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 Spiral technique 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 Arochlor-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 nitrohydrocarbons. 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 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1427-1433hughes/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 toxicants. Indigenous bacteria and fungi often utilize organic toxicants 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 toxicants, 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 BP 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 dimethyl sulfoxide (DMSO) used as a solvent for the plate incorporation mutagenicity bioassay (*41*) was a spectrophotometric grade. Both DCM and DMSO were obtained from Burdick and Jackson (Muskegon, Michigan). The water used for the bioavailability experiment was deionized-distilled, sterilized municipal

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Abbreviations used: 50:50, 50% water:50% salt water; BP, biopile extract; BS, bioslurry; CMP, compost; CV, percent coefficient of variation (standard deviation/mean × 100%); DCM, dichloromethane; DMSO, dimethyl sulfoxide; LRB, lab solvent blank in DCM; LT, land treatment; OECD, Organisation for Economic Co-operation and Development; PAH, polycyclic aromatic hydrocarbon; RTS, Reilly Tar site, St. Louis Park, Minnesota; S9, Aroclor-induced rat liver; UTS, untreated creosote-contaminated soil.

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 danthron (TA102). All control chemicals were obtained from the Sigma Chemical Company (St. Louis, Missouri).

Reilly Tar Site Soils

Untreated 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:phosphorus. 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 ostrates*).

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., Stead 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 1:1000 in water; sterile water; artificial seawater; a mixture of 50% water and 50% artificial seawater (50:50); 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 µ) overlaid with 1 to 2 cm sodium sulfate to dry the extract, and concentrated to 25 ml in a rotoevaporation 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 Soil Extracts

Preliminary mutagenic evaluation of the five RTS 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 microgam) 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.[a]

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
YG1042	–	6.7	NM	1.4	NM	NM	NM
	+	4.3	2.2	NM	NM	NM	NM
TA98	–	NM	NM	0.7	NM	NM	NM
	+	2.3	NM	NM	NM	NM	NM
TA100	–	NM	1.3	1.1	NM	0.7	NM
	+	NM	1.9	NM	NM	NM	NM
TA97	–	0.6	NM	NM	NM	NM	NM
	+	0.8	NM	NM	NM	NM	NM
TA102	–	0.8	NM	NM	NM	1.0	NM
	+	NM	1.4	NM	0.6	0.6	NM
TA104	–	NM	NM	NM	NM	NM	NM
	+	NM	NM	NM	NM	NM	NM

NM, not mutagenic. [a]Mutagenic potencies from the Bernstein linear regression model (*51*). Mutagenic potencies less than 0.5 and those that were nonsignificant are noted as NM.

Table 2. Mutagenic potencies of the extracts from the Reilly Tar site in the plate incorporation mutagenicity assay.

	Revertants/µg in two strains[a]			
	YG1041		YG1042	
Samples	–S9	+S9	–S9	+S9
UTS	NM	1.2	NM	0.17
LRB	NM	NM	NM	NM
BS	38.6[b]	31.4[b]	18.0[b]	8.9[b]
BP	3.0[b]	5.0[b]	1.3[b]	1.7[b]
CMP	NM	2.3[c]	NM	0.2
LT	0.16	1.8[c]	NM	0.2

[a]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. [b]Mutagenic response, which was significantly different from the UTS (>2-fold difference). BP values in YG1042 were significant because the values for UTS were not mutagenic (–S9) and there was a 10-fold difference in the +S9 comparison. [c]Elevated mutagenic response above UTS +S9 slope.

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.

	Revertants/µg[a]			
Sample	Assay 1	Assay 2	Assay 3	Assay CV[b]
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: scheme CV[b]	9.1	15.5	13.6	

[a]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. [b]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

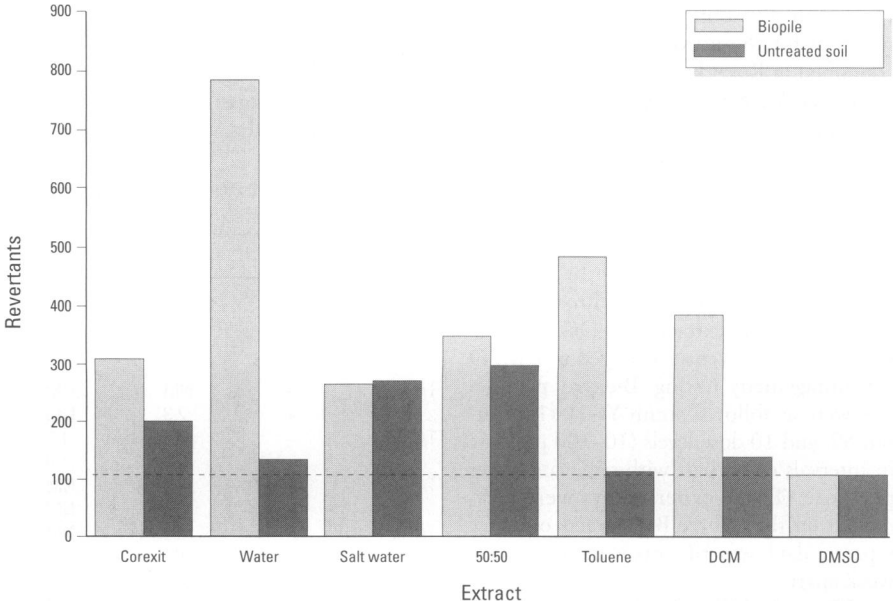

Figure 1. Mutagenic activity in *Salmonella* strain YG1041 of untreated soil and biopile soil extracts (100 µg/plate) from the Reilly Tar site tested without S9 and extracted with six different solvents.

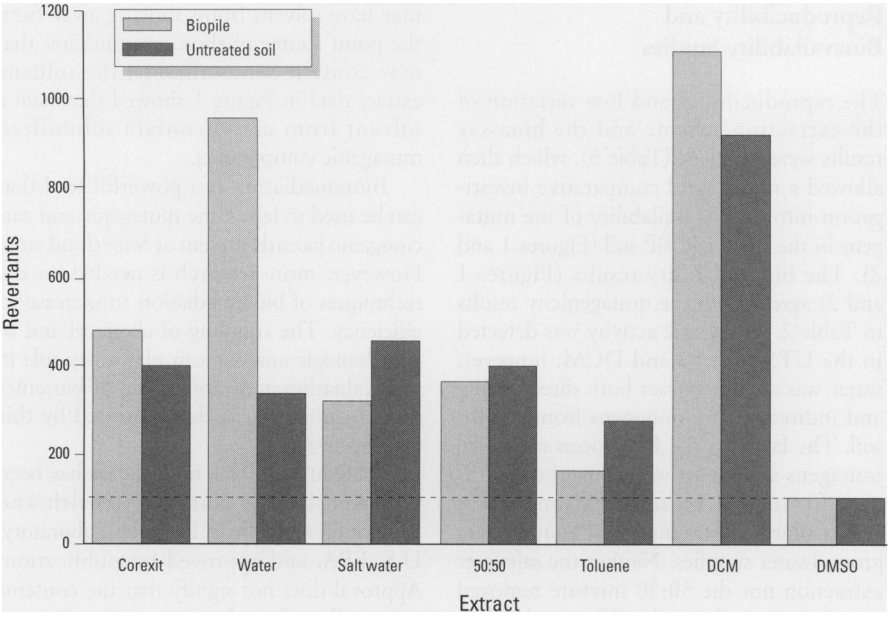

Figure 2. Mutagenic activity in *Salmonella* strain YG1041 of untreated soil and biopile soil extracts (100 µg/plate) from the Reilly Tar site tested with S9 and extracted with six different solvents.

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-aminoacridine, 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 mutagens in the UTS and BP soil (Figures 1 and 2). The bioavailability results (Figures 1 and 2) agreed with the mutagenicity results in Table 2. Mutagenic activity 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 vitro* 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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