Changes in Microbial Community Composition and Function during a Polyaromatic Hydrocarbon Phytoremediation Field Trial

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The purpose of this study was to investigate the mechanism by which phytoremediation systems promote hydrocarbon degradation in soil. The composition and degradation capacity of the bulk soil microbial community during the phytoremediation of soil contaminated with aged hydrocarbons was assessed. In the bulk soil, the level of catabolic genes involved in hydrocarbon degradation (ndoB, alkB, and xylE) as well as the mineralization of hexadecane and phenanthrene was higher in planted treatment cells than in treatment cells with no plants. There was no detectable shift in the 16S ribosomal DNA (rDNA) composition of the bulk soil community between treatments, but there were plant-specific and -selective effects on specific catabolic gene prevalence. Tall Fescue (Festuca arundinacea) increased the prevalence of ndoB, alkB, and xylE as well as naphthalene mineralization in rhizosphere soil compared to that in bulk soil. In contrast, Rose Clover (Trifolium hirtum) decreased catabolic gene prevalence and naphthalene mineralization in rhizosphere soil. The results demonstrated that phytoremediation systems increase the catabolic potential of rhizosphere soil by altering the functional composition of the microbial community. This change in composition was not detectable by 16S rDNA but was linked to specific functional genotypes with relevance to petroleum hydrocarbon degradation.

Reduction of hydrocarbons in soil is enhanced in the presence of plants (3), but the mechanisms by which plants enhance hydrocarbon removal are not completely understood. The uptake of hydrocarbons from contaminated soils by plants (phytoaccumulation) is limited by the high lipophilia of hydrocarbons (24), and there is little data available on plant-induced sequestration (phytostabilization) of hydrocarbons in soil (20). The mechanism responsible for the phytoremediation of contaminated soil is thought to be an increase in microbial activity. Supporting this hypothesis, the population levels of contaminant-degrading bacteria and the potential of soil to degrade contaminants typically increases during phytoremediation (18, 22).

We hypothesize that there may be two mechanisms by which plants increase catabolic activity in the rhizosphere or the bulk soil. Increases in catabolic activity may result from an enhancement of general microbial activity. For example, plants sometimes increase catabolic activity in rhizosphere soil (10, 18) independent of contaminants. This suggests that the enhancement of catabolic activity may simply be the result of microbial activity increasing due to the release of plant lysates and root exudates by what is commonly termed the rhizosphere effect. Additionally, increases in catabolic activity may result from the proliferation of specific microbial groups as the microbial community size increases due to the rhizosphere effect. This is the reasoning behind the use of microbial inoculants that stimulate phytoremediation (7) and also alter the diversity of the root-associated community (27). In this scenario, the microbial community has little inherent ability to degrade hydrocarbons, and it requires either the selective enrichment or addition of specific microbial species before significant remediation activity is observed.

In this study we assessed the impact of phytoremediation on the composition of the bulk soil microbial community and the ability of that community to degrade hydrocarbons. We postulated that phytoremediation treatments would have an effect beyond that of the rhizosphere and would alter bulk soil microbial processes in such a way as to promote hydrocarbon degradation. The catabolic genes encoding alkane monoxygenase (alkB) (36), naphthalene dioxygenase (ndoB) (31), and catechol-2,3-dioxygenase (xylE) (16) were used to assess the prevalence of bacteria involved in petroleum hydrocarbon degradation. The potential of the indigenous soil microbial population to mineralize representative polycyclic aromatic hydrocarbons (PAHs) was also assessed. The purpose of this study was to determine if phytoremediation treatments increased the catabolic potential of bulk soil by altering the taxonomic structure of the soil microbial community or by stimulating a specific functional genotype in the bulk soil microbial community. It is unlikely that these processes are mutually exclusive, but we wished to determine which process dominates during the phytoremediation of aged hydrocarbons in soil.

MATERIALS AND METHODS

Site description. The phytoremediation study site was located at the Department of Defense National Test Site, Port Hueneme, Calif. The ability of this phytoremediation project to reduce contaminant concentrations and toxicity was...
previously documented (4). The study site, which was approximately 20 by 35 m, was divided into 12 plots in a randomized block design. The soil was contaminated with 1.5 g of total petroleum hydrocarbons kg\(^{-1}\) that originated from diesel fuel and heavy oil use over a 20-year period. Treatment plots with four replicate plant mixtures were placed on a fine sandy loam soil of the regional association. Phytovolatilization was assessed by monitoring the potential of soil microorganisms to degrade hydrocarbons. Soil (20 g) was placed in 100-ml serum bottles and was amended with ca. 100,000 dpm of either 2 mg of hexadecane kg\(^{-1}\) labeled with 14C at the C-1 position (Sigma, Oakville, Ontario, Canada) with a specific activity of 4.1 mCi/mg, 2 mg of naphthalene kg\(^{-1}\) uniformly labeled (99% purity; Sigma Chemicals) or 2 mg of phenanthrene kg\(^{-1}\) labeled with 14C at the C-9 position (99% purity; Sigma Chemicals) with a specific activity of 46.9 mCi/mg. One milliliter of 0.5 N KOH was added to a small test tube placed inside the microcosm to trap 14CO\(_2\), and the microcosm was sealed. We used 2 weeks of incubation at room temperature, the KOH was removed, 1 ml of 0.5 N KOH was used to wash the inside of the small test tube, and the resulting 2 ml of KOH was transferred to a scintillation vial. Radioactivity was determined in a Tri-Carb liquid scintillation counter model 2100TR (Packard Instruments Co., Meriden, Conn.). Selected microcosms were fertilized with a commercial fertilizer containing 20% (wt/wt) inorganic nitrogen (ammonium nitrate), 20% (wt/wt) organic nitrogen (urea), and 20% (wt/wt) phosphorous (potassium phosphate) to a final concentration of 1,250 mg kg\(^{-1}\) of soil (wt weight) (37).

Chemical gene probe analysis of the cultured and uncultured community. Bacteria were extracted from the root interior, rhizosphere, and bulk soil and were resuspended in 0.1% (wt/vol) tetraethylpyrophosphate (pH 7.0) (28). A 0.1-ml aliquot of serial dilutions in tetraethylpyrophosphate (10\(^{-2}\), 10\(^{-3}\)), and 10\(^{-4}\) was spread plated onto triplicate plates containing (per liter of tap water) 250 mg of yeast extract (Becton Dickinson, Cockeysville, Md.), 250 mg of tryptone (Difco Laboratories, Detroit, Mich.), 250 mg of soluble starch (Anachemia, Montreal, Quebec, Canada), and 15 g of granulated agar (YTS 250 medium; Becton Dickinson). Bacterial colonies were counted after incubation at room temperature (21 to 24°C) for 2 weeks and then were lifted onto nylon membranes. Cells adhering to membranes were lysed and the DNA was denatured, fixed, and cross-linked to the membrane and hybridized to the alkB, ndoB, or xylE gene probes (9).

Total microbial community DNA was extracted from bulk soil by chemical lysis (9) and was purified on polystyrenepolyisoprene spin columns (5), and 100 ng of the purified extract was dot blotted in triplicate on Zeta-Probe membranes (Bio-Rad Laboratories, Hercules, Calif.) (30). The concentration of total microbial community DNA applied to the membranes was determined by agarose gel electrophoresis. Scintillation counting of cut dot blot membranes after overnight hybridization (65°C) with 32P-labeled probes was performed with a Tri-Carb scintillation counter. Standard curves, constructed with total genomic DNA of Pseudomonas oleovorans (ATCC 29347) for alkB, Pseudomonas putida strain G7 for ndoB (ATCC 17484), and P. putida mt-2 (ATCC 33015) for xylE used for hybridization and the concentration of the extracted DNA was determined by comparing the integrated data with that obtained using standards from a pure culture of each strain. Total microbial community DNA was extracted from bulk soil of each treatment as described above and was amplified with eubacterial primers (8F-GC and 519R [6]) in triplicate. A hot start, touchdown protocol (annealing temperature of 65°C, touching down to 55°C in 10 cycles followed by 15 cycles at 55°C) was used to amplify community DNA as previously described (29). Triplicate reactions of DNA from the same soil extraction were used to minimize the stochastic biases present in PCRs. Reaction mixtures were concentrated 7.5 times by ethanol precipitation and were loaded onto a 6% polyacrylamide gel containing a 35 to 65% urea-formamide denaturing gradient. The denaturing gradient was prepared and allowed to polymerize for 1.5 h. A spacer gel (6% acrylamide, 0% denaturant, approximately 7.5 mm in height) into which the 20-well comb was inserted was applied on top of the denaturant gel to minimize denaturant gradient disturbance during comb insertion and was allowed to polymerize for 0.5 h. Wells were washed with buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and an approximately 350 ng of amplified PCR product was loaded per lane and run at 80 V for 16 h at 60°C. The resulting gel was stained in 1 mg of ethidium bromide liter\(^{-1}\) in Tris-acetate-EDTA buffer and was photographed. Banding patterns were analyzed by using a ChemiImager (Alpha Innotech, Mississauga, Ontario, Canada), and specific bands were removed by using a scalpel. DNA fragments from the denaturing gradient gel electrophoresis (DGGE) were reamplified with primers 8F and 519R and were sequenced as previously described (12). Briefly, DNA from DGGE bands was amplified on a thermocycler in a 40-μl PCR reaction volume. The PCR cocktail at 96°C for 5 min, after which Taq DNA polymerase was added at 80°C followed by 30 cycles of 1 min at 94°C, 30 s at 60°C, and 30 s at 72°C. The
reamplified bands were purified by using the QIAQuick PCR purification kit (Qiagen, Mississauga, Ontario, Canada) and were sequenced by using the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Montreal, Quebec, Canada) and the ABI Prism 377 automated fluorescence sequencer (Applied Biosystems, Foster City, Calif.). Sequences were compared to those in the GenBank databases by using the BLAST algorithm (1).

**Nucleotide sequence accession numbers.** Sequences were submitted to GenBank and were assigned accession numbers AF417499 to AF417506.

**RESULTS**

The phytoremediation treatments successfully decreased total petroleum hydrocarbon (TPH) concentrations in bulk soil by 30% after 2 years (Fig. 1). There was little difference in TPH degradation between the two types of phytoremediation systems used, i.e., native grasses or a grass-legume mixture. Both systems degraded approximately 3.7 mg of TPH kg\(^{-1}\) month\(^{-1}\), which is double that observed for the nonvegetated cells: 1.9 mg of TPH kg\(^{-1}\) month\(^{-1}\). If this degradation rate continued, one would expect complete removal of TPH from the phytoremediation cells within 5 years compared to over 10 years for the nontreated cells. There was little difference between the above-ground biomass or root surface area between the two phytoremediation treatments, so the results are combined for these treatments. These plant parameters did not display a temporal trend with the above-ground biomass displaying the typical trends expected of the California ecosystem, fluctuating around 460 g m\(^{-2}\). Below-ground root surface area remained relatively constant at around 312 dm\(^{2}\) cm\(^{-3}\).

Supporting the chemical analysis, more \(alkB\), \(ndoB\), or \(xylE\)-positive bacteria were isolated from the planted treatments compared to controls for the majority of the sampling times (Fig. 2). The trend was at its maximum during the summer of 1999, when \(alkB\) and \(xylE\) gene-positive bacteria were as much as two orders of magnitude greater in the planted versus nonplanted cells. However, by January 2000 this difference had decreased to less than one order of magnitude. In all treatments there was a significant \((P < 0.01)\) decrease over time in the number of bacteria containing these catabolic genes. For all sampling dates except June 1998 and June 1999, \(alkB\) and \(xylE\) prevalence in the cultured community was significantly \((P < 0.05)\) greater in the planted versus nonplanted cells. The prevalence of \(xylE\) was only sig-
In the uncultured community, catabolic gene prevalence in the phytoremediation treatments peaked in January and April 1999 (Fig. 3). There were significant ($P < 0.001$) treatment-time interactions for the dot blot results, with native grass mixture 2 peaking in January 1999 and grass-legume mixture 1 peaking in April 1999. For the January and April 1999 samples, ndoB and xylE prevalences were significantly ($P < 0.05$) greater in the planted versus nonplanted cells. The prevalence of alkB was only significantly greater in planted cells for the January 1999 sampling date. In general, the prevalence of catabolic genotypes in the total extracted community DNA declined with time. When both planted treatments were combined and the last two sampling points were averaged, the prevalence of catabolic genotypes was lower (5, 8, and 6% for alkB, ndoB, and xylE, respectively) compared to the average of the first three sampling points (11, 13, and 11 for alkB, ndoB, and xylE, respectively). In contrast, the nonplanted treatments did not show a similar trend, remaining at approximately 7% for the duration of the experiment. The results with total community DNA had a much lower degree of precision, with a coefficient of variation, which is the standard deviation expressed as a percentage of the mean, of 24% compared to a coefficient of variation of 5% for the colony lifts with 37,000 colonies probed. This difference in precision may be related to the selective properties of culturing in which only a small portion of the community is being monitored.

The diversity of the total microbial community in the bulk soil, as assessed by DGGE of 16S ribosomal DNA, was not altered by the phytoremediation treatments. For the first two sample periods there was little difference between replicates of the same treatment taken at the same sampling time. Thus, results are presented from one replicate of each treatment taken over the 2-year period (Fig. 4). No particular band present in the DGGE was only present in a particular treatment, with the majority of bands being present in almost all lanes, except band 9 (93% identity with AF058380 [35]), which was found only three times out of a maximum of 15. The remainder of the sequences obtained from the DGGE gel were closely related to sequences obtained during other experiments investigating contaminant degradation (band 2 had 92% identity with AB013416, from Aquaspirillum spp. [33]; band 4 had 98% identity with AB013429, from an unidentified ß-proteobacterium [33]; and band 5 had 95% identity with AJ233553, from an uncultured eubacterium [19]) or bacteria closely linked to plant-root systems (band 1 had 98% identity with AJ232881, from Flavobacterium indologenes [14]; band 6 had 99% identity with X67035, which is from Pseudomonas, Burkholderia, or Ralstonia solanacearum [26], and band 7 had 85% identity with U28230 from B. solanacearum strain R633 [34]). Band 10 had the same sequence as band 1, and band 11 had the same sequence as band 2. These results suggest that the bulk soil community of planted versus nonplanted treatment was highly similar and remained relatively stable over the study period.

In the bulk soil of the control and mixture 1, the potential to mineralize hexadecane was similar if the microcosms were not fertilized. Similarly, the mineralization potential of mixture 2 was only 84% that of the control soil (data not shown). When microcosms were fertilized, the planted treatments mineralized significantly more hexadecane and phenanthrene than the unplanted control (Fig. 5). This suggests that the hydrocarbon-degrading populations were higher in the planted soils but that they were nutrient limited. Soil chemical analysis indicated that, despite repeated fertilization of all treatments, the avail-
able N and P were significantly lower in the planted treatments than in the nonplanted treatments. Grass-legume mixture 1 contained 7.5 μg of N kg⁻¹ and 13 μg of P kg⁻¹, the native grasses mixture 2 contained 3.8 μg of N kg⁻¹ and 13 μg of P kg⁻¹, and the nonplanted control contained 18 μg of N kg⁻¹ and 23 μg of P kg⁻¹ 24 months after planting.

We also assessed plant-specific effects by harvesting selected species from the planted treatment plots and assessing their mineralization potential as well as the prevalence of catabolic genes. Mineralization of naphthalene in Tall Fescue (F. arundinacea) rhizosphere soil was significantly (P < 0.05) greater than naphthalene mineralization in the bulk soil (Fig. 6). In contrast, Rose Clover (T. hirtum) significantly (P < 0.05) decreased naphthalene mineralization in rhizosphere soil compared to that in bulk soil. For the three dates examined, July 1998, January 1999, and April 1999, there were no treatment-time interactions with each plant consistently increasing or decreasing rhizosphere naphthalene mineralization compared to that for bulk soil. The increase in Tall Fescue rhizosphere soil naphthalene mineralization was reflected by a 45% increase in the prevalence of ndoB-positive bacteria in the rhizosphere. Similarly, the decrease in naphthalene mineralization in Rose Clover rhizosphere soil was reflected by a 67% decrease in the prevalence of ndoB-positive bacteria in the rhizosphere. The number of culturable rhizosphere bacteria was similar in both plant species, averaging 1.3 × 10⁹ CFU g⁻¹ of rhizosphere soil for the duration of the experiment. This pattern was not observed with mixture 2, in which mineralization activity increased despite decreases in catabolic gene prevalence.

**DISCUSSION**

This study demonstrated that phytoremediation systems that substantially decrease aged TPHs in soil do so by increasing bacteria containing hydrocarbon catabolic genes in the bulk and rhizosphere soil. Both planted treatments increased numbers of bacteria harboring specific catabolic genes in the bulk

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**FIG. 4.** 16S-DGGE analysis of total community DNA present in bulk soil from the unvegetated soil (C, control), soil planted with grasses or legumes (M1, mixture 1), and soil planted with native grasses (M2, mixture 2) over a 2-year period. Arrows indicate the band sequenced, and their closest identities are provided in the text.

**FIG. 5.** Potential of fertilized soil to mineralize phenanthrene or hexadecane in soil after 7 days of incubation at room temperature. Soil was fertilized with commercial fertilizer (20:20:20) to a final concentration of 250 mg of N/kg of soil (wet weight) and was spiked with C¹⁴-labeled phenanthrene or hexadecane. Error bars represent one standard error.
controls under planted treatment cells compared to those of nonplanted phenanthrene and hexadecane mineralization in the bulk soil sphere soil, but phytoremediation treatments also doubled. Was the effect of phytoremediation systems evident in rhizosphere interactions are important during phytoremediation. Not only observed with Rose Clover, con was observed in the rhizosphere soil compared to that in the similar, in this study increased mineralization of naphthalene higher pyrene mineralization activity than bulk soil (23). Similarly found a strong species dependence on the ability of phytoremediation systems to promote hydrocarbon degradation. Microbial communities in petroleum-contaminated soils commonly have the potential to degrade hydrocarbons but require nutrients for this potential to be realized (37). Furthermore, the influence of plants on microbial diversity is dependent on the nutrient status of the soil and the indigenous microbial community. Our results suggest that phytoremediation systems can be considered as a method of increasing the potential of soil to degrade hydrocarbons but that other site management techniques, such as increased fertilization, will be required to realize this potential.

The effectiveness of the phytoremediation system was plant species dependent, with Tall Fescue stimulating catabolic activity and Rose Clover depressing it. Other investigators have also found a strong species dependence on the ability of phytoremediation systems to promote hydrocarbon degradation (13, 38). This may be due to alterations in root exudate patterns (8) but may also be due to differences in root architecture (2). Tall Fescue has a fibrous root system, whereas Rose Clover has a coarse, woody root system. The alteration of microbial diversity by plants is not only dependent on nutrient status but is also known to be dependent on the genetic composition of the plant (32) as well as the diversity of organisms already present in the soil (17). Typically, plant-dependent changes in microbial functionality are the result of some form of communication between the associated microorganisms and the plant. For example, bacterial products, such as lumichrome, stimulate root respiration and thereby increase the availability of root exudates for bacteria (21). This effect of lumichrome has been demonstrated with alfalfa, which is a plant shown to promote pyrene degradation in the rhizosphere (21). A present challenge in phytoremediation research is to identify the appropriate plant species that can beneficially alter microbial...
diversity for a specific soil contamination scenario or, alternatively, that is susceptible to manipulation by the appropriate bacterial inoculant.

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