Technol 21:57–64 (1987).

8. Schoor WP, Williams DE, Takahashi N. The induction of cytotoxicity with 1.45-IA1 in juvenile fish by creosote-contaminated sediment. Arch Environ Contam Toxicol 20:497–504 (1991).

9. Schoket B, Hewer A, Grover PL, Phillips DH. Covalent binding of components of coal-tar, creosote and bitumen to the DNA of the skin and lungs of mice following topical application. Carcinogenesis 9:1253–1258 (1988).

10. Malins DC, Krah MM, Myers MS, Rhodes LD, Brown DW, Krome DW, McCain BB, Chan SL. Toxic chemicals in sludge and biota from a creosote-polluted harbour: relationships with hepatic neoplasms and other hepatic lesions in English sole (Parophrys vetulus). Carcinogenesis 6:1463–1469 (1985).

11. Vogelbein WK, Fournier JW, Van Veld PA, Huggett RJ. Hepatic neoplasms in the Mummichog Fundulus heteroclitus from a creosote-contaminated soil. Cancer Res 50:5978–5986 (1990).

12. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 35: Polynuclear Aromatic Compounds: Part 4: Bitumens, Coal-Tars and Derived Products, Shale-Oils and Soots. Lyon:International Agency for Research on Cancer, 1985.

13. Middaugh DP, Mueller JG, Thomas RL, Lantz SE, Hemmer MH, Brooks GT, Chapman PJ. Detoxification of pentachlorophenol and creosote contaminated groundwater by physical extraction: chemical and biological assessment. Environ Contam Toxicol 21:233–244 (1991).

14. U.S. Environmental Protection Agency. National priorities list for uncontrolled hazardous waste sites; final rule and proposed rule. Fed Reg 61:67655–67677 (1996).

15. Barbee GC, Brown KW, Donnelly KC. Fate of mutagenic chemicals in soil amended with petroleum and wood preserving sludge. Waste Manage Res 10:73–85 (1992).

16. Mueller JG, Middaugh DP, Lantz SE, Chapman PJ. Biodegradation of creosote and pentachlorophenol in contaminated groundwater: chemical and biological assessment. Appl Environ Microbiol 57:1277–1285 (1991).

17. Pereira W, Rosado CE, Garbarino JR, Hult MF. Groundwater contamination by organic bases derived from coal-tar wastes. Environ Toxicol Chem 2:283–294 (1983).

18. Stinson MK, Skovronek HS, Ellis WD. EPA site demonstration of the BioTrol soil washing process. J Air Waste Manage Assoc 42:96–103 (1992).

19. U.S. EPA. Presumptive Remedies for Soils, Sediments and Sludges at Wood Treater Sites. EPA-54-R-95-128. Washington:U.S. Environmental Protection Agency, 1995.

20. Fayad NM, Edora RL, El-Mubarak AH, Polancos AB Jr. Effectiveness of a bioremediation product in degrading the oil spilled in the 1991 Arabian Gulf War. Bull Environ Contam Toxicol 49:787–796 (1992).
21. Claxton LD, Houk VS, Williams R, Kremer F. Effect of bioremediation on the mutagenicity of oil spilled in Prince William Sound, Alaska. Chemosphere 23:643–650 (1991).

22. Sayler GS, Layton A, Lajoie C, Bowman J, Tschantz M, Fleming JT. Molecular site assessment and process monitoring in bioremediation and natural attenuation. Appl Biochem Biotechnol 54:277–290 (1995).

23. Swannell RP, Lee BK, McDonagh M. Field evaluations of marine oil spill bioremediation. Microbiol Rev 60:342–365 (1996).

24. Knapp RB, Faison BD. A bioengineering system for the in situ bioremediation of contaminated groundwater. J Ind Microbiol Biotechnol 18:189–197 (1997).

25. Shannon MJR, Unterman R. Evaluating bioremediation: distinguishing fact from fiction. Ann Rev Microbiol 47:715–738 (1993).

26. Hughes JB, Beckles DM, Chandra SD, Ward CH. Utilization of bioremediation processes for the treatment of PAH-contaminated sediments. J Ind Microbiol Biotechnol 18:152–160 (1997).

27. Mueller JG, Devereux R, Santavy DL, Lantz SE, Willis SG, Prichard PH. Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils. Antonie van Leeuwenhoek 71:329–343 (1997).

28. Govindaswami M, Feldhake DJ, Kinkle BK, Mindell DP, Loper JC. Phylogenetic comparison of two polycyclic aromatic hydrocarbon-degrading Mycobacteria. Appl Environ Microbiol 61:3221–3226 (1995).

29. Geiselbrecht AD, Herwig RP, Deming JW, Staley JT. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediments. Appl Environ Microbiol 62:3344–3349 (1996).

30. Stringfellow WT, Atiken MD. Competitive metabolism of naphthalene, methyl-naphthalenes, and fluorene by phenanthrene-degrading Pseudomonads. Appl Environ Microbiol 61:357–362 (1995).

31. Bogan BW, Lamar RT. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by Phanerochaete chrysosporium. Appl Environ Microbiol 61:2631–2635 (1995).

32. Barr DP, Aust SD. Pollutant degradation by White Rot Fungi. Rev Environ Contam Toxicol 138:49–72 (1994).

33. Bonaventura C, Johnson FM. Healthy environments for healthy people: bioremediation today and tomorrow. Environ Health Perspect 105(Suppl 1):5–20 (1997).

34. Bilva V, Szymańska K, Tichy R, Triska J. Microbiological, chemical and toxicological characterization of contaminated soils in Czechoslovakia. Sci Total Environ(Suppl):185–193 (1993).

35. Lebłowska M, Karwowska E, Miaskiewicz E. Isolation and identification of bacteria from petroleum derivatives contaminated soil. Acta Microbiol Pol 44:297–303 (1995).

36. Vogel TM. Bioaugmentation as a soil bioremediation approach. Curr Opin Biotechnol 7:311–319 (1996).

37. Hanson KG, Nigam A, Kapadia M, Desai AJ. Bioremediation of crude oil contamination with Actinobacter sp. A3. Curr Microbiol 35:191–193 (1997).

38. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Rev 61:47–64 (1997).

39. Deschenes L, Lafrance P, Villeneuve J-P, Samson R. Adding sodium dodecyl sulfate and Pseudomonas aeruginosa UG2 biosurfactants inhibits polynuclear aromatic biodegradation in a weathered creosote-contaminated soil. Appl Microbiol Biotechnol 46:638–646 (1996).

40. Zhang Y, Miller RM. Enhanced octadecane dispersion and biodegradation by a Pseudomonas Rhamnolipid surfactant (bio-surfactant). Appl Environ Microbiol 58:3276–3282 (1992).

41. Zafiro MA, Rabinovitch J, Reed CA, Prichard PH. Revised methods for the Salmonella mutagenicity test. Mutat Res 113:173–215 (1983).

42. Houk VS, Early G, Claxton LD. Use of the spiral Salmonella assay to detect the mutagenicity of complex environmental mixtures. Environ Mol Mutagen 17:112–121 (1991).

43. Brooks L, Hughes TJ, Claxton LD, Austern B, Brenner R, Kremer F. Bioassay-directed fractionation and chemical identification of mutagens in bioremediated soils. Environ Health Perspect 106(Suppl 6):1435–1440 (1998).

44. OECD. OECD Guidelines for Testing of Chemicals. Section 3, Degradation and Accumulation, Method 301C. Ready Biodegradation: Modified MITI Test (I), and Method 302C. Inherent Biodegradability: MITI Test (II), Paris:Organisation for Economic Co-operation and Development. 1981.

45. Glaser JA, McCauley PT, Herrmann R, Dosani M, Zafiro A, Meier JR, Chang L, Brenner RC. Unpublished data.

46. Porter CL, Glaser JA, Dosani MA, Krishnan ER, Brenner RC. Treatment of soil contaminated by polynuclear aromatic hydrocarbons. Environ Sci Technol (in press).

47. Haught RC, Neogy R, Vonderhaar SS, Krishnan ER, Saffer RN, Ryan J. Land treatment alternatives for bioremediating wood preserving wastes. J Hazard Waste Hazard Material 12(4):329–344 (1995).

48. Saffer RN, Lamar RT, Vonderhaar SS, Neogy R, Haught RC, Krishnan ER. Treatability study using Phanerochaete sordida for the bioremediation of DDT contaminated soil. Toxicol Environ Chem 50:237–251 (1995).

49. Hinomura Y, Watanabe E, Oda Y, Sofuni T,Nohmi T. Specificity and sensitivity of Salmonella typhimurium YG1042 and YG1042 strains possessing elevated levels of both nitroreductase and aceetyltransferase activity. Mutat Res 291:171–180 (1993).

50. Claxton LD, Allen J, Auletta A, Mortelmans K, Nestmann E, Zeiger E. Guide for the Salmonella typhimurium/mammalian microsome test for bacterial mutagenicity. Mutat Res 189:83–91 (1987).

51. Bernstein L, Kaldor J, McCann J, Pike M. An empirical approach to the statistical analysis of mutagenic data from the Salmonella test. Mutat Res 97:267–281 (1982).

52. Hughes TJ, Lewtas J, Claxton LD. Development of a standard reference material for diesel mutagenicity in the Salmonella plate incorporation assay. Mutat Res 391:243–258 (1997).

53. Strand A, Hasselbald V, Creason J, Claxton L. Modeling the Ames Test. Mutat Res 85:13–27 (1981).

54. Claxton LD, Creason J, Nader J, Potet W, Orr J. GeneTox manager for bacterial mutagenicity assays: a personal computer and minicomputer system. Mutat Res 342:87–94 (1995).

55. Available upon written request from L. Claxton, U.S. EPA, MD68, Research Triangle Park, NC 27711.

56. Rosenkranz HS, Potet W. Development of the mutagenicity and DNA-modifying activity in microbial systems of carcinogens and non-carcinogens. J Natl Cancer Inst 62:873–892 (1979).

57. Hughes T. Unpublished data.

58. Myers LE, Adams NH, Hughes TJ, Williams LR, Claxton LD. An interlaboratory study of an EPA Ames/Salmonella test protocol. Mutat Res 182:121–133 (1987).

59. Claxton LD, Houk VS, Monteith LG, Myers LE, Hughes TJ. Assessing the use of known mutagens to calibrate the Salmonella typhimurium mutagenicity assay. I: Without exogenous activation. Mutat Res 253:137–147 (1991).

60. Claxton LD, Houk VS, Warner JR, Myers LE, Hughes TJ. Assessing the use of known mutagens to calibrate the Salmonella typhimurium mutagenicity assay. II: With exogenous activation. Mutat Res 253:149–159 (1991).

61. Rosenkranz HS, Mermelstein R. Mutagenicity and genotoxicity of nitroarenes: all nitro-containing chemicals were not created equal. Mutat Res 114:217–267 (1983).

62. Bodzek D, Janoszka B, Dobosz C, Warzecha L, Bodzek M. Determination of polycyclic aromatic hydrocarbons and heavy metals in sludges from biological sewage treatment plants. J Chromat 74:177–192 (1997).

63. Chapman PJ, Shelton M, Griffoll M, Selinonov S. Fossil fuel biodegradation: laboratory studies. Environ Health Perspectives 103:79–83 (1995).