Multi-Species Ecotoxicity Assessment of Petroleum-Contaminated Soil

Kathleen Duncan,^{1*} Eleanor Jennings,¹ Paul Buck, and Harrington Wells

Department of Biological Sciences

Ravindra Kolhatkar² and Kerry Sublette

Department of Chemical Engineering

William T. Potter

Department of Chemistry, University of Tulsa,
600 S. College Ave., Tulsa, OK 74104

Timothy Todd

Department of Plant Pathology, 4024
Throckmorton Hall, Kansas State University,
Manhattan, KS 66506

In 1992, a study was begun to compare the effect of landfarming vs. natural attenuation on the restoration of soil that had been contaminated with crude oil. Each of three lysimeters was filled with a sandy loam topsoil, and crude oil was applied to two of the lysimeters. One of the contaminated

lysimeters was tilled, watered, and received a one-time application of fertilizer (N, P, K). No amendments were added to the second contaminated lysimeter, and the third was left uncontaminated. The lysimeters were monitored for 6 months and then left unattended.

In 1995 and again in 1997 we sampled these lysimeters to evaluate the long-term effects of contamination and bioremediation. In 1995 we found marked effects on soil chemistry, bacterial, fungal, nematode, and plant populations and a higher rate of bioremediation in the fertilized-contaminated lysimeter (Lawlor et al., 1997). Data from 1997 and previously unreported data from 1995 are the subject of the current report. In 1997, low densities of hydrocarbon-degrading bacteria were found in all the lysimeters and little loss of TPH from the two contaminated lysimeters, suggesting a decreased rate of bioremediation. Nevertheless, there were increases in diversity and number of functional groups of bacteria, nematodes, and native plant species. However, molecular analyses revealed marked differences remained in the composition of dominant eubacterial species, and tests of soybeans indicated field conditions remained unsuitable for these plants.

* corresponding author.

¹ Present address: Department of Botany and Microbiology, The University of Oklahoma, 770 Van Vleet Oval, Norman, OK 73019. Phone: 405-325-4892. Fax: 405-325-3180. Email: kathleen.e.duncan-1@ou.edu

² Present address: BP America Inc., Naperville, IL 60563.

Key Words: *crude oil contamination, restoration of chemically contaminated soil, biological indicators, nematodes, bioremediation, disturbed habitats.*

INTRODUCTION

The restoration of chemically contaminated soil is the goal of various bioremediation techniques. Success is frequently measured by chemical analysis of the soil matrix after a period of microbial activity, and by determining whether the level of certain chemicals fall below regulatory toxicity standards (Pope and Matthews, 1993; Huesemann, 1994). These standards were typically derived from tests of acute toxicity using a limited number of test species (Munkittrick *et al.*, 1991). The limitations of these techniques, including synergetic effects of mixtures of chemicals, absorption by soil humic acids, and variation in the physiology or modes of exposure of the test organism are well known (Baker, 1970; Wang and Bartha, 1990; Munkittrick *et al.*, 1991). Microbial degradation of chemical compounds can produce striking alterations of the soil ecosystem, including changes in soil gas levels of oxygen and carbon dioxide, variation in pH levels, and the production of toxic intermediates (Bossert and Compeau, 1995; Renoux *et al.*, 1995; Madsen, 1997). These alterations can be expected to have a direct impact on soil biota and associated ecosystem functions such as carbon and nitrogen cycling. However, indirect effects such as the stimulation/inhibition of prey populations for nematodes or alterations of plant-mycorrhizal associations may be just as important (Goldstein *et al.*, 1985; Hetrick *et al.*, 1990; van der Heijden *et al.*, 1998; Joner *et al.*, 2001). The cascade of impacts that stem from the initial chemical contamination means that neither standard chemical analytical techniques nor toxicity measurements is sufficient to fully gauge the impact on environmental receptors. We also predict that the “restoration” of the soil ecosystem (defined as a return to precontaminated conditions) will not proceed at the same rate for all components of the ecosystem (e.g., lack of synchronization), and that restoration will show a time lag relative to the rate of loss of the chemical contaminant—making it even more difficult to determine acceptable end-points solely from chemical concentration.

Given the complexity of the problem, how can we evaluate the impact of chemical contamination on relevant ecological receptors? In this study, we traced the effect of contamination by crude oil and remediation on a soil ecosystem over a period of 5 years as an illustration of a multipronged approach. An important component is the emphasis on species diversity and composition of indigenous, multispecies communities.

Previous History

In 1992, the Amoco Production Company initiated a study of soil contaminated with Michigan Silurian Reef crude oil (48.98 mole % C₄–C₁₀, see Fisher and King, 1994; Lawlor *et al.*, 1997 for more details). Three lysimeters were constructed, each consisted of a reinforced concrete container measuring (inner dimensions) 2.8

m by 2.8 m by 0.91 m deep, filled with 7.1 m³ of soil, and drained by a pipe into a collection pond. The surface of the soil inside the lysimeters was the same level as that outside. The soil, collected from northwest Tulsa County, is representative of the loamy Okay series (Cole *et al.*, 1977) and was not contaminated with salt or hydrocarbons. 79.5 L (1.7 % wt or 17,000 mg/kg) of crude oil were applied to two of the three lysimeters by hand-spraying evenly over the surface and tilling to a depth of 30.5 cm. The third plot was left uncontaminated and hereafter is referred to as the “C” (e.g., “Control”) plot. Fertilizer was added to one of the contaminated lysimeters (managed bioremediation, hereafter “OF”, e.g., “oil and fertilizer”) to provide nitrogen (0.73 kg-N, in the form of urea), phosphate (0.18 kg-P, as P₂O₅), and potassium (0.18 kg-K as K₂O), to give an initial C:N:P ratio (wt) of approximately 195:4:1. The soil in the OF lysimeter only was watered to maintain soil moisture at 80% of container capacity. Neither fertilizer nor water, other than naturally occurring rainfall, was added to the C or unmanaged contaminated lysimeter (hereafter “O”, e.g. “oil”). BTEX and total petroleum hydrocarbons (TPH-IR, EPA Method 418.1) were monitored from all three lysimeters for 190 days. By the end of this period TPH-IR in soil collected from the top 30.5 cm of the O lysimeter decreased to 12,580 mg/kg and decreased in the OF lysimeter to 2040 mg/kg (Fisher and King, 1994). All BTEX compounds except xylenes at 11.64 ppm in the O lysimeter were below the limit of detection (<0.50 ppm) after 190 days. At the conclusion of this phase the lysimeters were unattended and exposed to the elements.

In 1995, 3 years after the original contamination of the lysimeters, we initiated a project to evaluate the long-term effects of crude oil contamination and bioremediation on the soil ecosystem (Lawlor *et al.*, 1997). The three lysimeters were compared on the basis of soil chemistry; bacterial, fungal, and nematode populations at different depths; and the plant life that had become established on the soil surface of each lysimeter. There were still marked effects at 3 years posttreatment. The OF lysimeter appeared to have sustained a higher rate of biodegradation relative to the O lysimeter and had greater nematode diversity, higher plant species diversity, and greater plant biomass. However, restoration was incomplete: both O and OF showed reduced pH and higher densities of fungi, sulfate-reducing bacteria (SRB), and hydrocarbon-degrading bacteria, and lower nematode and plant species diversity compared with C.

Current Study

A posttreatment study was undertaken 5 years after the original application of crude oil in order to determine the time course and synchrony of the return to uncontaminated conditions. Previously unpublished plant bioassay results from 3 years posttreatment are also presented.

MATERIALS AND METHODS

Soil Sampling for Chemical Analysis

2.54-cm-diameter soil cores were taken in a five-spot pattern from each lysimeter from the surface to 61 cm below the surface using a soil corer. A spatula, rinsed with ethanol between samples, was used to subdivide cores by sample depth: L1, surface to 7.6 cm; L2, 7.6 to 30.5 cm; and L3, 30.5 to 61 cm. The five replicate samples from a single lysimeter at one depth, obtained after subdivision of the cores by sample depth, were composited in a metal bucket, then packed into glass jars with TeflonTM-lined lids and immediately placed on ice.

Soil Chemical Analysis

Composited soil samples were analyzed for total petroleum hydrocarbons by both gas chromatography (TPH-GC, EPA Method 8015M) and by infra-red absorbance of a solvent extract (TPH-IR, EPA Method 418.1). Samples were shipped on ice by overnight delivery in completely filled glass jars with TeflonTM-lined lids to Soil Analytical Services, Inc., College Station, TX for analysis.

Microbial analysis

Soil Sampling for Microbial Analysis

A set of soil cores were taken in a five-spot pattern from each lysimeter from the surface to 61 cm below the surface using a soil corer. Each core was placed in a sterile Whirl-pakTM bag and placed on ice until returned to the lab for subdivision and compositing. Each of the five cores from a single lysimeter was subdivided as for the chemical analyses above, then the five replicate subsamples were composited in an autoclaved glass beaker by mixing thoroughly with an autoclaved spoon. Each of the nine composited samples was subdivided into two sterile 50-mL polyethylene centrifuge tubes and stored until used: one tube stored for 5 days at 3°C for all aerobic microbial analyses and one tube stored at -80°C for DNA extraction. Several studies (van Elsas and Smalla, 1997) found little if any change occurred in viable microbial populations if soil was stored at 3°C for no more than 7 to 21 days. The percentage of moisture in the soil was estimated gravimetrically from oven-drying a 10-g sample. All bacterial counts are expressed as per gram of soil (dry weight).

Aerobic Heterotrophic Bacteria and Hydrocarbon Degraders

A soil suspension was made for a series of 10-fold dilutions by removing 2.5 g of soil from each 50-mL tube and placing it into a new 50-mL tube containing 25 mL

of sterile isotonic saline (0.85% NaCl, pH 7.0) with 100 µg/mL cycloheximide (“cyx”, Sigma Chemical Company, St. Louis, MO) as an antifungal agent. After mixing thoroughly by vortexing, the suspension was diluted and spread (three replicates) on the following types of media: Plate Count Agar with cyx (“PCA”, Difco Co., Detroit, MI) for total aerobic heterotrophs; mineral salts (MS) agar with trace metals (Duncan *et al.*, 1999), and 100 µg/mL cyx containing one of the following aromatic hydrocarbons: (1) toluene added as a liquid to filter paper placed on the bottom of a glass petri plate lid for toluene-degraders (TOL), (2) naphthalene added as crystals on the bottom of the plate lid for naphthalene-degraders (NAP), (3) MS agar containing 0.8% phenanthrene for phenanthrene degraders (PHE). All plates were incubated at room temperature. Colonies on PCA plates were counted after 48 h. growth, while colonies on MS plates were counted at 1 week and again at 2 weeks to conform to previous methods (Lawlor *et al.*, 1997). Individual colonies were transferred to two different sets of plates, one containing the same medium as the original plate, the other consisting of MS agar without any added hydrocarbon to confirm that the hydrocarbon was necessary for growth.

Nitrogen-Cycling Bacteria

Inoculations from the dilution tubes above were made into microtiter plate wells (12 × 8 array) at 1/10 dilution. The plate wells contained one of the following: (1) nitrate broth (for enumerating assimilatory and dissimilatory nitrate reducers), (2) ammonium mineral salts medium (ammonium oxidizers), or (3) nitrite mineral salts medium (nitrite oxidizers), following the techniques of Jones *et al.* (1991). Three rows of wells per type of media were inoculated using a total of 12 dilutions of the original soil sample. The microtiter plates were incubated in the dark at room temperature for the following periods then examined for conversion of the initial nitrogenous compound using standard colorimetric tests for the detection of nitrite (Jones *et al.*, 1991): 14 days (nitrate reducers and denitrifiers), or 42 days (ammonium and nitrite oxidizers). The most probable number (MPN) of bacteria in each category was estimated from standard tables for a three-tube MPN (Rodina, 1972).

DNA Extraction and Purification

DNA was extracted from two replicate 0.5-g samples composited from each soil sample. The FastDNA® SPIN Kit for Soil (Quantum Biotechnologies, Inc., Carlsbad, CA) was used to extract and purify DNA from the soil using a bead-loading method to lyse bacterial cells and release DNA (Borneman *et al.* 1996).

Amplification of 16S rRNA

Universal eubacterial primers, 341F-GC and 907R (Casamayor *et al.*, 2000), targeting two conserved regions (341–357 and 907–926, using the *E. coli* numbering system), were used to amplify a 585-bp region of the 16S rRNA gene. 341F-GC contains an additional 40–base “GC clamp” at the 5' end (Muyzer *et al.*, 1993).

Primers were synthesized by Gibco BRL (LifeTechnology). Other reagents and enzymes were purchased from Fisher Scientific. PCR amplification was performed in a Stratagene Robocycler (Stratagene, La Jolla, CA) and followed the touch-down procedure described in Muyzer *et al.* (1993).

Separation of 16S rRNA Fragments by DGGE

Denaturing gradient gel electrophoresis (DGGE) was performed using a D-Code 16/16-cm gel apparatus (Bio-Rad, Hercules, CA), at a constant voltage of 65 V for 16 h and a constant temperature of 60°C. The gradient was formed of 7% polyacrylamide in TAE buffer with between 30 and 60% denaturant (7M urea and 40% formamide defined as 100% denaturant). After electrophoresis, the gel was stained in a solution of ethidium bromide, destained in deionized water, and a permanent image captured by the NucleoCam Digital Image Documentation System (NucleoTech Corp., San Mateo, CA).

Statistical Analysis

The number of aerobic heterotrophic and aromatic hydrocarbon-degrading bacteria (toluene, naphthalene, and phenanthrene) were log transformed and analyzed using a repeated measures (same lysimeters sampled each year) nested (level and treatment within a year) ANOVA model (MANOVA, Fall and Lehman, 1996). The effect of the different treatments (uncontaminated, crude oil, oil plus fertilizer) on the number of specific groups of bacteria was compared for samples taken from three different levels and both sampling events (3 years posttreatment, 1995, and 5 years posttreatment, 1997). Mean numbers from the crude oil (O) and crude oil plus fertilizer (OF) treatments were compared with those obtained from the control (C) lysimeter using Dunnett's test (Zar, 1984) to test for specific differences when significant interactive effects precluded separate analysis of year and year $\times$ treatment effects.

Nematode Species Composition and Diversity

Five samples per lysimeter were collected from the soil surface down to about 4 in (10.2 cm), separately placed in one-quart Ziploc® bags, and held on ice until returned to the lab. The samples were shipped by overnight express to Kansas State University for analysis. Nematodes were extracted from 100-g subsamples of soil using a modified Christie-Perry technique (Christie and Perry, 1951; Lawlor *et al.*, 1997) then identified to the taxonomic level of genus or subfamily using microscopy at 100 $\times$ magnification, and then assigned to trophic groups based on Yeates *et al.* (1993). The Shannon diversity index (H'), (Shannon and Weaver, 1949), was used to estimate the diversity of each nematode taxon and trophic group. Community maturity was estimated by the maturity index (MI):

$$MI = (\sum V_i f_i) / n$$

where V_i is the colonizer-persister value assigned to taxon i , f_i is the frequency of taxon i in the sample, and n is the total nematode population density (Bongers, 1990).

Plant Bioassays

Plant Species Diversity

No seeds had been deliberately sown in the lysimeters after concluding the experiments in 1992 and 1995, instead revegetation occurred due to the germination of seeds either present in the lysimeter soil or originating from the surrounding fields. In 1995 and in 1997, all vegetation, including roots, was removed from each lysimeter after the soil samples had been taken (mid-July, 1995; June 26, 1997). Voucher specimen identification was made using current regional floras and manuals (Correll and Johnston, 1970; Waterfall, 1979; Great Plains Flora Association, 1986; Diggs et al. 1999). Individual plant specimens representing each species were pressed for herbarium processing and the remaining plants were air-dried for 20 h at 60°C. Pairwise similarity between sites in terms of plant species composition was calculated as the Jaccard index of similarity:

$$J = n_{xy} / (n_{xx} + n_{xy} + n_{yy})$$

where n_{xy} is the number of plant species found in both lysimeters x and y , and $(n_{xx} + n_{xy} + n_{yy})$ is the total number of different species found in lysimeters x and y .

Biotoxicity 3 Years Posttreatment (1995)

Soybeans were used to evaluate the ecotoxicity of the soil, as they are a recommended crop for the type of soil used in the lysimeters (Cole *et al.*, 1977). Within 24 h after each lysimeter was completely denuded of plants and their roots; soybean seeds were planted in four rows of 36 seeds for a total of 144 seeds per lysimeter. The number of visible plants was recorded 14, 21, 29, 38, and 43 days after planting. Visible plants were marked on day 29 by placing toothpicks next to them. Records were made during each visit of any traces of disturbance by animals.

A germination test in the laboratory was performed concurrently with the field study, using an aqueous extract from the soil of each of the three lysimeters or tap water (as a control) to water the seeds. Wet paper towels lined the bottom of a large glass finger bowl, and three layers of five seeds were separated by wet towels for a total of 15 seeds per bowl (Hooft, 1987; Renoux *et al.*, 1995). Duplicate bowls were prepared for each test condition, for a total of eight bowls. The aqueous extract was prepared by adding 1.4 g of soil from the surface of each lysimeter to 14 mL of deionized water in a 15-mL centrifuge tube. The tube was rotated

continuously for 72 h. at room temperature and then centrifuged to remove particulates. The supernatant was used to wet the paper towels in the finger bowls. The tap water control extract was prepared by rotating 1.4 mL of tap water and 12.6 mL of deionized water for 72 h. Clear plastic wrap was placed over each of the eight finger bowls, which were then placed in the same south-facing window. The percentage of seeds that germinated (e.g., showed visible initial roots and/or stems) was recorded 14 days after watering.

Biotoxicity 5 Years Posttreatment (1997)

A field test of soybean germination and growth was performed in 1997. All indigenous plants were removed as described above, then soybeans were planted in two groups of five rows in each lysimeter, 20 seeds per row, for a total of 200 seeds per lysimeter. The number of plants that had emerged was recorded 10, 13, 21, and 24 days after planting, and a galvanized nail was placed next to each newly emerged plant. Ten weeks after planting, the plants were harvested and returned to the lab where the dry soybean biomass for each lysimeter was determined, and the plants were examined for the presence of large root nodules, indicating the establishment of a symbiosis with nitrogen-fixing bacteria. Large nodules were cut open and examined for the pink coloration due to the presence of leghemoglobin characteristic of active nitrogen-fixation (Madigan *et al.*, 1997).

RESULTS

Soil Chemistry

The TPH extracted from soil samples collected from lysimeters O and OF in 1995 was seen to be heavily weathered (Figure 1). GC chromatograms of TPH from lysimeter O showed the loss of light hydrocarbons and significant losses of the lower weight components of the n-alkane series indicative of natural attenuation by volatilization and intrinsic biodegradation. The GC chromatogram of TPH from lysimeter OF indicated extensive biodegradation. The same analysis in 1997 indicated continued weathering of TPH from lysimeter O. A comparison of the TPH-IR measurements (Figure 2A) shows that hydrocarbon concentrations had decreased substantially from 1995 to 1997 in the near-surface soil (L1—surface to 7.6 cm below surface) in both O and OF, but increased in L3 (30.5 to 61 cm below the surface). A similar loss from near-surface soils and some increase in concentration at the lower depths was seen for O for the TPH fraction analyzed as TPH-GC (Figure 2B). OF showed an increase in the TPH-GC fraction concentration at all depths.

For the purpose of calculating total losses of TPH between 1995 and 1997 in the entire 61 cm sampled, the measurements were weighted in proportion to the height of the soil core taken from each level. In total, TPH-IR levels decreased by

FIGURE 1

Chromatograms of TPH extracted from composited soil samples collected from the lysimeters in 1995. The o-terphenyl internal standard peak is indicated on each figure. (A) OL1: O lysimeter, level 1, surface to 7.6 cm below the surface. (B) OFL1: OF lysimeter, level 1, surface to 7.6 cm below the surface.

FIGURE 2

Measurements of TPH-IR and TPH-GC (as mg/kg soil) of composited soil samples taken from three different levels in the lysimeters. OL1: O lysimeter, level 1, surface to 7.6 cm below surface. OL2: O lysimeter, level 2, 7.6 to 30.5 cm below surface. OL3: O lysimeter, 30.5 to 61 cm below surface. OF: OF lysimeter, levels as designated for O. Solid bars: samples collected in 1995. Open bars: samples collected in 1997.

approximately 15% in O and 12% in OF over the 2-year period, and TPH-GC decreased by a similar percentage (14%) in O, but increased (approximately 200%) in OF. The ratio of TPH-GC/TPH-IR was approximately 1/3 for the O lysimeter in 1995 and 1997, and for the OF lysimeter in 1997, but less than 1/10 in 1995.

Microbial Analysis

Due to the inherent variation in bacterial numbers between samples taken in different years and among samples taken at different depths, an ANOVA was used

to determine whether there was significant variation in numbers due to the particular treatment (e.g., contamination with crude oil, with and without fertilizer). Significant three-way interaction terms for Treatment $\times$ Year $\times$ Level were found for the numbers of aerobic heterotrophic bacteria ($F_{8,32} = 12.23$, $P < 0.0001$), naphthalene- ($F_{8,34} = 10.17$, $P < 0.0001$) and phenanthrene-degrading bacteria ($F_{8,34} = 3.26$, $P = 0.007$) and for Year $\times$ Level ($F_{4,34} = 11.93$, $P < 0.0001$) and Year $\times$ Treatment ($F_{4,34} = 9.28$, $P < 0.0001$) for the toluene degraders, as described in more detail below.

Aerobic Heterotrophic Bacteria

Mean numbers of aerobic heterotrophic bacteria were generally higher in 1997 than in 1995 in C, suggesting that the soil environment was generally conducive to the growth of these microbes in 1997 (Table 1). However, O at all levels had significantly lower densities of aerobic heterotrophic bacteria than C even 5 years after contamination (in 1997). Results in 1997 for OF were mixed, with numbers lower than C in L1, and higher in L2.

Aromatic Hydrocarbon-Degrading Bacteria

Toluene-degrading bacteria were present at low numbers in all levels in each of the lysimeters, with little change in numbers from 1995 to 1997 (Table 1). There were significant year by level effects—higher numbers were found in the deeper levels in 1995, but the converse in 1997. The application of crude oil 3 or 5 years previously did not produce elevated numbers, with one exception (OF L2, 1997). These are the results expected if the numbers of toluene degraders track the presence of toluene, because no toluene was detected in soil samples taken in 1995 or in 1997.

Low numbers of naphthalene degraders were also found in the uncontaminated C lysimeter (Table 1). Although elevated numbers of naphthalene-degrading bacteria were seen in the OF lysimeter in 1995, the numbers on the whole in 1997 were lower and similar to those seen in C.

Phenanthrene-degrading bacteria were the most abundant of the hydrocarbon degraders assayed in both 1995 and 1997 in all the lysimeters (Table 1). Their number had appeared to be stimulated in 1995 in OF relative to C for all levels, but were significantly less abundant in 1997, except for O L1.

Nitrogen-Cycling Bacteria

More levels of the lysimeters had bacteria capable of contributing to a functioning nitrogen cycle in 1997 than in 1995. The numbers of nitrate-reducing bacteria varied widely in 1995, but few were found in O L3 and OF L3 (Table 2; and Jennings, 1997). They were relatively abundant in most samples in 1997, although still approximately 100-fold less abundant in O L2 and O L3. Nitrifying bacteria were more abundant and more widely distributed in 1997 than in 1995. In 1995, ammonium-oxidizing bacteria were only detected in C L1, C L2, and O L3 (Table

TABLE 1
Aerobic Heterotrophic and Hydrocarbon-Degrading Bacteria in Each
Lysimeter as ($\log_{10}$)/g soil (dry wt.), 3 and 5 Years Postcontamination
(1995 and 1997)

Level	L1	L2	L3	L1	L2	L3
Aerobic heterotrophs	3 yrs			5 yrs		
C	5.923	5.803	5.287	6.705	6.538	6.447
O	5.597*	5.203*	5.119	6.421*	5.882*	5.703*
OF	5.888	6.104*	5.586	6.457*	6.761*	6.604
Toluene-degrading	3 yrs			5 yrs		
C	3.811	4.057	4.100	3.857	3.167	2.918
O	3.524	3.599*	3.946	3.427	3.448	2.855
OF	3.416	3.729*	3.449*	3.864	3.808*	3.431
Naphthalene-degrading	3 yrs			5 yrs		
C	3.200	2.888	2.349	3.973	3.94	3.983
O	2.998	3.32	2.867	3.719	3.112*	4.37*
OF	4.323*	3.921*	3.496*	4.782*	3.802	4.019
Phenanthrene-degrading	3 yrs			5 yrs		
C	4.992	4.675	5.011	4.425	4.836	5.730
O	6.268	5.349*	5.131	5.205*	3.900*	4.854*
OF	6.939*	6.939*	5.599*	4.885	3.538*	4.619*

L1: level 1, surface to 7.6 cm below surface, composite of 5 soil samples

L2: level 2, 7.6 cm to 30.5 cm below surface, composite of 5 soil samples

L3: level 3, 30.5 cm to 61 cm below surface, composite of 5 soil samples

*: Values are significantly different from C value, 0.05 level (Dunnett's Method).

TABLE 2
Denitrifying, Ammonium-Oxidizing, and Nitrite-Oxidizing Bacteria in Each
Lysimeter as (log₁₀)/g soil (dry wt.), 3 and 5 Years Postcontamination
(1995 and 1997)

Level	L1	L2	L3	L1	L2	L3
Denitrifying	3 yrs			5 yrs		
C	11.301	11.845	8.342	6.403	6.453	6.464
O	11.301	11.301	6.588	6.519	4.223	4.130
OF	10.954	10.954	6.727	7.193	6.017	6.009
Ammonium-oxidizing	3 yrs			5 yrs		
C	4.00	2.00	BDL	ADL*	ADL	10.464
O	BDL**	BDL	2.00	BDL	ADL	12.724
OF	BDL	BDL	BDL	ADL	11.342	13.033
Nitrite-oxidizing	3 yrs			5 yrs		
C	BDL***	BDL	BDL	BDL	BDL	BDL
O	BDL	BDL	BDL	BDL	BDL	BDL
OF	BDL	BDL	BDL	BDL	2.439	3.356

L1: level 1, surface to 7.6 cm below surface, composite of 5 soil samples

L2: level 2, 7.6 cm to 30.5 cm below surface, composite of 5 soil samples

L3: level 3, 30.5 cm to 61 cm below surface, composite of 5 soil samples

ADL*: above the dilution limit of approximately 10¹³/g soil.

BDL**: below detection limit for ammonium-oxidizing bacteria, approximately 100/g soil.

BDL***: below detection limit for nitrite-oxidizing bacteria, approximately 200/g soil.

2; and Jennings, 1997). In 1997, ammonium-oxidizing bacteria were very abundant, near the upper limit of detection or above, in all soil samples except O L1 (Table 2). No nitrite-oxidizing bacteria were detected in any of the samples in 1995 (Jennings, 1997). In 1997, nitrite-oxidizing bacteria were detected in OF L2 and L3 at an estimated 275 and 2.3×10^3 nitrite-oxidizing bacteria per g soil, respectively (Table 2).

Microbial Species Diversity (DGGE)

Comparison of the banding pattern formed by the separation of amplified 16S rDNA of the bacterial sequences extracted from the soil revealed that the pattern differed by site and by level 5 years after contamination (1997, Figure 3). Although each sample clearly contained a large number of different sequences, the dominant sequences from each site were different on the whole, although some bands in lanes from different sites (e.g., lanes 5 and 6, OL2 and OFL2) appeared to coincide.

FIGURE 3

Denaturing gradient gel electrophoresis of 16S rDNA amplified from soil sampled 5 years postcontamination, using the eubacterial primers 341F-GC and 907R. Lane 1–9: C level 1, O level 1, OF level 1, C level 2, O level 2, OF level 2, C level 3, O level 3, OF level 3.

Nematode Species Composition and Diversity

Total nematode densities and number of taxa in the OF and C lysimeters in 1997 (Table 3) were similar to those observed in 1995 (Lawlor *et al.*, 1997). In the O lysimeter, however, twice the number of taxa were recovered in 1997 than were recovered in 1995. This increase in diversity in O from 1995 to 1997 was also reflected by the diversity indices, with equal or greater diversity occurring in O compared with OF. Diversity and maturity indices remained lower in 1997 for O and OF than for C, suggesting that the nematode community continued in 1997 to be impacted by the crude oil contamination.

TABLE 3
Nematode Trophic Structure and Diversity Indices for the
Nematode Community Isolated from L1 of the C, O, and OF
Lysimeters in 1997

	Lysimeter		
Trophic group	C	O	OF
Herbivores	25 (3)*	116 (2)	12 (2)
Fungivores	157 (6)	510 (4)	120 (4)
Microbiovores	306 (5)	344 (4)	125 (3)
Omnivores/predators	58 (1)	0	33 (1)
Index	C	O	OF
Taxon-level diversity	6.17 (0.82)**	4.52 (0.55)	3.69 (0.59)
Trophic-level diversity	2.68 (0.32)	2.30 (0.20)	2.4 (0.28)
Maturity index	2.71 (0.09)	1.98 (0.02)	2.18 (0.13)

*# nematodes/100 g soil (# taxa)

** Mean and (standard error) of five soil cores, collected in 1997.

Plant Species Composition and Diversity

Although the soil in the three lysimeters was from the same source, by 1997 soil characteristics differed among the lysimeters: the surface level of C was noticeably loose and sandy, O had a surface crust in areas and an odor of petroleum when the soil was disturbed, and OF had areas of extremely tight soil aggregates, which could have adversely impacted soil properties such as wettability. Plant distribution in 1997 reflected these characteristics: plants were most evenly distributed over the C lysimeter, had a highly scattered, patchy distribution over O, and a moderately patchy distribution in OF.

Table 4 lists the plant taxa identified from the lysimeters in 1997. The greatest number of taxa (15) was identified in OF, followed by O (11) and C (8). C was dominated by forbs, with *Plantago* spp. being especially common, while O was rich in grasses, particularly *Elymus*. In contrast, in 1995, C contained the most species (11), with OF intermediate (8) and O as the least diverse (5) (Lawlor *et al.*, 1997).

Sampling at a single point in time makes it difficult to compare the gain or loss of individual species between years, because some plants will have already completed their life cycle and died, while others have yet to germinate and grow. Comparisons within years, however, suggest that in 1997 OF had a plant species composition more like that of C than it did in 1995; the two lysimeters had more of the same plant species present (Table 5). The C and OF were also similar in 1997 in that they also lacked most of the grass species that were present in O, even though O was physically located between the C and OF lysimeters. Diversity increased in O from 1995 to 1997 due to an apparent increase in diversity of grasses. This is also largely responsible for the decreased similarity between C and O between 1995 and 1997. O did not appear to be becoming more like OF over this period, either (Table 5).

Plant Biotoxicity Assays

The laboratory germination test performed in 1995 did not indicate the presence of any water-soluble germination inhibitors in the soil: germination frequencies for 90 soybean seeds watered with a soil extract were as high or higher than those wet with tap water (% germinating: 73% C, 83% O, 73% F, 70% tap water).

Contrary to our expectations based on TPH levels and Microtox soil toxicity measurements (Lawlor *et al.*, 1997), seeds planted in the OF lysimeter in 1995 had the poorest germination record (% germinating: 57% C, 25% O, 3% OF). It was thought that rodents may have eaten the plants in OF before they could be counted, based on the visible signs of rodent activity. However, soybean seeds planted in the OF lysimeter in 1997 again had poor germination (33% C, 17.5% O, 3% OF), even though there was very low mortality of seedlings once germinated and little

TABLE 4
Plant Species Identified from Lysimeters in
1997

Plant species	Lysimeter		
	C	O	OF
I. Grasses			
<i>Bromus tectorum</i>	--	Yes	Yes ³
<i>Elymus</i> *	--	Yes ²	--
<i>Hordeum pusillum</i>	--	Yes	--
Grass sp. 1*	--	Yes	--
Grass sp. 2*	--	Yes	--
II. Forbs			
<i>Chenopodium</i> *	--	--	Yes
<i>Cirsium</i> *	--	--	Yes
<i>Croton</i> sp.*	--	--	--
Compositae	--	--	Yes
<i>Geranium</i> sp.*	Yes	Yes	Yes
<i>Lactuca</i> *	--	--	Yes
<i>Lepidium virginicum</i>	Yes	--	Yes
<i>Linaria canadensis</i>	--	Yes	Yes
<i>Oxalis</i> sp.*	Yes	--	Yes
<i>Plantago aristata</i>	--	Yes	--
<i>Plantago purshii</i>	Yes ¹	Yes	--
<i>Plantago virginica</i>	Yes	--	--

TABLE 4 (continued)

<i>Pyrrhopappus carolinanus</i>	Yes	Yes	Yes
<i>Rudbeckia hirta</i>	Yes	--	Yes
<i>Solidago</i> *	Yes	Yes	Yes
<i>Specularia</i> *	--	--	Yes
<i>Umbelliferae</i> **	--	--	Yes
<i>Veronica</i> *	--	--	Yes
Total number of species	8	11	15

*: unable to determine species.

** : unable to determine genus

yes: species present.

--: species not present.

1: dominant in C lysimeter

2: dominant in O lysimeter

3: dominant in OF lysimeter

TABLE 5
Plant Species Similarity

Similarity* (all plants)	C,O ¹	C,OF ²	O,OF ³
July 1995	0.46	0.24	0.27
June 1997	0.27	0.36	0.24
Similarity** (forbs only)	C,O	C,OF	O,OF
July 1995	0.45	0.27	0.30
June 1997	0.40	0.38	0.25

* $J = n_{xy} / (n_{xx} + n_{xy} + n_{yy})$, where n_{xy} is the number of plant species found in both lysimeters

x and y, and $(n_{xx} + n_{xy} + n_{yy})$ is the total number of different species found in lysimeters x and y. Plant species are from Table 4.

** : Similarities calculated as above, but excluding plants listed as "grasses".

1: Similarity of C to O

2: Similarity of C to OF

3: Similarity of O to OF

evidence of disturbance by animals. There were marked differences in the average weight of plants, as well as the percentage that attained a large enough size by 10 weeks to flower and establish a productive symbiosis with nitrogen-fixing bacteria—as shown by effective root-nodule formation (Table 6). Very few plants in O or OF were large enough to either flower or produce nodules at 10 weeks.

DISCUSSION

Time Course of Bioremediation

It has been shown repeatedly that after an initial lag period, the rate of bioremediation first increases then slows, with most activity taking place within a 2- to 5-month span or in 10 to 12 months for a soil low in organic matter, and little mineralization after that (Bossert *et al.*, 1984; Wang *et al.*, 1990; Wang and Bartha, 1990;

TABLE 6
Soybean Germination and Growth in the Field (1997)

<u>Lysimeter</u>	<u>C</u>	<u>O</u>	<u>OF</u>
Total # Plants ^a	55	19	3
# (%) Plants with flowers	50 (91%)	8 (42%)	1 (33%)
# (%) Plants with root nodules ^b	50 (91%)	4 (21%)	1 (33%)
# (%) Plants with active nodules ^c	33 (60%)	3 (16%)	1 (33%)
Total plant biomass (g)	82.3	15.7	2.7
<u>Mean biomass (g/plant)</u>	<u>1.50</u>	<u>0.83</u>	<u>0.90^d</u>

a: # plants 10 weeks post-planting.

b: Root nodules-characteristic swellings due to infection by symbiotic nitrogen-fixing bacteria. Plants that produced flowers were not necessarily the same as those that formed nodules.

c: Active nodules had a pink interior due to the production of leghemoglobin, and are presumed to be actively fixing nitrogen.

d: One of the three plants in OF weighed 1.67 g.

Salanitro *et al.*, 1997; Fisher and King, 1994; Duncan *et al.*, 1998). Bossert *et al.* (1984) noted an increase in humification after the active mineralization phase. Treatment of any kind, for example, tilling, watering, fertilization, etc., increased the rate of loss of hydrocarbons compared with no-treatment controls (Bossert *et al.*, 1984, Wang *et al.*, 1990; Wang and Bartha, 1990). Although in our study the greatest loss of TPH occurred within a 2- to 4-month time span, we found an indication of continued bioremediation even after 3 years (Lawlor *et al.*, 1997). The fate of the small fraction of TPH lost between 1995 and 1997 is not known; whether primarily leached from the lysimeter, incorporated into humic compounds, or mineralized. Most studies do not span several years, and hence there is little information on these rates after 1 to 2 years. The increase in TPH at some of

the lower levels sampled might reflect several processes—transport through a relatively unconsolidated soil (TPH-GC), or possibly the co-extraction of plant material by freon in EPA Method 418.1 and its incomplete removal by the subsequent silica gel treatment. However, the latter seems unlikely because lower levels of the C lysimeter did not have detectable levels of TPH-IR.

Lack of Synchrony of Recovery

The lack of synchrony by different groups of organisms in response to the loss of hydrocarbons that we saw has been observed in other studies (Hund and Traunspurger, 1994; Salanitro *et al.*, 1997; Sayles *et al.*, 1999). In general, the inhibition of plant growth persisted longer than the effects on invertebrate viability or toxicity measured by Microtox® (Salanitro *et al.*, 1997; Hund and Traunspurger, 1994). Hund and Traunspurger (1994) also found that nematodes and nitrification were very sensitive to petroleum contamination: nematodes were not found in heavily contaminated soils and were still at much reduced densities after 4 months, ammonium oxidation showed partial recovery after 7 months, and nitrite oxidation after 10 months of bioremediation. In our study the relative numbers of different classes of aromatic hydrocarbon-degrading bacteria reflected the expected sequence of loss of these hydrocarbons (monoaromatics first, followed by simple polyaromatics, then the more complex polyaromatics). Monoaromatic hydrocarbons were essentially gone from the lysimeters well before 1995, and toluene degraders were not found in elevated numbers in 1995 or 1997. Higher numbers of naphthalene- or phenanthrene-degrading bacteria were found in the O and OF lysimeters in 1995 than in the uncontaminated lysimeter, but were no longer at elevated numbers in 1997. The numbers of toluene-, naphthalene-, and phenanthrene-degrading bacteria may thus track rather well the presence of these compounds, which in turn reflects processes such as the evaporation of compounds with low boiling points and/or their K_{ow} (octanol-water partition coefficient) (Jensen *et al.*, 2000).

The complex pattern of eubacterial 16S rRNA sequences shown by DGGE attests that a fair degree of the species richness seen in the C lysimeter was also found in O and OF in 1997, although there were substantial differences in the position of the dominant bands. This species richness is unlike the low diversity of eubacterial species typically seen shortly after contamination (MacNaughton *et al.*, 1999; Cheung and Kinkle, 2001). Yin *et al.* (2000) proposed that the degree of bacterial functional redundancy might serve as an indicator of soil quality and ecosystem functioning. The eubacterial sequence composition and diversity seen in the current study therefore may represent an intermediate stage of recovery in which species diversity is fairly high, but restoration of functional redundancy is incomplete.

Plants as Ecotoxicity Indicators

Differences among the lysimeters in plant viability and species composition remained in 1997. Both the O and OF lysimeters had apparently made gains in species diversity relative to the C lysimeter, with OF more nearly approaching the species composition of C, and O apparently diverging from C. The soybean germination and growth tests indicated that conditions in the O and OF lysimeters were not suitable for these plants. Therefore, the plant community was shown to be a sensitive and quantifiable indicator of ecotoxicity.

It is not known whether the forbs absent from O were more sensitive to the toxic effects of crude oil. The phytotoxicity of petroleum products has been found to vary with the chemical composition of the petroleum, with smaller compounds of the same chemical class more toxic than larger compounds (Carr, 1919; Baker, 1970; Salanitro *et al.*, 1997). Of particular relevance to this study is the variation in sensitivity of different species of plants (Baker, 1970; Wang and Bartha, 1990; Hund and Traunsperger, 1994; Salanitro *et al.*, 1997) and the stimulation of growth of soybeans and corn at low concentrations of naphthalenic compounds (Carr, 1919; Baker, 1970; Salanitro *et al.*, 1997). This supports the interpretation that differences in the concentration and/or composition of petroleum hydrocarbons in the lysimeters may have affected plant species composition. However, petroleum hydrocarbons can also affect plant growth and survival indirectly through the effects on arbuscular mycorrhiza and rhizosphere bacteria (Joner *et al.*, 2001).

Species Composition and Diversity Measures

Potentially the most sensitive indicators of lack of ecotoxicity and/or evidence of restoration are measures of species composition and diversity. Nematode diversity and maturity indices, more so than total numbers of nematodes or number of species, showed a clear-cut difference between uncontaminated and contaminated lysimeters. Differences in plant species composition and dominant bacterial species, as demonstrated by DGGE, also provided measures rich in information. Two difficulties arise: (1) finding a suitable “control” for comparison—the C lysimeter was especially important for this study because its soil had also been subjected to disturbance, so the multitude of effects of mechanical soil disturbance would not be confounded with those due to oil application, and (2) how to interpret even statistically significant differences in terms of biological significance. The direct assessment of suitability for the specific intended function of an individual restored site is useful, but more information is needed before general conclusions about the degree of restoration of ecosystem functioning can be drawn from measurements of species composition and diversity.

CONCLUSIONS

The current low densities of hydrocarbon-degrading eubacteria and little loss from 1995 to 1997 of the total TPH mass in the lysimeters all suggest that bioremediation had greatly decreased in the lysimeters. Decreased TPH values in the uppermost layer sampled together with increased TPH in the lower layers indicate that some changes at least in distribution had also occurred. It is not known whether the remaining petroleum components are not susceptible to further biodegradation, or whether other factors, such as nitrogen availability, are limiting the activity of biodegradative microorganisms, especially at the lower levels of the lysimeters. However, after being in contact with the soil for this long, bioavailability of the hydrocarbons becomes an issue.

Although some anomalies exist (e.g., soybean germination and growth), the actively bioremediated OF lysimeter appeared to have not only lower levels of TPH but also a more fully restored ecosystem (e.g., numbers of heterotrophic bacteria, nitrifying bacteria, nematode trophic-level diversity and maturity index, plant species similarity) than did the O lysimeter. The active management of bioremediation, where practical, will not only speed the rate of degradation of crude oil contamination, as demonstrated by a variety of studies (Wang and Bartha, 1990; Wang *et al.*, 1990; Huesemann, 1994), but may also offer the benefit of more quickly producing a restored soil ecosystem. Restoration could be driven by lower levels of petroleum compounds in two ways: first, a decrease in the level of toxic compounds will allow the more sensitive microbes and meiofauna to return, and, second, once easily degradable hydrocarbons are consumed, the lack of stimulation of hydrocarbon-degrading bacteria allows more typical levels of oxygen and carbon flow to resume, which in turn will affect soil pH and the relative rate of aerobic/anaerobic decomposition. The latter parameters are known to have profound effects on biotic components of the soil ecosystem ranging from microorganisms to plants.

ACKNOWLEDGMENTS

We acknowledge Amoco Production Co., Tulsa, OK, for allowing us access to the site and especially J. Berton Fisher for facilitating this research. The research was made possible, in part, by a NSF-EPSCoR grant (Project # EPS9550478) to W. Potter and K. Duncan, which also provided funds to enhance the Oklahoma Mesonetwork.

REFERENCES

- Baker, J. M. 1970. The effects of oils on plants. *Environ. Pollution* **1**, 27–44.
Bongers, T. B. 1990. The maturity index: an ecological measure of environmental disturbance based on nematode species composition. *Oecologia* **83**, 14–19.

- Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., Nienhuis, J., and Triplett, E. W. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* **62**, 1935–1943.
- Bossert, I., Kachel, W. M., and Bartha, R. . 1984. Fate of hydrocarbons during oily sludge disposal in soil. *Appl. Environ. Microbiol.* **47**, 763–767.
- Bossert, I. D. and Compeau, G. C. 1995. Cleanup of petroleum hydrocarbon contamination in soil. 5. In: *Microbial Transformation and Degradation of Toxic Organic Chemical*, pp. 77–12. (Young, L. and Cerniglia, C., Eds.). New York, Wiley-Liss.
- Carr, R. H. 1919. Vegetative growth in soils containing crude petroleum. *Soil Sci.* **8**, 67–68.
- Casamayor, E., Schaffer, H., Baneras, L., Pedros-Alio, C., and Muyzer, G. 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **66**, 499–508.
- Christie, J. R. and Perry, V. G. 1951. Removing nematodes from soil. *Proceedings of the Helminthological Society of Washington* **18**, 106–108.
- Cole, E., Bartolina, D., and Swafford, B. 1977. *Soil Survey of Tulsa County, Oklahoma*. United States Department of Agriculture Soil Conservation Service.
- Cheung, P.-Y. and Kinkle, B. K.. 2001. *Mycobacterium* diversity and pyrene mineralization in petroleum-contaminated soils. *Appl. Environ. Microbiol.* **67**, 2222–2229.
- Corell, D. S. and Johnston, M. C. 1970. *Manual of the Vascular Plants of Texas*. Texas Research Foundation, Renner, Texas.
- Diggs, G. M. Jr., Lipscomb, B. L., and O'Kennon, R. J. 1999. *Shinners and Mahler's Illustrated Flora of North Central Texas*. Botanical Research Institute of Texas and Austin College, Fort Worth.
- Duncan, K. E., Kolhatkar, R., Subramanim, G., Narasimhan, R., Jennings, E., Hettenbach, S., Brown, A., McComas, C., Potter, W., and Sublette, K. 1999. Microbial dynamics in oil-impacted prairie soil. *Appl. Biochem. Biotech.* **77–79**, 421–434.
- Duncan, K., Jennings, E., Hettenbach, S., Potter, W., Sublette, K., Subramaniam, G., and Narasimhan, R. 1998. Nitrogen cycling and nitric oxide emissions in oil-impacted prairie soils. *Bioremed. J.* **1**, 195–208.
- Fall, F. and Lehman, A. 1996. *JMP IN*, SAS Institute, Inc. Ducksberry Press, Belmont, CA.
- Fisher, J. B. and King, G. 1994. *Monitoring of Hydrocarbon Bioremediation Progress with Microtox*. Report 940870004–APR. Amoco Production Company.
- Goldstein, R. M., Mallory, L. M., and Alexander, M. 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* **50**, 977–983.
- Great Plains Flora Association. 1986. *Flora of the Great Plains*. University of Kansas Press, Lawrence, KS.
- Hetrick, B. A. D., Wilson, G. W. T., and Todd, T. C. 1990. Differential responses of C₃ and C₄ grasses to mycorrhizal symbiosis, phosphorus fertilization, and soil microorganisms. *Can. J. Bot.* **68**, 461–467.
- Hooft, J. 1987. *Plants from Seeds*. Carolina Biological Supply Company. Burlington, NC.
- Huesemann, M. H. 1994. Guidelines for land-treating petroleum hydrocarbon-contaminated soils. *J. Soil Contamin.* **3**, 299–318.
- Hund, K. and Traunsprurger, W. 1994. Ecotox-evaluation strategy for soil bioremediation exemplified for a PAH-contaminated site. *Chemosphere* **29**, 371–390.
- Jennings, E. 1997. *The Long-Term Effects of Crude Oil Contamination and Bioremediation*. Unpublished thesis. The University of Tulsa, Tulsa, OK.
- Jensen, T. S., Arvin, E., Svensmark, B., and Wrang, P. 2000. Quantification of compositional changes of petroleum hydrocarbons by GC/FID and GC/MS during a long-term bioremediation experiment. *Soil and Sediment Contamin.* **9**, 549–577.

- Joner, E. J., Johansen, A., Loibner, A. P., Dela Cruz, M. A., Szolar, O. H. J., Portal, J.-M., and Leyval, C. 2001. Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAHs) in spiked soil. *Environ. Sci. Technol.* **35**, 2773–2777.
- Jones, R. A., Broder, M. W., and Stotzky, G. 1991. Effects of genetically engineered microorganisms on nitrogen transformation and nitrogen-transforming microbial populations in soil. *Appl. Environ. Microbiol.* **57**, 3212–3219.
- Lawlor, K., Sublette, K., Duncan, K., Levetin, E., Buck, P., Wells, H., Jennings, E., Hettenbach, S., Bailey, S., Fisher, J., and Todd, T. 1997. Long-term effects of crude oil contamination and bioremediation in a soil ecosystem. *Bioremed. J.* **1**, 41–51.
- MacNaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y.-J., and White, D. C. . 1999. Microbial population changes during bioremediation of an experimental oil spill. *Appl. Environ. Microbiol.* **65**, 3566–3574.
- Madigan, M.I., Martinko, J. M., and Parker, J. 1997. *Biology of Microorganisms*, 8th ed. Upper Saddle Rivers, N.J, Prentice Hall.
- Madsen, E. 1997. Methods for determining biodegradability. In: *Manual of Environmental Microbiology*, pp. 709–720. (Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenberg, L., and Walter, M.V., Eds.) ASM Press, Washington, D.C.
- Munkittrick, K. R., Power, E. A., and Sergy, G. A. 1991. The relative sensitivity of Microtox®, daphnia, rainbow trout, and fathead minnow acute lethality tests. *Environ. Toxic. Wat. Qual.* **6**, 35–62.
- Muyzer, G., De Waal, E. C., and Uitterlinden, A. G. 1993. Profiling of microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
- Pope, D. F. and Matthews, J. E. 1993. *Bioremediation Using the Land Treatment Concept*, EPA/600/R-93/164, Robert S. Kerr Environmental Research Laboratory, Ada, OK.
- Renoux, A., Tyagi, R., Roy, P., and Samson, R. 1995. Ecotoxicological assessment of bioremediation of a petroleum hydrocarbon contaminated soil. In: *Microbial Processes for Bioremediation*, pp. 259–264. (Hinchee, R.E., Brockman, F.T., and Vogel, C. M., Eds.). Columbus, Batelle Press.
- Rodina, A. G. 1972. *Methods in Aquatic Microbiology*. Baltimore, MD, University Park Press.
- Salanitro, J. P., Dorn, P. B., Huesemann, M. H., Moore, K. O., Rhodes, I. A., Jackson, L. M. R., Vipond, T. E., Western, M. M., and Wisniewski, H. L. 1997. Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. *Environ. Sci. Technol.* **31**, 1769–1776.
- Sayles, G. D., Acheson, C. M., Kupferle, M. J., Shan, Y., Zhou, Q., Meier, J. R., Chang, L., and Brenner, R. C. 1999. Land treatment of PAH-contaminated soil: performance measured by chemical and toxicity assays. *Environ. Sci. Technol.* **33**, 4310–4317.
- Shannon, C. E. and Weaver, W. 1949. *The Mathematical Theory of Communication*. 117 pp. University of Illinois Press, Urbana, IL.
- van der Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., and Sanders, I. R. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**, 69–72.
- Van Elsas, J. D., and Smalla, K. 1997. Methods for sampling soil microbes. In: *Manual of Environmental Microbiology*, pp. 383–390. (Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenberg, L., and Walter, M.V, Eds.). ASM Press, Washington, D.C.
- Wang, X. and Bartha, R. 1990. Effects of bioremediation on residues, activity and toxicity in soil contaminated by fuel spills. *Soil Biochem.* **22**, 501–505.
- Wang, X., Yu, X., and Bartha, R. 1990. Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. *Environ. Sci. Technol.* **24**, 1086–1089.
- Waterfall, U. T. 1979. *Keys to the Flora of Oklahoma*. Oklahoma State University, Stillwater, OK.

- Yeates, G. W., T. Bongers, R. G. M. de Goede, D. W. Freckman, and S. S. Geogieva. 1993. Feeding habits in soil nematode families and genera-an outline for soil ecologists. *J. Nematology* **25**, 315–331.
- Yin, B., Crowley, D., Sparovek, G., De Melo, W. J., and Borneman, J. 2000. Bacterial functional redundancy along a soil reclamation gradient. *Appl. Environ. Microbiol.* **66**, 4361–4365.
- Zar, J. H. 1984. *Biostatistical Analysis*. 2nd ed. Prentice-Hall, Inc. Engelwood Cliffs, N.J.