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Managed Bioremediation of Soil Contaminated with Crude Oil

Soil Chemistry and Microbial Ecology Three Years Later

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ABSTRACT

Analysis of samples taken from three experimental soil lysimeters demonstrated marked long-term effects of managed bioremediation on soil chemistry and on bacterial and fungal communities 3 yr after the application of crude oil or crude oil and fertilizer. The lysimeters were originally used to evaluate the short-term effectiveness of managed (application of fertilizer and water, one lysimeter) vs unmanaged bioremediation (one lysimeter) of Michigan Silurian crude oil compared to one uncontaminated control lysimeter. Three years following the original experiment, five 2-ft-long soil cores were extracted from each lysimeter, each divided into three sections, and the like sections mixed together to form composited soil samples. All subsequent chemical and microbiological analyses were performed on these nine composited samples.

Substantial variation was found among the lysimeters for certain soil chemical characteristics (% moisture, pH, total Kjeldahl nitrogen [TKN], ammonia nitrogen [NH_4-N], phosphate phosphorous [PO_4-P], and sulfate [SO_4^{-2}]). The managed lysimeter had 10% the level of total petroleum hydrocarbons (TPH-IR) found in the unmanaged lysimeter. Assessment of the microbial community was performed for heterotropic bacteria, fungi, and aromatic hydrocarbon-degrading bacteria (toluene, naphtha-

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lene, and phenanthrene) by dilution onto solid media. There was little difference in the number of heterotrophic bacteria, in contrast to counts of fungi, which were markedly higher in the contaminated lysimeters. Hydrocarbon-degrading bacteria were elevated in both oil-contaminated lysimeters. In terms of particular hydrocarbons as substrates, phenanthrene degraders were greater in number than naphthalene degraders, which outnumbered toluene degraders. Levels of sulfate-reducing bacteria seem to have been stimulated by hydrocarbon degradation.

Index Entries: Crude Oil; bioremediation; TPH; sulfate-reducing bacteria; nematodes; soil; fungi; hydrocarbon.

INTRODUCTION

It is well established that most of the hydrocarbons of crude oils are amenable to biodegradation by microorganisms indigenous to soil provided sufficient aeration, moisture, and mineral nutrients are available. The ultimate objective of *in situ* bioremediation of petroleum-contaminated soil is to return the economic and/or aesthetic value of the site. Clearly the long-term effects of the original contamination and the bioremediation process on the soil ecosystem are important determinants to the eventual postremediation use of the soil.

In 1992 Amoco Production Company initiated a study of managed and unmanaged bioremediation of soil contaminated with Michigan Silurian crude oil. Three lysimeters were filled with topsoil. One was left uncontaminated as a control, whereas the other two were contaminated with crude oil. Of the contaminated lysimeters, one received fertilizer and water (managed), and the other did not (unmanaged). The rates and extent of bioremediation in the contaminated lysimeters were then studied for 6 mos. After the conclusion of this work, the lysimeters were unattended and exposed to the elements.

Three years after the original contamination of the lysimeters, the work presented in this article was initiated with the objective of evaluating the long-term effects of crude oil contamination and bioremediation on soil chemistry and microbial populations. This study consisted of a comparison of the three lysimeters on the basis of soil chemistry and bacterial and fungal populations at different depths.

MATERIALS AND METHODS

Soil Lysimeters

The three soil lysimeters were originally set in place in 1992 at the Amoco Production Research Environmental Test Facility in Rogers County, OK. Each lysimeter measures 9.1 ft (2.8 m) by 9.1 ft (2.8 m) by 3 ft (0.91 m) (depth) and consists of a shallow, reinforced concrete container

Table 1
Analysis of Original Lysimeter Soil

Soil Moisture (%)	9.6
pH	7.0
Saturated Paste Moisture (%)	26.4
CEC (meq/100g)	4.8
NO_3 - N (ppm)	0.3
PO_4 - P (ppm)	30.2
EPTA K (ppm)	61.7
Soluble Cations (mg/L)	
Na	0.2
Ca	1.9
Mg	0.9
Exchangeable Sodium (%)	<1.0
TPH-IR	17.5
TPH-GC	4.1
Exchangeable Cations (meq/100g)	
Na	<0.1
Ca	2.1
Mg	1.0

Table 2
Type Analysis of Michigan Silurian Crude Oil

Carbon Number	Mole %
C4 - C10	48.98
C11 - C20	36.66
C21 - C30	10.26
C31 - C40	3.05
C41 - C53	1.05

containing 9.2 yd^3 (7.1 m^3) of soil. Each lysimeter is drained by a pipe into a collection pond. The soil used to fill the lysimeters was collected from northwest Tulsa County, and was representative of the loamy Okay series found in that area *(1)*. This soil was not contaminated with salt or hydrocarbon. An analysis of this soil as given by Fisher and King *(2)* is given in Table 1.

Twenty-one gallons of Michigan Silurian Reef crude oil (Table 2) were applied to two of the lysimeters by hand-spraying evenly over the surface and tilling to a depth of 1 ft (0.305 m). This gave an initial oil loading of 1.7% by weight. Fertilizer was added to one of the contaminated lysimeters (managed bioremediation) to provide nitrogen (0.73 kg, in the form of urea), phosphate (0.18 kg, as P_2O_5), and potassium (0.18 kg as K_2O). The managed lysimeter was watered as needed to maintain soil moisture at 80% of container capacity. Total petroleum hydrocarbons (TPH) were

monitored for 190 d. By the end of this period, TPH in the unmanaged lysimeter showed a 26% decrease (as determined by infrared absorbance) and an 88% decrease in the managed lysimeter.

Soil Gas Measurements

Three years after the original contamination with crude oil, the current project was initiated with a soil gas analysis in the control (C), oiled (O), and oiled and fertilized (OF) lysimeters. Soil gases (O_2, CO_2, volatile organic compounds, volatile organic compounds minus methane) were measured 12 in. (30.5 cm) below the surface at three (C, OF) or four (O) well-separated locations in each lysimeter. Soil vapors were sampled with an AMS Soil Gas Vapor Probe (SGVP; Forestry Suppliers, Jackson, MI). SGVP dedicated sampling tips, perforated with vapor inlet holes, were driven 12 in. (30.5 cm) into the subsurface. The SGVP drive tubes were removed, leaving the vapor tip probe imbedded at the desired sampling depth. A Teflon vapor tube, connected to the tip and extending to the surface, was used to sample soil gases near the tip. The VOCs in soil gases were measured using a Gastech Trace-Techtor hydrocarbon analyzer with range settings of 100, 1000, and 10,000 ppm. The analyzer was calibrated against hexane calibration gas (4350 ppm). Soil gas concentrations of CO_2 and O_2 were measured using a Gastech model 32520 × CO_2/O_2 analyzer. The CO_2 calibration was performed against atmospheric CO_2 concentration (0.05%) and a 2.5% standard. The O_2 was calibrated using an atmospheric standard (20.9%). Both analyzers had an internal vacuum pump for sampling soil gases.

Soil Sampling

Soil core probes were made of 3-cm (id) stainless-steel tubing. The inside surface of all probes was washed with methylene chloride to remove hydrocarbon residues. The probes were then sterilized by autoclaving at 300°C for 90 min. Soil cores were taken in a five-spot pattern from each lysimeter from the surface to 24 in. (61 cm) below the surface. The probes were brought back to the laboratory to be processed immediately after collection. Using a tubing cutter, each probe was cut into three sections: surface to 3 in. (7.6 cm) below surface (L1); 3 in. (7.6 cm) below surface to 12 in. (30.5 cm) below surface (L2); 12 in. (30.5 cm) below surface to 24 in. (61 cm) below surface (L3). Sterilized spoons were used to remove soil from each section of a probe. Soil samples were placed into pails that had previously been washed with methylene chloride and then sterilized by autoclaving. Composite soil samples were made by mixing with a sterile spoon the five like sections (in terms of sample depth) from each lysimeter in the sterilized pails, giving a total of nine samples, three from each lysimeter. The composited soil samples were then subdivided for chemical and microbial analysis.

Soil Chemical Analysis

The pH, % moisture, carbonate, bicarbonate, chloride, sulfate, sodium, calcium, magnesium, potassium, sodium adsorption ratio (SAR), cation-exchange capacity (CEC), total Kjeldhal nitrogen (TKN), nitrate nitrogen (NO_3-N), ammonia nitrogen (NH_4-N), total phosphorous, available phosphate phosphorous (PO_4-P), and total carbon (%) were determined for each of the composited soil samples. Composited soil samples were also analyzed for total petroleum hydrocarbons by both gas chromatography (TPH-GC) and by infrared absorbance of a solvent extract (TPH-IR), and for benzene, toluene, ethylbenzene, and xylenes (BTEX). Samples were shipped by overnight delivery in completely filled glass jars with Teflon-lined lids to Soil Analytical Services, College Station, TX for analysis.

Fungi

Nine milliliters of sterilized water were added to 1 g of soil, mixed thoroughly by vortexing, then diluted, and spread (three replicates) onto Malt Extract Medium (Difco, Detroit, MI) containing 300 μg/mL of the antibacterial antibiotic streptomycin (Sigma Chemical, St. Louis, MO). The plates were incubated at room temperature for 2 wk before colonies were counted.

Heterotrophic and Hydrocarbon-Degrading Bacteria

Ten milliliters of sterilized water were added to 1 g of soil, mixed thoroughly by vortexing, then diluted, and spread (three replicates) onto Plate Count Agar (PCA, Difco Co.) containing 40 μg/mL of the antifungal antibiotic cycloheximide (Sigma Chemical Co.), and onto a mineral salts agar with trace metals containing either toluene (TOL), naphthalene (NAP), or phenanthrene (PHE), to select for toluene-, naphthalene-, or phenanthrene-degrading bacteria, respectively. PCA media were incubated at room temperature for 48 h before colonies were counted. Each of the three hydrocarbons was separately administered in the vapor phase as crystals (NAP, PHE) or liquid on filter paper (TOL) placed on the inside of the Petri plate lid. Each type of hydrocarbon medium was sealed separately in a Rubbermaid container, and incubated for 36 at room temperature in an active chemical fume hood in order to prevent mixing of the vapors.

Sulfate-Reducing Bacteria

Three grams of composited soil were added to a bottle containing 9 mL of SRB medium (Bioindustrial Technologies, Austin, TX). The soil and medium in the tube were then thoroughly mixed by vortexing for 1 min. A 1-mL sample was then withdrawn with a sterile needle and syringe, and used to inoculate another 9-mL bottle of SRB medium. The procedure was

Table 3
Soil Gas Measurements[a]

Lysimeter	O_2 (%)	CO_2 (%)	VOC (ppm)	VOC-Me (ppm)
C	18-19	0.5-0.6	12-16.5	10-11.5
O	14.5-15.8	2.1-2.9	36-40.5, 119	30-37
OF	11.7-14	2.2-4.2	130-145	37-40

[a]Soil gases were measured 12 in. below the surface at three (C, OF) or four (O) well-separated locations in each lysimeter. The numbers span the range of values obtained.

ppm = parts per million; VOC = volatile organic compounds; VOC-Me = volatile organic compounds minus methane.

repeated for a total of 10 dilutions of the original soil sample, and performed in triplicate for each of the nine composited samples. The tubes were incubated for 25 d and then scored for growth and formation of a black precipitate (iron sulfide), indicating the presence of sulfate-reducing bacteria (SRB). The most-probable number (MPN) of SRB in the original samples was estimated from the characteristic number for a three-tube MPN *(3)*.

RESULTS AND DISCUSSION

Soil Gas

Results of soil gas analysis (at 1 ft or 30.5 cm) are summarized in Table 3. Total VOCs were highest in the fertilized and oiled (OF) lysimeter compared to the oiled (O) and control (C) lysimeters. However, as shown in Table 3, most of the VOC in the soil gas of the OF lysimeter was methane, suggesting that the fertilizer originally applied to this lysimeter has had a stimulating effect on methanogenesis in anaerobic zones or microenvironments. High levels of methane were also detected at one location in the O lysimeter. The substrates for methanogenesis could be products of aerobic degradation of the hydrocarbons. Elevated nonmethane VOCs in the O and OF lysimeters were accompanied by reduced oxygen (O_2) and elevated carbon dioxide (CO_2) concentrations, indicative of aerobic biodegradation of petroleum hydrocarbons. The greater CO_2 concentrations and lower O_2 concentrations in the OF lysimeter compared to the O lysimeter again suggest a stimulation of bioactivity 3 yr after application of the fertilizer.

Hydrocarbon Analysis

As expected, no BTEX was detected in any composited soil samples. Results of analysis of composited soil samples for TPH are given in Table 4. For comparison, the TPH-IR levels in 12-in. (30.5-cm) composited samples 190 d after oil application were (as given by Fisher and King [2]): 17.5

Table 4
Total Petroleum Hydrocarbon Analysis of Composited Soil Samples[a]

Lysimeter	Level	TPH-IR (mg/kg)	TPH-GC (mg/kg)
C	L1	< 10	< 25
	L2	< 10	< 25
	L3	< 10	< 25
O	L1	17237	6000
	L2	1452	249
	L3	30.7	60
OF	L1	1633	84
	L2	863	75
	L3	< 10	< 25

[a]C = control lysimeter; O = oiled lysimeter; OF = oil and fertilized lysimeter; L1 = 0–3 in. depth composite; L2 3–12 in. depth composite; L3 = 12–24 in depth composite.

mg/kg (C), 8440–16,277 mg/kg (O), and 1511–2169 mg/kg (OF). The data given in Table 4 suggest that although the soil in each oiled lysimeter was tilled to 1 ft after application, most of the oil remained near the surface. If the TPH-IR data in Table 4 for L1 and L2 are averaged over a 1-ft (30.5-cm) depth, the weighted averages are 5400 mg/kg (O) and 1060 mg/kg (OF). Therefore, in both the O and OF lysimeters, additional TPH-IR reduction has been realized since the 190-d analysis. It is interesting to note though that if the TPH-GC is taken to be the lighter fraction of the total hydrocarbons in the soil, then the hydrocarbons in the oil and fertilized lysimeter (OF) have been significantly enriched for the heavier components compared to the oiled lysimeter (O). The soil gas analysis suggests that these heavier components or residual partially oxidized products of the original oil in place are still undergoing active aerobic biodegradation.

Soil Chemistry

Results of the chemical analysis of composited soil samples from the three lysimeters are given in Table 5. The following observations are made based on the comparative soil chemistry of the lysimeters:

1. The capacity of the soil for holding moisture correlated somewhat with TPH. Higher TPH-IR resulted in greater moisture retention in the soil.
2. There was a reduction in pH, which correlated to greater hydrocarbon biodegradation. This is attributed to the production of organic acids as intermediates of hydrocarbon degradation, perhaps because of local oxygen limitations.

Table 5
Lysimeter Soil Chemistry

Lysimeter	Level	pH	% Moisture	Soluble cations and anions (meq/L) CO_3^{-2}	HCO_3^-	SO_4^{-2}	Cl^-	Na^+	Ca^{+2}	Mg^{+2}	K^+
C	L1	6.3	5.0	< 0.1	2.0	0.3	0.7	0.1	1.2	0.5	0.1
	L2	6.6	11.5	< 0.1	1.3	0.5	1.4	0.5	0.8	0.5	0.1
	L3	6.7	15.8	< 0.1	1.2	0.5	0.8	0.4	0.8	0.5	0.1
O	L1	6.0	7.8	< 0.1	0.6	0.7	0.2	0.1	1.1	0.4	< 0.1
	L2	6.1	15.4	< 0.1	1.0	0.4	0.2	0.1	0.9	0.4	< 0.1
	L3	6.8	18.1	< 0.1	2.4	0.3	0.2	0.1	1.6	0.5	0.1
OF	L1	5.5	4.9	< 0.1	0.9	0.4	0.4	0.1	1.0	0.4	0.2
	L2	5.8	12.5	< 0.1	0.9	0.5	0.2	0.1	0.9	0.3	0.2
	L3	6.3	15.2	< 0.1	1.4	0.4	0.3	0.2	1.0	0.4	0.1

Lysimeter	Level	SAR	CEC (meq/100g)	TKN (mg/L)	NO_3-N (mg/L)	NH_4-N (mg/L)	Total P (mg/L)	Avail-PO_4-P (mg/L)	LECO Total C (%)
C	L1	0.1	4.1	155	< 1.0	5.9	252	39.1	0.2
	L2	0.6	4.2	218	< 1.0	3.1	198	39.0	0.2
	L3	0.5	4.0	112	< 1.0	8.1	302	42.4	0.1
O	L1	0.1	4.7	1160	< 1.0	10.6	404	26.8	1.2
	L2	0.1	5.7	290	< 1.0	9.7	301	36.2	0.3
	L3	0.1	5.8	154	< 1.0	8.3	283	35.7	0.2
OF	L1	0.1	5.7	554	< 1.0	14.7	284	69.0	0.6
	L2	0.1	5.4	359	< 1.0	14.7	246	51.8	0.4
	L3	0.2	5.3	308	< 1.0	12.9	276	33.8	0.3

3. The oiled and fertilized (OF) lysimeter still contained elevated levels of NH_4-N, available PO_4-P, and TKN relative to the C and O lysimeters 3 yr after application.
4. The OF lysimeter still contained significant levels of total carbon compared to the O lysimeter, out of proportion to the relative TPH levels. This suggests that high concentrations of partially degraded hydrocarbons that are not measured as TPH remain in the OF lysimeter that continue to fuel biological activity as indicated by the soil gas analysis.

Fungi

Fungi were must abundant in the uppermost level of all lysimeters, with higher concentrations in the O and OF lysimeters ($5–7 \times 10^4$ CFU/g soil) than in C (2×10^4 CFU/g soil), suggesting that one effect of crude oil contamination and subsequent bioremediation was to promote the growth of soil fungi. The positive effect seen in O and OF may be partially owing to the crude oil acting as a carbon and energy source for fungi that can degrade oil, since fungi were abundant on media containing aromatic hydrocarbons. Also, the low pH in the O and OF lysimeters (Table 5) may have favored fungi at the expense of bacteria, since fungi are more tolerant of low pH.

Bacteria

Mean Heterotrophic Bacteria

Aerobic heterotrophic bacteria were more abundant in the two uppermost levels of the lysimeters, but differed little among the lysimeters. The mean concentration of viable bacteria (CFU)/g soil (dry wt) for each level of the three lysimeters was approx 10^6. Crude oil contamination seemed to have relatively little effect after 3 yr on densities of bacteria assayed on this medium, unlike the soil fungi.

Hydrocarbon-Degrading Bacteria

Toluene-degrading bacteria were present in all lysimeters and all levels at approximately the same low concentration ($4 \times 10^3–10^4$ CFU/g soil). The concentration of naphthalene degraders was somewhat elevated in OL2 and OL3 (10^3 CFU/g soil) above the corresponding levels in C ($7 \times 10^1–8 \times 10^2$ CFU/g soil), whereas the concentration in OF ($4 \times 10^4–2 \times 10^6$ CFU/g soil) was elevated for all levels. Phenanthrene degraders were much more abundant in OF ($4 \times 10^5–2 \times 10^7$ CFU/g soil) and O ($10^5–2 \times 10^6$ CFU/g soil) than in C ($3 \times 10^3–7 \times 10^4$ CFU/g soil). The relative densities of these hydrocarbon degraders (PHE > NAP > TOL) probably reflect the current level of these classes of compounds as the hydrocarbon is enriched for the heavier components. Fungi were also abundant on these plates, but have not yet been examined.

Table 6
Concentration (Most Probable Number/g Soil)
of Viable Sulfate-Reducing Bacteria in Soil from Each Lysimeter

Level	Lysimeter C	O	OF
L1	2.5×10^2	9×10^5	1.5×10^6
L2	2.5×10^2	5×10^6	5×10^6
L3	10^5	10^9	10^9

Sulfate-Reducing Bacteria

The mean concentration of sulfate-reducing bacteria (most probable number/g) in composited samples from each level of each lysimeter is shown in Table 6. A higher concentration of SRB was found in L2 and L3 of each lysimeter than in L1, as expected for these anaerobic bacteria. Higher concentrations of SRB were found in each level in the lysimeters contaminated with crude oil than in the control. It appears that aerobic degradation of hydrocarbons in L1 has stimulated the growth of SRB, especially in L3.

CONCLUSIONS

Three soil lysimeters, originally used to study the short-term degradation of crude oil in soil, have been re-examined 3 yr after the application of oil. The application of crude oil and subsequent bioremediation (managed and unmanaged) have had marked effects on soil chemistry and microbial populations. Soil analysis showed that the fertilized lysimeter still contained elevated levels of NH_4-N, available PO_4-P, and TKN relative to the control lysimeter (C) and the lysimeter (O), which received oil only. The application of fertilizer to a crude oil-contaminated lysimeter (managed bioremediation—OF) compared to unmanaged bioremediation (O) has resulted after three years in:

1. Greater degradation of hydrocarbons (TPH);
2. Greater rates of oxygen consumption in aerobic zones; and
3. Increased methanogenesis.

However, although the OF lysimeter TPH was much reduced compared to the O lysimeter, the OF lysimeter still contained significantly higher levels of total carbon than the O or C lysimeters, suggesting high concentrations of partially degraded hydrocarbons. The soil gas analyses strongly suggest that these compounds continue to fuel high levels of bioactivity. Both the O and OF lysimeters exhibited reduced pH and increased levels of fungi, SRB, and hydrocarbon degraders compared to C.

In summary, three years after crude oil contamination and subsequent application of fertilizer (managed bioremediation), microbial populations and microbial activity still remain stimulated, and bioremediation of the soil continues. However, more time and/or active intervention will be required for this soil to return to normal as defined by the uncontaminated lysimeter.

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