The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil

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Plant-specific changes in microbial populations on roots affect degradation of petroleum hydrocarbons in contaminated soil.

Abstract

Enhanced rhizosphere degradation uses plants to stimulate the rhizosphere microbial community to degrade organic contaminants. We measured changes in microbial communities caused by the addition of two species of plants in a soil contaminated with 31,000 ppm of total petroleum hydrocarbons. Perennial ryegrass and/or alfalfa increased the number of rhizosphere bacteria in the hydrocarbon-contaminated soil. These plants also increased the number of bacteria capable of petroleum degradation as estimated by the most probable number (MPN) method. Eco-Biolog plates did not detect changes in metabolic diversity between bulk and rhizosphere samples but denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified partial 16S rDNA sequences indicated a shift in the bacterial community in the rhizosphere samples. Dice coefficient matrices derived from DGGE profiles showed similarities between the rhizospheres of alfalfa and perennial ryegrass/alfalfa mixture in the contaminated soil at week seven. Perennial ryegrass and perennial ryegrass/alfalfa mixture caused the greatest change in the rhizosphere bacterial community as determined by DGGE analysis. We concluded that plants altered the microbial population; these changes were plant-specific and could contribute to degradation of petroleum hydrocarbons in contaminated soil.
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Keywords: DGGE; Ecology; Petroleum contaminated soil; Phytoremediation; Rhizosphere

1. Introduction

Phytoremediation is the use of plants to remediate contaminated matrices such as soil, sediment, surface and ground water. Metals and radionuclides can be extracted from the contaminated matrix, translocated within the plant and concentrated in the shoots for recovery (Kumar et al., 1995; Salt et al., 1995; Blaylock et al., 1997; Siciliano and Germida, 1998; Khan et al., 2000). Organic contaminants, such as petroleum hydrocarbons, poly-cyclic aromatic hydrocarbons (PAHs), organic solvents (trichloroethylene or TCE) and pesticides can be stabilized within the soil matrix, taken up by plants and transformed or stored in a non-phytotoxic form. Plants can also stimulate the rhizosphere microbial community that is capable of degrading organic contaminants (Siciliano and Germida, 1998; Macek et al., 2000).

Laboratory, greenhouse and pilot scale studies have shown increased degradation of pollutants in planted systems over non-planted systems (Aprill and Sims, 1990; Wiltse et al., 1998; Siciliano et al., 2003). The plant rhizosphere can support a microbial community of several orders of magnitude higher than bulk soil (Curl and Truelove, 1986; Siciliano and Germida, 1998; Macek et al., 2000). Plants also influence the structure of

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microbial communities through the release of root exudates (Grayston and Campbell, 1996; Grayston et al., 1996; Grayston et al., 1998; Kozdroj and van Elsas, 2000; Macek et al., 2000) and by providing surfaces for colonization. However, it is not understood how specific plants increase the remediation of contaminated soils.

In this study, we assessed the microbial community in the rhizosphere of perennial ryegrass (*Lolium perenne* Variety Affinity) and alfalfa (*Medicago sativa* L.) and perennial ryegrass grown together with alfalfa to determine if the microbial community structure and functional diversity in a petroleum-contaminated soil changed with the presence of plants. Community structure is defined for the purpose of this paper as the community fingerprint obtained during DGGE analysis and functional diversity is defined as the number of carbon substrates utilized by a microbial population (Derry et al., 1998; Derry et al., 1999). Plants were selected based on previous research showing their phytoremediation potential (Gunther et al., 1996; Reilley et al., 1996; Nichols et al., 1997) and the ability to germinate and grow in a petroleum-contaminated soil (Kirk et al., 2002). Microbial populations were assessed numerically using plate counts on selective media and functionally using community level physiological profiling (CLPP) and the most probable number (MPN) method for estimating the number of microorganisms capable of utilizing petroleum as a source of carbon and energy (Wrenn and Venosa, 1996). Denaturing gradient gel electrophoresis (DGGE) was used to determine the influence of plants on microbial community structure in petroleum-contaminated soil. We hypothesized the presence of plants would increase the numerical and functional diversity and alter the bacterial community structure in the rhizosphere and that this effect was dependent on plant species.

Plants increase the microbial numbers in the rhizosphere, a phenomenon termed the rhizosphere effect. In phytoremediation, it is not known if this increase in microbial numbers is responsible for the increased degradation of contaminants or if plants are selectively increasing certain populations of microorganisms. This experiment was therefore designed to study the influence that two different plant types (perennial ryegrass, a monocot with a fibrous root system and alfalfa, a dicot with a tap root system) have on the microbial population in the rhizosphere in an attempt to understand how different plants influence microbial populations in contaminated soils.

2. Materials and methods

2.1. Soil preparation and analysis

Weathered petroleum hydrocarbon-contaminated soil was collected from a decommissioned petroleum storage site in Southern Ontario, Canada as described in Kirk et al. (2002). Contaminated soil was removed from 15 to 46 cm depth layer and sieved through a 2-mm screen. Soil was stored at 4 °C in the dark until required. Total petroleum hydrocarbon (TPH) content in soil samples was determined using GC/FID by the Environmental Chemistry Laboratory at Water Technology International (WTI), Burlington, Ontario, Canada. Briefly, 20 g of soil were spiked with 100 μl of 2000 ppm *o*-terphenyl, as an internal standard. Soil was extracted by shaking vigorously on an orbital shaker with 80 ml dichloromethane for 5 h. The extract was dried by passing through anhydrous sodium sulphate and concentrated. The concentrated extracts and standards were analysed by GC/FID for TPH content using a Hewlett Packard 5890 Series II and a 30 m DB-5 (0.25 I.D. $\times$ 0.25 μm film thickness) chromatography column.

Soil and nutrient analyses of plant available phosphorous (colorimetric autoanalyzer), potassium and magnesium (atomic absorption spectrophotometer), total carbon, organic carbon, inorganic carbon, total nitrogen (Leco furnace), NH_4-N, NO_3-N, NO_2-N (KCl extractable) and pH (soil slurry method) were conducted by the Soil and Nutrient Laboratory, Laboratory Services Division, University of Guelph, Guelph, ON, Canada, using standard methods.

Four hundred and fifty g dry weight of contaminated soil were placed in 10-cm diameter ceramic pots with a drainage hole in the bottom, and brought to approximately 13–15% gravimetric water content with sterile distilled water. Pots were equilibrated in a growth room under 16-h of light at 24 °C and 8-h of dark at 18 °C for 7 days prior to planting. Soil was kept moist throughout this equilibration period by addition of sterile distilled water. Soil moisture was maintained in the 13–15% range for gravimetric water content. Sampling of the soil microbial community started one day before planting. Sampling was done at weekly intervals following planting for seven weeks (see below).

Twenty-four hours prior to planting and after initial sampling, each pot was fertilized with 75 ml of sterile liquid fertilizer solution containing 100 ppm of potassium, supplied as potassium sulfate, and 100 ppm of nitrogen, supplied as ammonium nitrate. Two hundred ppm of solid calcium phosphate powder (1.02 g per pot) was also mixed thoroughly into each pot.

2.2. Seeds and planting

Perennial Ryegrass (*Lolium perenne* var. "Affinity") and Alfalfa (*Medicago sativa* L.) (Oseco, Brampton, ON, Canada) seeds were stored at 4 °C in the dark until required. Twenty-four hours prior to surface disinfection, the seeds were placed at room temperature (22–24 °C) in the dark. Surface disinfection of seeds was conducted to prevent the addition of non-indigenous

microbes to the treatments. Seeds were surface-disinfected by shaking at 200 rpm at room temperature for 15 min in a solution of 20% (v/v) Javex (5.25% sodium hypochlorite) in sterile distilled water. Seeds were rinsed five times with sterilized distilled water and germinated in the dark at room temperature on sterile filter paper soaked in 5 ml of sterilized deionised water. Germination occurred after 2 days for alfalfa and 4 days for perennial ryegrass. Eighteen seedlings per pot were planted in two concentric circles, a larger circle surrounding a smaller circle. The approximate density of plants was 4 per cm^2. There were four treatments each with three replicates. The four treatments were: (i) perennial ryegrass; (ii) alfalfa; (iii) perennial ryegrass grown (nine seedlings) together with alfalfa (nine seedlings) in the same pot; and (iv) bulk soil (no plants).

2.3. Sampling

At sampling time (T=0) prior to planting, 1–2 g of soil were removed using a sterile spatula. Soil was weighed in a sterile weighing dish and placed in a 125-ml Erlenmeyer flask containing 45 ml of sterile 0.1% w/v sodium pyrophosphate (pH adjusted to 7.0) and 4 g of sterile 3-mm diameter glass beads. Flasks were shaken for one hour at 140 rpm. For all subsequent sampling times, bulk soil was sampled using the above technique. For the other three treatments, plant(s) were randomly removed from the soil. One plant at each sampling time was removed for the perennial ryegrass and alfalfa treatments. For the treatment containing both plants, one plant of each species growing in close proximity to each other was randomly selected.

Plants removed from the soil were shaken to dislodge excess soil adhering to the roots. Roots and shoots were separated and weighed in sterile pre-weighed aluminium weighing dishes. Roots with the rhizosphere soil were placed in 125-ml Erlenmeyer flasks containing 45 ml of 0.1% w/v sodium pyrophosphate solution (pH adjusted to 7.0) and 4 g of sterile glass beads as above and were shaken at 140 rpm for 1 h. Roots were aseptically removed from the solution, blotted dry and weighed. The difference in root weight from pre- and post-shaking was considered to be the amount of wet rhizosphere soil from which the microbial populations were extracted. A 3–5 g sample of soil from the site at which the plant was removed was dried at 105 °C for 48 h to determine the soil moisture content. Plant roots and shoots were weighed and dried at 105 °C for 24 h to determine the dry phytomass. Soil pH and conductivity were determined at T=0 just prior to planting and at 7 weeks by adding 20 ml of deionized water to 10 g of soil, stirring for 3 min and left unstirred for 1 h at room temperature. Samples were stirred briefly and the measurements taken.

Serial dilutions, up to 10^{-10} in sterile 0.1% (w/v) sodium pyrophosphate (pH 7.0) were used for enumeration of microbial populations by plate counts and the MPN methods, and for assessment of metabolic potential using the Eco-Biolog plates.

2.4. Microbial plate counts

2.4.1. Bacteria

Culturable, aerobic heterotrophic bacterial cells were enumerated in triplicate using the drop plate method of Cassidy et al. (2000) with 20 μl per drop over a range of serial dilutions on tryptic soy agar (TSA) supplemented with 75 ppm cycloheximide to inhibit fungal growth. Plates were incubated at 30 °C for 24 h in the dark and colonies counted.

2.4.2. Fungi

Total fungal counts were performed in triplicate using 100 μl of each dilution spread plated on malt extract agar supplemented with 100 ppm of chloramphenicol and 50 ppm of Rose Bengal. Rose Bengal was used to slow the growth and spread of fast growing fungi so slower growing fungi could be enumerated. The malt extract agar contained, per litre, 20 g of malt extract, 15 g of agar and 1 g of yeast extract. Petri plates were incubated at 28 °C in the dark for 14 days and colonies counted.

2.5. Culturable petroleum-degrading microbial counts

2.5.1. Bacterial counts

Petroleum degrading aerobic bacteria were enumerated in triplicate using the drop plate method (20 μl per drop) (Cassidy et al., 2000) over a range of dilutions on Atlas oil agar. Composition of this agar per liter was: Bushnell-Haas (BH) Agar 990 ml (15.0 g of agar, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeCl_3$ and 0.02 g $CaCl_2 \cdot H_2O$ adjusted to pH 7.0) and 10.0 ml of filter sterilized F2 Oil (Esso Heating Oil) added to sterile BH Agar which had been cooled to 60 °C.

2.5.2. Fungal counts

Culturable petroleum degrading fungi were enumerated following the method of Oudot et al. (1993) with some modifications. Sample dilutions (100 μl) were spread plated onto a medium selective for petroleum degrading fungi supplemented with 100 ppm of chloramphenicol. Composition of this medium per litre was 250 mg KCl, 1 g each of NaH_2PO_4 and NH_4NO_3, 0.5 g $MgSO_4$ and 20 g agar. Chloramphenicol and 0.2 ml of filter sterilized F2 Oil (Esso) were added to the medium once it cooled to 50 °C. Plates were incubated at 28 °C for 21 days in the dark before enumerating petroleum-degrading fungi.

2.6. Most probable number (MPN) method: enumeration of F2, PAH and hexadecane degraders

The MPN procedure was used to estimate the numbers of aromatic and aliphatic hydrocarbon degrading bacteria present in soil samples. This method was adapted from Wrenn and Venosa (1996), with some modifications. Each 96-well microtiter plate contained either F2 oil (Esso), n-hexadecane (Sigma, St. Louis, USA) or a mixture of polycyclic aromatic hydrocarbons (PAH) (PAH mixture was dissolved in pentane and consisted of 10 g/l phenanthrene (Sigma), 1 g/l anthracene (Sigma), 1 g/l fluorene (Aldrich, Milwaukee, USA) and 1 g/l dibenzothiophene (Aldrich) and were used to enumerate total petroleum degraders, alkane degraders and PAH degraders, respectively. The composition of the Bushnell-Haas (BH) medium used for all three hydrocarbon degrading MPN procedures was per litre: 1.0 g each of KH_2PO_4, K_2HPO_4, and NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $FeCl_3$ and 0.02 g $CaCl_2 \cdot 2H_2O$.

Microtiter plates to enumerate total petroleum and alkane degraders were prepared by adding 5 μl of filter-sterilized carbon source, either F2 oil or n-hexadecane, to each well containing 180 μl BH medium. The PAH microtiter plates were prepared by first adding 10 μl of the PAH solution in pentane to each well, allowing the pentane to evaporate thereby coating the well with PAHs and then adding 180 μl of BH medium. Twenty μl of ten-fold serial dilutions of each treatment were added starting in row 1 with the undiluted extract and continuing until well 11 with 10^{-10} dilution. Row 12 was an uninoculated sterile control consisting of BH medium and 20 μl of sodium pyrophosphate (pH 7.0). The alkane and total hydrocarbon plates were incubated at room temperature. After 2 weeks, 50 μl of filter sterilized iodonitrotetrazolium violet (INT) solution (3 g/l) was added to each well. INT is metabolically reduced to an insoluble purple formazan precipitate that can be visually scored. Positive scores were recorded after 24 h incubation at room temperature. PAH degraders were enumerated after 3 weeks in the dark at room temperature. INT was not used since intermediates of PAH degradation can be detected visually by colour change in the absence of INT. Positive scores of PAH degradation were wells with a yellow or brown colour caused by the intermediates of partial degradation of PAHs. A computer program (most probable number calculator V 4.04, 1996 Albert J. Klee, United States Environmental Protection Agency, Cincinnatti, OH, USA) was used to calculate the MPN of degraders.

2.7. Eco-Biolog plates to assess substrate utilization patterns for metabolic diversity

The initial soil extract for each treatment was centrifuged for 2 min at 500×g to remove soil and root particles. The supernatant was decanted into a second sterile centrifuge tube and centrifuged for 10 min at 8000×g to pellet microbial cells. The sterile sodium pyrophosphate was decanted and replaced with the same volume of sterile 0.85% (w/v) NaCl. Tubes were vortexed for 2 min to suspend the cells.

The optical density of each treatment extract resuspended in NaCl was determined at 600 nm. If necessary, the sample was diluted with sterile 0.85% (w/v) NaCl to an optical density of approximately 0.05 to standardize the inoculum size to similar values at time zero.

All original and diluted samples were inoculated into an Eco-Biolog plate (150 μl/well) to determine the metabolic potential of the microbial population over time. The Eco-Biolog plate contains 31 different carbon sources, replicated three times and was created specifically for microbial community analysis and ecology studies. Samples were incubated at 30 °C and analyzed at 595 nm using a Bio-Rad microplate reader (Model 3550-UV) at 1, 2, 3, 7 and 14 days after inoculation. Only data from the 72-h reading will be presented.

The Shannon diversity (H') index of catabolic diversity was used to measure species metabolic diversity and was calculated according to the formula $H' = -\Sigma p_i \ln p_i$. The value p_i was calculated by subtracting the blank from each substrate's absorbance at 595 nm and then dividing this value by the total colour change recorded for all 31 substrates. The calculated H' values were then subjected to principle component analysis (PCA) as described by Derry et al. (1999) and Garland and Mills (1991).

2.8. Denaturing gradient gel electrophoresis (DGGE)

DNA was extracted directly from rhizosphere and bulk soil samples using the Ultra Clean Soil DNA Kit (Bio/Can Scientific, Mississauga, ON, Canada) as per the manufacturer's instructions with one modification. A bead beater (Bio101 Fast Prep FP120, Holbrook, NY, USA) was used for 30 sec at speed setting 5.5 (m/s) instead of shaking for 10 min. DNA extracts were cleaned using Wizard DNA Clean-up System (Promega, Madison, WI, USA). Oligonucleotide primers able to amplify a fragment spanning nucleotide positions 341–534 of E. coli 16S rDNA were synthesized by GIBCO Life Technologies (Burlington, ON, Canada). The nucleotide sequences of the primers were: 341F, 5′-GC clamp-CCTACGGGAGGCAGCAG-3′ and 534R, 5′-ATTACCGCGGCTGCTGG-3′. These primers have been used previously for bacterial community analysis using DGGE (Muyzer et al., 1993; MacNaughton et al., 1999). A 40 bp GC clamp at the 5′ end of the forward primer (341F) had the sequence: 5′-GGA GCC CGG CGA CCG GCG CGC GCG GCG GCA CGG GGG C. The GC clamp ensures the DNA fragment is

not completely separated into single strands so that migration in the polyacrylamide gel was based mainly on melting behaviour of the DNA fragment.

PCR was conducted in a Perkin Elmer Gene Amp PCR System 2400 with the following program: 4 min at 94 °C, and 40 cycles of 94 °C for 30 s, 63 °C for 1 min and 72 °C for 30 s followed by 10 min at 72 °C and then held at 4 °C. Fifteen μl of the PCR product were loaded with 5 μl of loading dye into a 10% polyacrylamide gel with 35–80% denaturant gradient for DGGE. Three replicate samples were run on this gradient and on gradients of 45–65 and 35–75%. Although banding patterns were similar among replicates and data trends were the same, the 35–80% gradient provided the best resolution of the banding patterns of the bacterial community and therefore the results are presented and discussed. The 100% denaturant solution had 40 ml of formamide and 42 g of urea in 100 ml. DGGE was run on a Biorad DeCode system for 4 h at 180 volts at 60 °C. Gels were stained for 30 min in 50 μg/ml solution of ethidium bromide and 0.5×TAE buffer. Gels were destained for 15 min in deionized water and visualized on an Ultralum UV transmitter using Kodak Electrophoresis Documentation and Analysis System 120.

DGGE banding patterns were analysed on BioNumerics Software version 1.5 (Applied Maths, Kortrijk, Belgium). The gel image was digitized and corrected for background using the rolling ball method. DNA bands were automatically detected with band filtering parameters set at: 5.0% minimum profiling, 2.0% minimum area and a shoulder sensitivity of 3%. The Dice coefficient (a measure of similarity) was determined from banding patterns measuring the similarity between each treatment (Gelsomino et al., 1999; Simpson et al., 2000).

3. Results

3.1. Plant growth

Perennial ryegrass and alfalfa germinated in petroleum-contaminated soil but growth of these seedlings was stressed and stunted. Total plant biomass (dry weight) ranged from an average of 5, 1 and 3 mg/plant at week one to 105, 24 and 356 mg/plant at week 7 for perennial ryegrass, alfalfa and perennial ryegrass and alfalfa grown together, respectively.

3.2. Total microbial plate counts

3.2.1. Bacteria

After 7 weeks of incubation in the TPH contaminated soil, heterotrophic bacterial numbers in the rhizosphere of perennial ryegrass was 3.49×10^9 CFU/g dry soil, which was significantly higher than bulk soil with

4.49×10^7 CFU/g dry soil (P < 0.05). The rhizosphere in the remaining plant treatments all exhibited increased heterotrophic microbial numbers over the bulk soil, however, this difference was not statistically significant (Fig. 1A). Although highly variable, the number of heterotrophic bacteria in the bulk soil remained relatively constant throughout the 7 weeks of the experiment (about 10^7/g). All three plant treatments led to increased heterotrophic bacterial numbers in the rhizosphere within 1 week of the start of the experiment (ranging from approximately 10^8–10^{10} CFU/g). Bacterial numbers in the rhizosphere of alfalfa and perennial ryegrass grown together with alfalfa fluctuated throughout the experiment while the heterotrophic bacterial numbers in the rhizosphere of perennial ryegrass continued to increase over the duration of the experiment (Fig. 1A).

The number of petroleum degrading bacteria was significantly higher in the rhizosphere of perennial ryegrass (4.18×10^9 CFU/g dry soil) compared to all other treatments (P < 0.05) after 7 weeks. A trend of increased microbial numbers in the rhizosphere of other plant treatments compared to bulk soil was observed, although this difference was not statistically significant
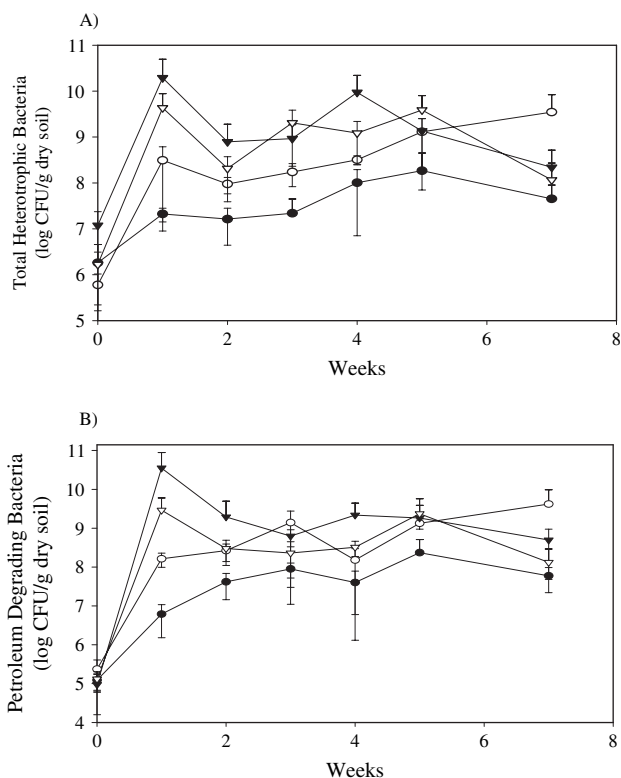

Fig. 1. Total (A) culturable heterotrophic bacteria; and (B) petroleum degrading bacteria from the rhizospheres of three plant treatments and bulk soil contaminated with 31,000 ppm TPH as determined by plate counts on (A) TSA; and (B) Atlas oil agar. Symbols: ● bulk soil; ○ perennial ryegrass; ▼ alfalfa; ▽ perennial ryegrass grown with alfalfa. Data are the means±SD (n=9).

($P < 0.05$) (Fig. 1B). The number of petroleum degrading bacteria in the rhizosphere of all three-plant treatments increased significantly in the first week of the experiment. After this period, bacterial numbers in all three plant treatments fluctuated, but were still above those of the bulk soil (Fig. 1B).

Over the 7 weeks of treatment, the heterotrophic bacterial numbers increased by 25-, 5817-, 18- and 80-fold for bulk soil, perennial ryegrass, alfalfa and perennial ryegrass grown together with alfalfa, respectively. The rhizosphere effect, defined as the influence of the plant over the bulk soil, calculated by CFU of rhizosphere sample (week 7)/CFU of bulk soil (week 7), for the plant treatments was 78, 5 and 3 for perennial ryegrass, alfalfa and perennial ryegrass grown together with alfalfa, respectively. The number of petroleum degrading bacteria increased over the 7 weeks by 485, 17,787, 5299 and 1008 for bulk soil, perennial ryegrass, alfalfa and perennial ryegrass grown together with alfalfa, respectively, giving a rhizosphere effect of 70, 8 and 2.

3.2.2. Fungi

Seven weeks after planting, there was no statistically significant difference between plant treatments for both heterotrophic and petroleum degrading fungi. There was however, a significant increase in heterotrophic fungal numbers from $(3.36 \pm 1.4) \times 10^4$ to $(4.32 \pm 5.5) \times 10^6$ (CFU/g dry soil) over 7 weeks ($P < 0.001$). There was no difference in the number of petroleum degrading fungi in any of the plant treatments. Estimates of petroleum degrading fungi ranged from 2.66×10^6 to 1.41×10^7.

3.3. Bacterial counts estimated by most probable number method (MPN)

The number of bacteria capable of utilizing F2 diesel fuel (Fig. 2A), hexadecane (Fig. 2B) or a mixture of PAHs (Fig. 2C) was estimated using MPN and did not differ significantly between plant treatments. At seven weeks, the bulk soil and rhizosphere microbial populations showed a statistically significant increase in the number of bacteria capable of utilizing the three carbon sources relative to time 0 ($P < 0.05$). The mean number of F2-utilizing bacteria estimated in the rhizosphere of perennial ryegrass, alfalfa, and perennial ryegrass and alfalfa grown together was higher by an order of magnitude than in the bulk soil, although variability in this data was too high for this difference to be statistically significant (Fig. 2A). The rhizosphere of perennial ryegrass also appeared to host a larger microbial population capable of utilizing hexadecane, however, variability was too high for this difference to be statistically significant (Fig. 2B).

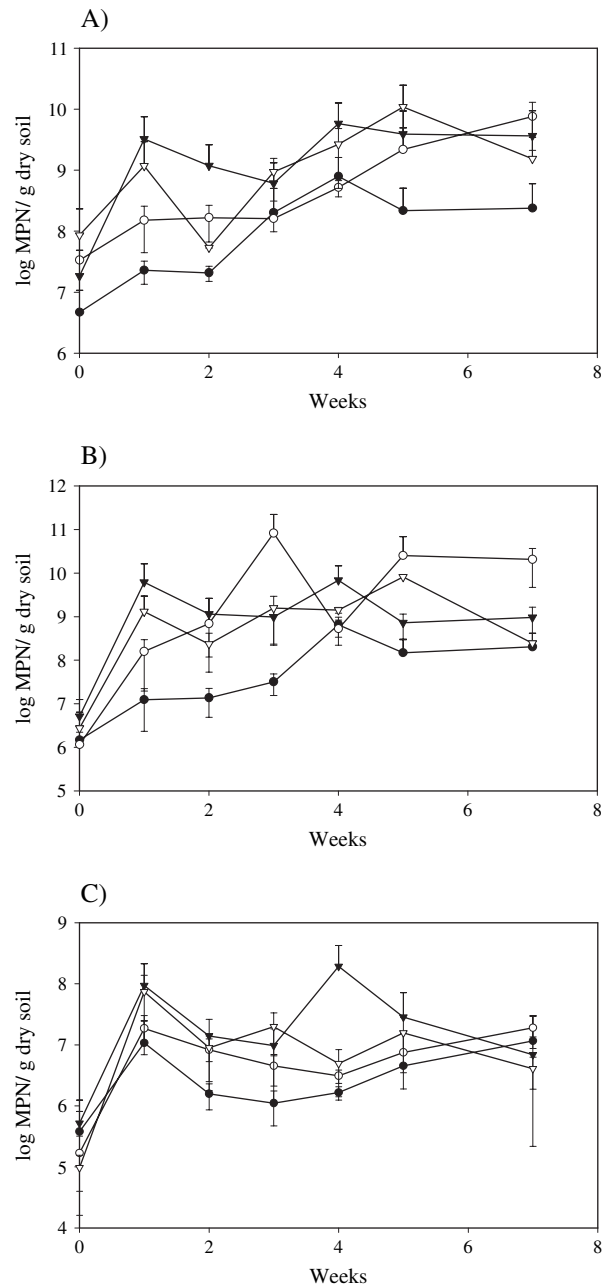Over the 7 weeks of the experiment, the number of F2 degraders estimated by the MPN method increased by

Fig. 2. MPN estimates of bacteria capable of utilizing: (A) F2 diesel; (B) hexadecane; and (C) a PAH mixture. Data are the means±SD ($n = 3$). Symbols: ● bulk soil; ○ perennial ryegrass; ▼ alfalfa; ▽ perennial ryegrass grown with alfalfa.

51-, 230-, 200- and 17-fold for bulk soil, perennial ryegrass, alfalfa and the perennial ryegrass/alfalfa mixture, giving a rhizosphere effect of 32, 15 and 6, respectively. The number of hexadecane degrading microorganisms increased by 133-, 17,500-, 185- and 89-fold for bulk soil, perennial ryegrass, alfalfa and perennial ryegrass/alfalfa mixture over the 7 weeks of the experiment. This corresponds to a rhizosphere effect of 105, 5 and 1 for perennial ryegrass, alfalfa and perennial ryegrass/alfalfa mixture, respectively.

3.4. Functional diversity of microbial communities measured by Eco-Biolog plates

Before plants were introduced into the contaminated soil the microbial communities of all treatments clustered together (PCA analysis of data from Eco-Biolog plates), with the exception of two outlying points from the alfalfa rhizosphere treatment (Fig. 3A). After 7 weeks, the functional diversity of the microbial communities began to change and cluster by plant species, although an outright distinction was not apparent (Fig. 3B). If plants were less stressed (as evidenced by stunted growth) because of soil conditions and the rhizospheres were more mature, or older, a more distinct relationship between plant species and metabolic functional diversity may have been detected.

Shannon diversity indices of the weighted colour development for the utilization of 31 carbon sources

showed no difference between the bulk soil and soil with plants. Of the 30 substrates, 28 were used by microbes in all treatments. The two substrates not utilized by the microbial communities in all treatments were α-ketobutyric acid and 2-hydroxybenzoic acid. The metabolic potential diversity of the communities had mean Shannon indices of 3.26, 3.28, 3.28 and 3.3, for bulk soil, and soils planted with perennial ryegrass, alfalfa and perennial ryegrass/alfalfa mix, respectively.

3.5. Structure of the microbial community measured by denaturing gradient gel electrophoresis (DGGE)

DGGE profiles differed among all treatments (Fig. 4). The number of distinct DNA bands ranged from 11 ± 1 in perennial ryegrass/alfalfa rhizosphere to 14 ± 1 in the bulk soil after 7 weeks. The largest difference in banding patterns was between bulk soil and the rhizospheres of perennial ryegrass [Dice co-efficient 50.0 ± 14.4 ($n=3$)] and perennial ryegrass/alfalfa [Dice coefficient 47.8 ± 2.0 ($n=3$)]. Clustering analysis of bulk soils at weeks 0 and 7 had a similarity (based on Dice coefficient) of banding patterns of $61.6 \pm 10.3\%$ ($n=3$), while the alfalfa and perennial ryegrass/alfalfa mixture showed a similarity of $82 \pm 11.2\%$ ($n=3$). All rhizosphere banding patterns displayed a similarity to bulk soils banding pattern of about 60% while perennial ryegrass had a similarity of 60.9 ± 5.6 to alfalfa and 55.6 ± 4.0 to perennial ryegrass/alfalfa mixture.

4. Discussion

Several methods were used to determine if selected plants influenced the numerical, structural and/or functional diversity of the microbial communities in the rhizosphere of a petroleum-contaminated soil. By using more than one method to study the microbial population, a more complete picture of the microbial population can be obtained (Kirk et al., 2004). The results from plate counts, MPN and DGGE indicated that a shift in the rhizosphere microbial populations occurred in the presence of plants, and that these shifts were plant-specific. However, it should be noted that all plants in this experiment were stressed in the contaminated soil. This could be from direct toxicity of the petroleum hydrocarbons in the soil or a result of the contaminant properties. Often, contaminants such as petroleum hydrocarbons alter the physical and chemical properties of the soil. Hydrophobic contaminants can change the water/soil interactions that would normally occur, thereby potentially affecting oxygen transfer, available water uptake, and nutrient mobility.

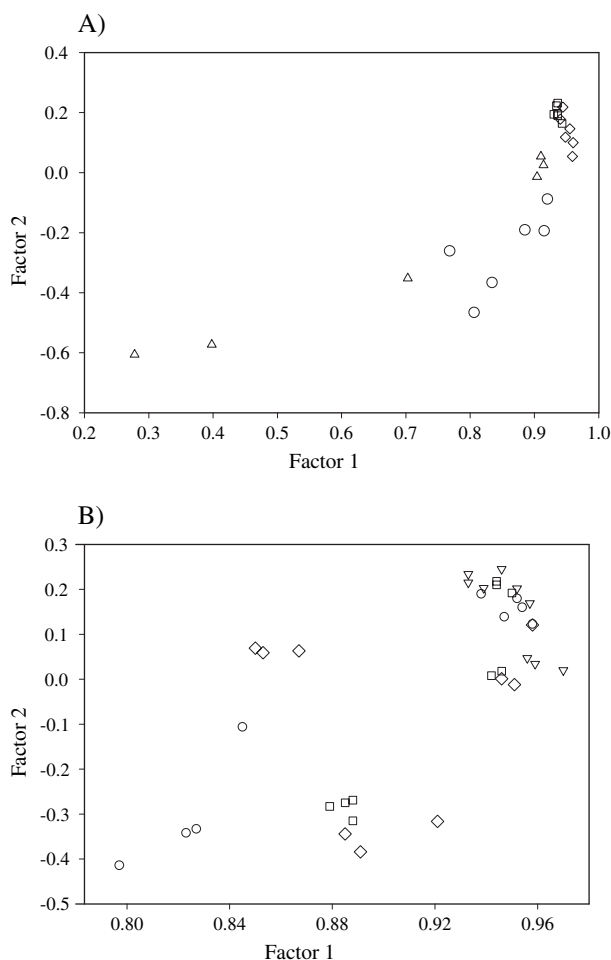Perennial ryegrass altered the rhizosphere by hosting 233 times more culturable heterotrophic bacteria and

Fig. 3. Principle component analysis of potential functional diversity of microbial communities in bulk soil and in rhizosphere samples of plants grown in petroleum-contaminated soil using Eco-Biolog carbon source utilization patterns at: (A) Time=0; and (B) Time=7 weeks. Symbols: ○ bulk soil; □ perennial ryegrass; ▽ alfalfa; ◇ perennial ryegrass grown with alfalfa (*n*=9 per treatment).

A)

B)

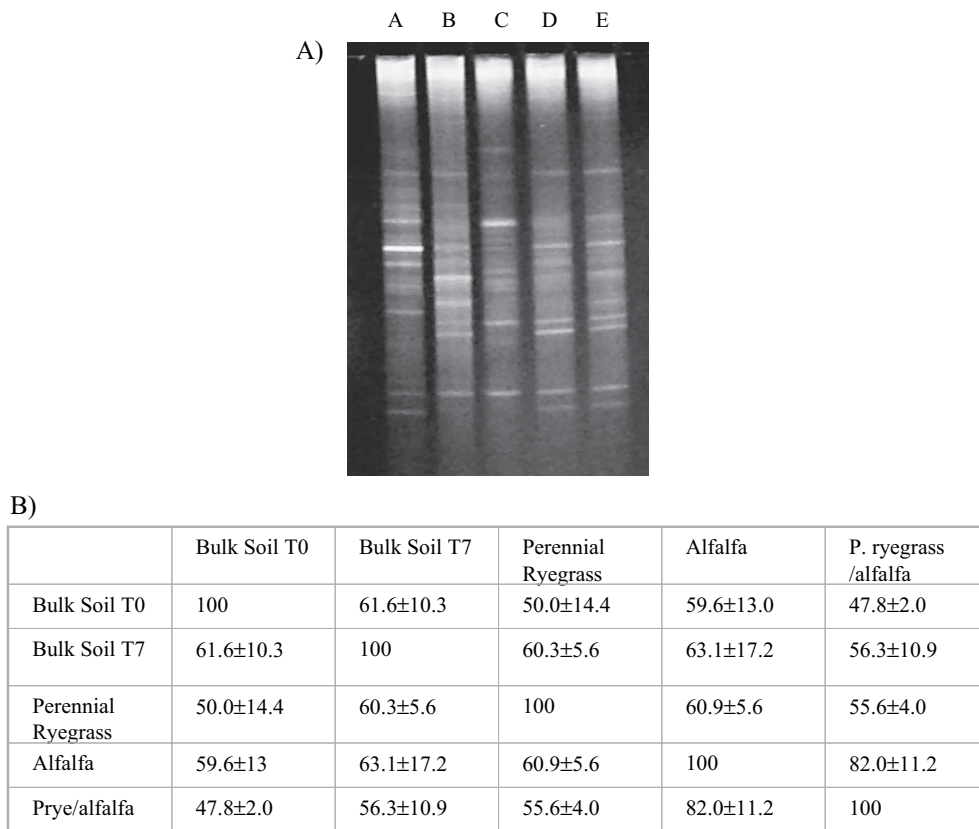|	Bulk Soil T0	Bulk Soil T7	Perennial Ryegrass	Alfalfa	P. ryegrass /alfalfa
Bulk Soil T0	100	61.6±10.3	50.0±14.4	59.6±13.0	47.8±2.0
Bulk Soil T7	61.6±10.3	100	60.3±5.6	63.1±17.2	56.3±10.9
Perennial Ryegrass	50.0±14.4	60.3±5.6	100	60.9±5.6	55.6±4.0
Alfalfa	59.6±13	63.1±17.2	60.9±5.6	100	82.0±11.2
Prye/alfalfa	47.8±2.0	56.3±10.9	55.6±4.0	82.0±11.2	100

Fig. 4. (A) Representative DGGE banding patterns of partial 16S rDNA sequences of bacteria from bulk and rhizosphere soils; and (B) dice coefficients of DGGE analysis ($n=3$). Treatments (A) original bulk soil (time 0); (B) bulk soil (7 weeks); (C) perennial ryegrass (7 weeks); (D) alfalfa (7 weeks); and (E) perennial ryegrass/alfalfa mixture (7 weeks).

37 times more petroleum degrading bacteria in the rhizosphere after 7 weeks compared to the bulk soil. This suggested the petroleum degrading microbial population in the rhizosphere was increased by the presence of perennial ryegrass, as was a general increase in microbial numbers (rhizosphere effects of 78 and 70 for culturable, aerobic heterotrophic and petroleum degraders, respectively).

Alfalfa increased the number of both culturable, aerobic heterotrophic and petroleum degrading bacteria in the rhizosphere with a rhizosphere effect at 7 weeks of 5 and 8, respectively. Although the rhizosphere effect for alfalfa was not as substantial as perennial ryegrass, probably due to the different root morphologies, the results are interesting. Perennial ryegrass seemed to support a general increase in microbial numbers, the rhizosphere effects were similar for both heterotrophic bacteria (rhizosphere effect of 78) and petroleum degrading bacteria (rhizosphere effect of 70) and there was a large increase in bacteria capable for degrading hexadecane (rhizosphere effect of 105). Alfalfa produced a rhizosphere effect for heterotrophic bacteria of 5 but had a rhizosphere effect of 8 for petroleum degrading bacteria as estimated by plate counts. The MPN method estimated the rhizosphere effect of alfalfa to be 5 for

hexadecane degraders and 15 for those organisms capable of degrading the F2 diesel. These results indicate both plants increased rhizosphere bacterial numbers and petroleum degraders. At week 7, perennial ryegrass led to a larger increase in hexadecane degraders over other carbon sources and alfalfa led to a larger increase in microorganisms capable of degrading F2 diesel than other carbon sources. Although both plant species increased the degrading population, the apparent selection of specific groups of degraders was not uniform throughout the experiment. This relationship of plants seemingly selecting certain microbial populations at week 7 might be an artefact of sampling and may not have occurred if the plants were more mature, less stressed and if the soil conditions were more conducive to plant growth. On the other hand, as the rhizosphere matures over time, we may actually see a more distinct selection of microbial populations by the plant. A similar study with a longer growing season may answer the question of whether this selection seen at 7 weeks is a plant specific selection of the microbial population to enhance petroleum degraders. Nichols et al. (1997) observed an increase in microorganisms capable of utilizing a mixture of organic chemicals [hexadecane, (2,2-dimethylpropyl)benzene, *cis*-decahy-

dronaphthalene, benzoic acid, phenanthrene and pyrene] in the rhizosphere of alfalfa grown in soil contaminated with these organics using the MPN method. Other researchers have also found that certain plant species are more efficient at phytoremediation of hydrocarbons than others (Wiltse et al., 1998; Liste and Alexander, 2000). Siciliano et al. (2003) reported the effectiveness of phytoremediation to be plant specific with Tall Fescue stimulating catabolic activity of soil microorganisms and Rose clover inhibiting it. Further research will be needed to determine if perennial ryegrass non-selectively increases, and alfalfa selectively increases the petroleum degrading bacterial community in the rhizosphere. These changes in the microbial population could simply be a result of differential plant exudates due to species or plant developmental stage that would occur whether petroleum was present or not.

Total heterotrophic and petroleum degrading fungal populations did not differ between plant treatments and bulk soil, although the number of fungi did increase significantly in all treatments. This result should be interpreted with caution because dilution and plating favour the species that produce large numbers of spores (Curl and Truelove, 1986). Depending on the soil, plant and sampling method, fungi in the rhizosphere could be 3- to 200-fold higher than bulk soil (Curl and Truelove, 1986). The lack of an apparent plant effect on the number of rhizosphere fungi could be a combination of soil, contaminant or experimental conditions favouring growth of bacteria over fungi.

The MPN method was used to assess the number of bacteria capable of utilizing F2-diesel, hexadecane and a mixture of PAHs as the sole source of carbon and energy. Analysis of the contaminated soil revealed the petroleum compounds resembled a diesel fuel. Therefore, it is not surprising that the plant treatments contained a large number of aerobic, heterotrophic bacteria capable of utilizing F2 (10^9–10^{10} per g dry soil) (Fig. 2A).

Although functional diversity did not change during the experiment and there was no difference in Shannon diversity indices, microbial populations did seem to be differentiating based on plant species. At the beginning of the study, with the exception of two outliers, all samples clustered together (Fig. 3). After week 7, although many rhizosphere samples had not differentiated from time zero as seen by clustering in the PCA analysis, some samples had started to differ and it appeared that these differences could be attributed to plant species. This differentiation however is not definitive and there are a number of possible explanations. Firstly, the soil was compacted with a poor ability to hold moisture, making it difficult for plants to establish. Second, the rhizosphere was immature and the plants poorly developed in the soil. It would be expected that

as the rhizosphere populations established, the communities would cluster by plant species.

For comparison, Shannon diversity indices for this study were 3.3 for all treatments (bulk soil, perennial ryegrass, alfalfa and perennial ryegrass/ alfalfa mixture) compared to values of 2.7 for a scarified mineral forest soil, 3.0 for a timber mineral forest soil, 3.5 for a burned mineral forest soil and 3.8 for a clear-cut mineral forest soil (Staddon et al., 1996). CLPP has been shown to be sensitive enough to differentiate between microbial populations in the rhizosphere of different plants (Grayston and Campbell, 1996; Grayston et al., 1998; Grayston et al., 2001).

Results from DGGE analysis suggested the plants altered the composition of the microbial community. Clustering analysis indicated that the microbial composition in plant rhizospheres were different from bulk soils. Since alfalfa and perennial ryegrass/alfalfa mixture clustered together and showed similarity in DNA banding patterns, this suggested alfalfa had a greater influence on the composition of the dominant rhizosphere bacterial population. Of all plant treatments, the DGGE fingerprint of perennial ryegrass and of perennial ryegrass/alfalfa mixture differed the most from the original bulk soil (Dice coefficients of 50.0 ± 14.4 and 47.8 ± 2.0, respectively), indicating the greatest shift in the microbial community structure as detected by DGGE.

To our knowledge, there is no consensus on the level of differences or similarity needed to reveal a difference in the microbial populations using DGGE. Smit et al. (1999) observed a similarity of 60 and 75% of banding patterns of rhizosphere soils to bulk soil and concluded that the plant was influencing fungal diversity. DGGE has been suggested to be able to detect 1-2% of the dominant microbial community. This typically represents the most abundant DNA extracted from the soil that contain sequences of the primers used (MacNaughton et al., 1999). As more bacterial species are characterized and as more genes from viable but non-culturable bacteria are sequenced, we may find that the "universal bacterial primers" are not as non-selective as once thought. It is possible that abundant species are present whose DNA was not easily amplified by the universal primers. It is therefore possible that a small change could reflect a significant change in the bacterial community.

Plants can alter rhizosphere microbial populations and increase degradation or immobilization of contaminants. This could minimize exposure to contaminants and subsequent toxicity to the plant. Plants could selectively increase a degrading population in its rhizosphere by altering exudation, could simply cause a non-specific increase in microbial numbers some of which may be degraders, or could have some other mechanism of protection against the toxic compound

(i.e. metabolism, sequestering). Siciliano and Germida (1997) found that only five of 16 plants tested were tolerant to 2-chlorobenzoic acid, and only 3 out of the 5 showed increased degradation of 2-chlorobenzoic acid over controls. The two plants not showing increased degradation may have had a different mechanism of tolerance than hosting a microbial population capable of degrading the contaminant. Siciliano et al. (2001) found that some plants, when exposed to different contaminants, selectively enriched catabolic genotypes of microorganisms living within the rhizoplane, this mechanism would not only protect the plants from the toxic effect of the contaminant, but also contribute to phytoremediation.

The results of our study suggest that perennial ryegrass and alfalfa may utilize different mechanisms to facilitate phytoremediation. Perennial ryegrass seems to support a general increase in microbial activity and numbers in the rhizosphere, some of which have catabolic activity towards petroleum hydrocarbons. Alfalfa, on the other hand, seems to specifically increase the number of microorganisms capable of degrading more complex hydrocarbons. A longer study period with more replicates to reduce variation may provide more conclusive results to determine if this pattern seen after 7 weeks is indeed occurring. Our results do indicate there are plant-induced changes in the soil microbial communities. Perhaps by researching direct effects of contaminants on root exudation, insights may be gained on how plants are able to alter microbial populations. It may also be possible to genetically engineer a plant to release extracellular compounds or to stimulate specifically the degrading microbial populations in the rhizosphere.

Acknowledgements

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