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Rhizosphere Microbial Characterization in Petroleum-Contaminated Soil

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Contamination of soil with petroleum compounds is of concern worldwide. Although there are a variety of physical and chemical technologies available to remediate petroleum waste sites, biological methods are often used due to lower cost and public acceptance. Growth and enhanced activity of microbial communities in contaminated soil is a key factor for the success of bioremediation. Establishing vegetation in petroleum-contaminated soil may enhance microbial activity and remediation success even further by providing root exudates to the rhizosphere microorganisms. In this study, microorganisms were characterized in petroleum-contaminated soils and sediments quantitatively and qualitatively based on enumeration and metabolic diversity assessments. Contaminated soils and sediments were obtained from a phytoremediation field demonstration project in California. Microbial numbers in the unvegetated soil, based on plate counts and most probable number of hydrocarbon degraders, were significantly lower than the vegetated soils. Metabolic microbial characterization using BIOLOG was also conducted and based on principle component analysis (PCA), there was a distinct difference between the metabolic diversity of microbial communities in vegetated and unvegetated soils. Results from this research indicate that the presence and type of plants, and level of contamination may greatly influence microbial communities in polluted soils.

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Introduction

CONTAMINATION of groundwater, surface water, and soil with petroleum compounds is widespread in the U.S. Bioremediation, the use of plants and microorganisms to reduce contaminants in soil and water, is a cost-effective, low maintenance solution to this problem. Petroleum hydrocarbons can be used by soil microorganisms as a carbon source (Austin *et al.,* 1977), and vegetation and fertilization of petroleum-contaminated soil have been shown to further enhance degradation of the contaminants (Hutchinson *et al.,* 2001). The success of these biological remediation methods depends primarily on the microbial growth environment (Applied Biotreatment Association, 1989). Therefore, studies investigating the characteristics of microbial communities in contaminated soils provide important information that can be used to improve existing biological remediation methods.

Phytoremediation, the use of plants to enhance degradation of contaminants in the rooting zone, has been shown to be an effective remediation strategy for petroleum contaminants (Schnoor *et al.,* 1995; Gunther *et al.,* 1996). The area surrounding plant roots is a specialized habitat for soil microorganisms, usually containing increased microbial populations. The community of root-associated microorganisms depends on the plant species, plant nutrition, soil moisture, soil organic matter, temperature, and light supply to plants (Gerhardson and Clarholm, 1986).

Accurate enumeration of soil microorganisms is difficult for a number of reasons. Soil microbes are in a constant state of flux and the microbial cells comprise a small portion of the total soil biomass. There are limitations to the traditional methods of rhizosphere microbial enumeration (plate counts, respiration methods, direct counts); therefore, functional diversity assessments have been used to analyze microbial community structure based on carbon source utilization (Garland and Mills, 1991; Winding, 1993; Zak *et al.,* 1994; Haack *et al.,* 1995). Evaluation of the characteristics of microbial communities in vegetated and unvegetated petroleum-contaminated soils will allow us to understand more completely the basis for the community structure development associated with maximum rates of degradation.

Materials and Methods

Site Description

Target soil samples were obtained from vegetated and unvegetated plots, and sediment samples were taken from adjacent wetland areas. The goal was to evaluate the effect of established vegetation on the quality and quantity of rhizosphere microorganisms. Samples were obtained from a petroleum refinery site in

northern California (Table 1). In mid-summer, four experimental plots were established at this site. The four plots included vegetation treatments of tall fescue, a mixture of cool season grasses and legumes recommended for erosion control, a mixture of California native perennial grasses, and an unvegetated control. A fifth adjacent wetland plot was planted with a native bulrush species. Sampling for this study was conducted 1 year after plant establishment.

Seeds germinated and established a nearly complete canopy in all vegetated plots. All plots had good biomass production. The tall fescue plot was almost exclusively dominated by tall fescue. The dominant plant species in the plot seeded with the native California grass mix was a rye grass. The erosion control mix plot was vegetated by three dominant species: meadow barley, rabbitsfoot grass, and rye grass. For this study, a total of ten soil samples were obtained from each of the five plots, representing high and low levels of contamination, based on previous total petroleum hydrocarbon (TPH) measurements. The high TPH samples were chosen from an area containing >2000 mg/kg TPH (range of 2846 mg/kg to 10,875 mg/kg). The low TPH area had <2000 mg/kg TPH (range of 846 mg/kg to 1675 mg/kg). The soil samples were taken directly from the rhizosphere, and were immediately shipped on ice to the laboratory at Kansas State University, where analyses were conducted after no more that 1 week of refrigeration.

For microbial enumeration using the plate count technique, three replicates of each of the ten soils were used. For hydrocarbon degraders and BIOLOG analysis, three replicates of three vegetation treatment types (tall fescue, wetland, and unvegetated) and two contamination levels (high and low) were used.

Contaminant Analysis

The sludge was analyzed for various chemical and physical properties by the Kansas State University Soil Testing Laboratory (Table 1) (Brown, 1997). Petroleum compounds were extracted from the soils and sediments using a sequential shaking extraction (Schwab *et al.,* 1999). Samples were air dried and mechanically ground to pass through a 0.25-mm mesh sieve. One gram of air dried sample was spiked with 100 mL of 100 mg L^{-1} tetracosane and shaken on a reciprocal shaker with 10 mL Optima grade dichloromethane (Fisher Scientific, St. Louis, MO) for 30 min, then centrifuged at 2000 rpm for 10 min to separate solids. The supernatant was collected in 60-mL glass jars. This procedure was repeated twice, and the mass of the final extractant was recorded. A 1.5-mL sample was transferred into a 2-mL gas chromatography (GC) vial, spiked with 10 µL of 1000 mg L^{-1} α-androstane, capped with a Teflon® lined septa, and stored at 4°C until analysis by gas chromatography.

Gas Chromatography. All extracted samples were analyzed on a Hewlett-Packard (HP) 5890A Gas Chromatograph (GC) equipped with a flame ionization detector

TABLE 1
Soil Characteristics for Sampling Areas

Soil Type	pH	K (mg/kg) (extractable)	P (mg/kg) (extractable)	OM (%)	CEC (cmol(+)/kg)	N(%) (Total)	Sand	Silt	Clay
Tall Fescue, high TPH	6.8	278	6	3.6	13.5	0.08	62	32	6
Tall Fescue, low TPH	7.0	131	22	3.1	14.3	0.09	62	22	16
Native grasses, high TPH	7.0	105	32	4.4	13.9	0.11	54	30	16
Native grasses, low TPH	7.0	121	27	4.6	13.1	0.11	60	24	16
Erosion mix, high TPH	7.0	123	20	4.4	14.0	0.11	52	30	18
Erosion mix, low TPH	7.2	127	14	3.2	13.9	0.11	48	32	20
Wetland, high TPH	6.9	100	4	2.5	13.5	0.06	44	16	10
Wetland, low TPH	6.7	125	1	3.8	13.6	0.10	52	42	6
Unvegetated, high TPH	7.3	127	52	3.4	12.2	0.07	58	26	16
Unvegetated, low TPH	7.3	119	35	3.7	12.5	0.09	56	28	16

(FID), HP Chemstation Integration Software, and an HP7673A autosampler utilizing a DB-TPH column. The DB-TPH column, designed specifically for analysis of TPH, had an inside diameter of 0.32 mm, a length of 30 m, and a 0.25-μm film thickness. The fuel and carrier gas for the FID was H_2. The carrier gas was delivered at 67 μL s^{-1} and the fuel gas at 670 μL s^{-1}. Nitrogen was used as the make-up gas at a flow rate of 530 μL s^{-1}. Air was supplied as the oxidant at a rate of 7.0 μL s^{-1}. The initial oven temperature was maintained at 40°C for 2 min, then increased at 0.2°C s^{-1} to 320°C and maintained at 320°C for 10 min. The temperature of the injection port was 250°C, the detector temperature was 350°C, and the splitless injection volume was 2 μL. Total petroleum hydrocarbon concentrations were measured using integrated GC areas and then converted to concentrations using external standard calibration curves.

Microbial Enumeration

Dilution plate counts and most probable number (MPN) of hydrocarbon degraders were selected to estimate the total number of microorganisms and hydrocarbon degraders in the samples. Of note is that both methods have limitations and underestimate the actual number of microorganisms in the soils. However, the techniques are useful for comparisons between samples.

Dilution Plate Counts

Plate counts were used to enumerate total aerobic heterotrophic bacteria. Ten grams of soil or sediment was weighed for moisture content determination. The sample was weighed after drying at 100^0C for 3 days. The 10 g of sample was placed in 95 mL of sterile 0.85% NaCl solution with 0.1 mL of Tween 40, a surfactant commonly used to remove microbes from soil. The sample was shaken for 30 min. The particles were allowed to settle for 2 min and 1 mL of the supernatant was transferred into a test tube containing 9 mL of sterile 0.85% NaCl solution. After vortexing for 1 minute, serial dilution continued to the 10^{-5} or 10^{-6} dilution level.

Tryptic soy agar plates (Fisher Scientific, St. Louis, MO) were prepared and allowed to get at 4°C for 24 h. Three prepared nutrient agar plates were inoculated with 0.1 mL of sample for the two highest dilutions. The agar plates were incubated at room temperature (23° to 25°C) for 3 days. Colonies on the plates were counted after the incubation period. For plates containing colonies between 30 and 300, the number of colony forming units per gram soil (CFU g^{-1} soil) was calculated using the following formula:

$$\frac{(\#\,\mathrm{CFU/plate})/[(\text{soil wet wt.})(\text{dilution factor})(\text{vol. of solution plated})]}{1+(\%\ \text{moisture}/100)}$$

Hydrocarbon Degraders

Sterile 96-well microtiter plates were prepared for samples with three columns serving as controls (Wrenn and Venosa, 1996). A stock solution of diesel fuel was dissolved in water. Fifty liters of the stock diesel fuel solution was placed into each column of a single plate except for the column that served as one of the three controls. Next, 180 µL of a Bushnell-Hass (B-H) broth/0.85% NaCl solution was added to each column except for the control. A small amount of sample solution at the 10^{-1} dilution level was placed in a sterile petri dish, 20 µL added to control column 12, and 20 µL is added to column 1. Tenfold serial dilutions were completed and each plate was labeled and wrapped in parafilm. Plates were incubated in the dark at 25 ± 0.2°C for 3 weeks and were then checked for positive results. Positive results were determined by the appearance of an orange color that is produced by partial oxidation of petroleum and intermediate byproducts. Enumeration was done statistically by counting the number of positive results in extinction dilutions. Population estimates were based on the pattern of attribute occurrence across a serial dilution from MPN tables (Halvorson, 1933).

BIOLOG Procedure

Soil preparation and dilution procedures for the BIOLOG method were similar to the dilution plate count method. Ten grams of soil or sediment was blended with 9 mL of sterile 0.85% NaCl solution and 0.01 mL Tween 40. The sample was shaken for at least 30 min. After the large particles settled, 1 mL of the supernatant was transferred to 9 mL of 0.85% sterile NaCl solution, followed by a dilution to 10^{-3} that was placed in three BIOLOG Gram-negative (GN) microplates (BIOLOG, Inc.). The first well on the plate was a water blank control. Dried substrate and redox dye (tetrazolium) were present in the other wells for evaluation of microbial respiration.

Plates were incubated at 25°C. Readings of color change in the wells of the BIOLOG plates were taken at 24-h intervals for 4 to 5 days. A photometer (Titertek Multiskan), designed to measure absorbance of the microtitration plates, was used to evaluate quantitative readings of the color changes in the wells of the microplates. An interference filter at a wavelength of 595 nm was used. Absorbance readings greater that 40% of the control well were considered positive (Marello and Bochner, 1989).

Principal component analysis was performed on the BIOLOG data using PC-SAS. The PCA for the BIOLOG color responses allows for comparison of microbial communities in different samples (Haack et al. 1995). Data representing the pattern of sole carbon source utilization of each plate was expressed by the transformed data. The transformed data is calculated by dividing the raw difference value for each well by the average well color development (AWCD). The AWCD

is equal to the sum of (C – R) divided by 95, where C is control well and R is sole carbon source. The PCA of the transformed data can represent significant structure in the data set as a result of differences in the utilization of sole carbon sources among samples (Garland and Mills, 1991).

Results and Discussion

A plate count technique to quantify the total number of aerobic heterotrophic bacteria and an MPN method for enumeration of hydrocarbon degraders were used to estimate the microbial population in petroleum-contaminated soils and sediments. Three to four replicates of the each sample were analyzed for statistical relevance. To allow for relevant comparisons, percent removal of TPH for each treatment is shown in Table 2. The native California and erosion control mixture plots showed the lowest TPH reduction of the vegetated plots. The fescue plot had a high TPH reduction of 65% over the 15-month study period. The wetland plot had a higher TPH reduction than both the California and erosion control mixture plots. A complete description of the field demonstration results and methods can be found in Banks *et al*. (1997).

The microbial plate counts in the lower contaminated samples were significantly higher than those found in the highly contaminated material (Table 3). The effect of vegetation on the enumerated microbial populations was different for the two levels of contamination. Microbial numbers for the low TPH samples are shown in Table 4. Results indicate that soils vegetated with tall fescue and erosion control grasses have significantly higher numbers of microbes than other types of vegetation. The unvegetated low TPH samples had significantly lower microbial numbers than the vegetated samples. In the moderately impacted soils and sediments, it appears that the limiting factor for microbial establishment is available growth substrates, not inhibition due to contamination.

For the high TPH soils and sediments, the native grasses, tall fescue, and wetland vegetation had a significantly higher number of microbes than other treatments (Table 5). Microbial numbers in soils vegetated with tall fescue, erosion control mixture, and in unvegetated soils were not statistically different. Variability of microbial numbers in the different vegetation treatments may be attributed to the characteristics of the vegetation and the concentration of the contaminants.

The MPN technique was used to enumerate hydrocarbon degraders in the petroleum field samples. After 1 week of incubation, significant color change was not evident in the MPN tubes. Therefore, data after 2 weeks of incubation were used to determine the number of hydrocarbon degraders per gram of soil. There was high variability in the number of degraders within replicates of samples for both vegetated and unvegetated samples (Table 6). The average number of microbes in the unvegetated soils was less than that in the vegetated soils and sediments by an order of magnitude. However, because of the high variability in

TABLE 2
Reduction in TPH in Phytoremediation Field Study

Plant Species	15 months	*Statistical Significance
Native California Mixture	11.8%	a
Tall Fescue	65.2%	b
Erosion Control Mixture	18.1%	a
Unplanted	22.3%	a
Wetland	31.1%	a
LSD	25%	

* Means with the same letter are not significantly different ($P < 0.05$).

TABLE 3
Microbial Plate Counts for High and Low Levels of Petroleum Contaminated Field Samples

field samples

Samples	Avg. CFU/g soil	Statistical significance*
Low level TPH	4.6×10^6	a
High level TPH	2.0×10^6	b
LSD	0.8×10^6	

* Means with the same letter are not significantly different ($P<0.05$).

TABLE 4
Microbial plate counts for Low TPH Level Soils with Treatments

Samples	Avg. CFU/g soil	Stat. significance*
Tall fescue	1.3×10^7	a
Native grasses	6.5×10^6	b
Wetland	3.3×10^6	c
Erosion mix	2.5×10^6	c,d
Unvegetated	0.5×10^6	d
LSD	2.5×10^6	

* Means with the same letter are not significantly different ($P<0.05$).

TABLE 5
Microbial Plate Counts for High TPH Level Soils with Treatments

Samples	Avg. CFU/g soil	Stat. significance*
Native grasses	4.1×10^6	a
Tall fescue	2.6×10^6	b
Wetland	1.6×10^6	b
Unvegetated	0.8×10^6	c
Erosion Mix	0.6×10^6	c
LSD	0.9×10^6	

*Means with the same letter are not significantly different ($P<0.05$).

TABLE 6
Hydrocarbon Degraders in Petroleum
Contaminated Field Samples with Treatments

Samples	Avg. #cells/g soil	Stat. Significance*
Tall fescue	3.1×10^7	a
Wetland	2.5×10^7	a
Unvegetated	0.4×10^6	a
LSD	4.8×10^7	

* Means with the same letter are not significantly different (P<0.05).

the replicates, the LSD is high and the differences between vegetated and unvegetated samples were not statistically significant. Also, there was no statistically significant difference between the number of degraders in the high and level TPH soils (Table 7).

The BIOLOG method was conducted to detect utilization patterns of 95 sole carbon sources on BIOLOG GN plates by soil microbial communities. Using this technique, the microbial metabolic diversity of petroleum-contaminated field samples was assessed. The selected terrestrial plant for assessment was tall fescue. Both high and low level TPH soils were tested from the unvegetated-, wetland-, and tall fescue-treated plots. The percent substrate utilization over the incubation period is shown in Figure 1. A lag period of 24 h for all samples was evident. Between 24 and 72 h, the percent substrate utilization of the wetland and vegetated samples increased to values between 55 and 70%. A longer lag period and lower

TABLE 7
Hydrocarbon Degraders in High and Low Petroleum-Contaminated Samples

Samples	Avg. #cells/g soil	Stat. Significance*
High level TPH	2.9×10^7	a
Low level TPH	8.9×10^6	a
LSD	3.9×10^7	

*Means with the same letter are not significantly different (P<0.05).

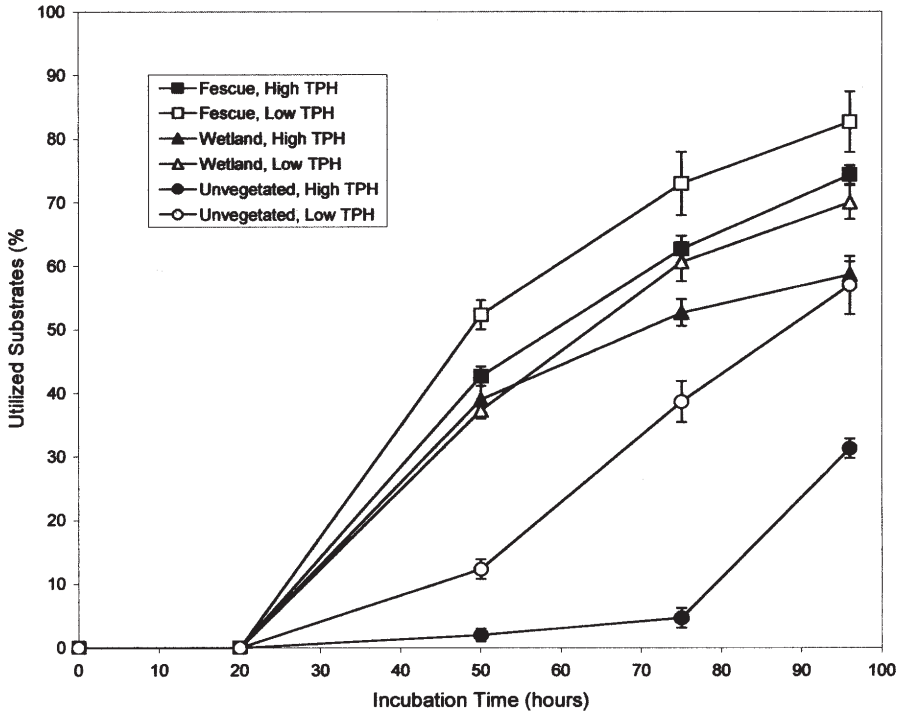

FIGURE 1

Substrate utilization over the incubation period for soil samples contaminated with petroleum. (Error bars represent standard deviation.)

substrate utilization was evident for the unvegetated samples, especially for those with high levels of TPH, indicating the presence of a lower density of microbes capable of utilizing the target carbon sources.

Principal component analysis was performed on the 72-h data for wetland and fescue samples, and on the 96-h data for unvegetated samples. The first principle component (PC) accounted for 18% of the variation in the data, and the second PC accounted for 14% of the variation in the data. The individual substrates that were highly correlated with the first PC (>0.85) were L-arabinose, D-galactose, D-mannitol, sucrose, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, and L-sernine. Substrates positively correlated with the second PC (>0.70) were lactulose, α-hydroxybutyric acid, L-theronine, and thymidine.

A plot of first and second PCs shows distinct differences between the microbial communities of vegetated and unvegetated samples (Figure 2). Microbial communities in the low and high TPH vegetated samples and high TPH wetland samples

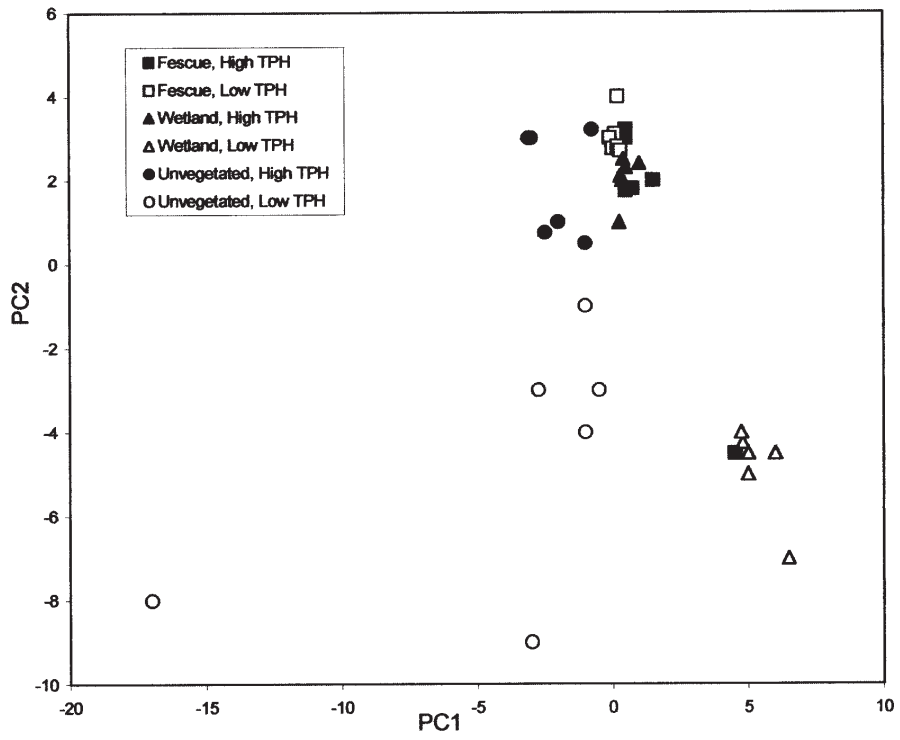

FIGURE 2

Principal component analysis of fescue, wetland, and unvegetated petroleum-contaminated field samples

showed similar carbon utilization patterns. However, microbial communities in the low TPH wetland samples showed significant differences from the other treatments. Differences were also evident in the microbial community carbon utilization from high and low TPH unvegetated samples, indicating variation in the functional diversity of microbial communities due to type of vegetation, environmental conditions (wetland vs. terrestrial), and contamination level.

CONCLUSIONS

Results from this study indicate quantitative and qualitative differences exist between microbial communities of vegetated and unvegetated contaminated soils and sediments. Microbial enumeration using plate counts showed that microbial numbers in petroleum-contaminated unvegetated soils are significantly lower than

those in vegetated soils. Principal component analysis also identified distinct differences in carbon utilization patterns between vegetated and unvegetated soils, specifically, microbial communities capable of utilizing carbon sources in the BIOLOG GN plates were lower in the unvegetated soils than those found in vegetated soils.

In addition, high contaminant concentrations in petroleum-contaminated soil were shown to significantly affect microbial populations. Higher numbers of microorganisms were found in lower contaminated samples when compared to highly contaminated material. Also, PCA indicates differences in metabolic diversity between microbial communities in high and low contaminated soils. Results from this study showed that petroleum-contaminated soil microbial communities are quantitatively affected by the presence of vegetation and concentration of contaminants, and vegetated soils may have a more diverse microbial community capable of utilizing a variety of carbon sources.

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