Environ. Eng. Res. Vol. 12, No. 2, pp. 37~45, 2007
Korean Society of Environmental Engineers

ENHANCED BIOREMEDIATION AND MODIFIED BACTERIAL COMMUNITY STRUCTURE BY BARNYARD GRASS IN DIESEL-CONTAMINATED SOIL

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(received September 2006, accepted April 2007)

Abstract : Phytoremediation has been used effectively for the biodegradation of oil-based contaminants, including diesel, by the stimulation of soil microbes near plant roots (rhizosphere). However, the technique has rarely been assessed for itsinfluence on soil microbial properties such as population, community structure, and diversity. In this study, the removal efficiency and characteristics of rhizobacteria for phytoremediation of diesel-contaminated soils were assessed using barnyard grass (*Echinochloa crusgalli*). The concentration of spiked diesel for treatments was around 6000 mg kg^{-1}. Diesel removal efficiencies reached 100% in rhizosphere soils, 76% in planted bulk soils, and 62% in unplanted bulk soils after 3weeks stabilization and 2 months growth (control, no microbial activity: 32%). The highest populations of culturable soil bacteria (5.89×10^8 per g soil) and culturable hydrocarbon-degraders (5.65×10^6 per g soil) werefound in diesel-contaminated rhizosphere soil, also yielding the highest microbial dehydrogenase. This suggests that the populations of soil bacteria, including hydrocarbon-degraders, were significantly increased by a synergistic rhizosphere + diesel effect. The diesel treatment alone resulted in negative population growth. In addition, we investigated the bacterial community structures of each soil sample based on DGGE (Denaturing Gel Gradient Electrophoresis) band patterns. Bacterial community structure was most influenced by the presence of diesel contamination (76.92% dissimilarity to the control) and by a diesel + rhizosphere treatment (65.62% dissimilarity), and least influenced by the rhizosphere treatment alone (48.15% dissimilarity). Based on the number of distinct DGGE bands, the bacterial diversity decreased with diesel treatment, but kept constant in the rhizosphere treatment. The rhizosphere thus positively influenced bacterial population density in diesel-contaminated soil, resulting in high removal efficiency of diesel.

Key Words : Phytoremediation, Rhizosphere, Bulk, Population, Diversity, DGGE (Denaturing Gel Gradient Electrophoresis)

INTRODUCTION

Phytoremediation is defined as the use of plants to remediate contaminated sites such as soil, sediment, surface and groundwater. In soil, organic contaminants, such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), organic solvents (trichloroethylene or TCE) and pesticides can be treated by combinations of the following mechanisms: stabilization within the soil matrix, take-up by plants, transformation into or storing in non-phytotoxic forms, and stimulation of soil microbes for biodegradation.[1,2)]

The rhizosphere is the soil region that is influenced by plant roots and is characterized by high microbial activity.[3)] In this region, the

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microbial population density can be several orders of magnitude higher than in the bulk soil.[1,2,4] The structure of microbial communities can be modified through the release of root exudates[1,5,6] and rhizodeposits.[7-9]

In this study, we examined the microbial communities within the rhizosphere and bulk soil of barnyard grass (*Echinochloa crusgalli*) in the presence or absence of diesel. In addition, we tested the diesel removal efficiencies of these microbial communities. Bacterial population densities were assessed numerically using plate counts, and the number of microorganisms capable of utilizing diesel as a carbon and energy source was estimated using the most probable number (MPN) method.[10] Dehydrogenase activity was quantified for evaluating soil microbial activity (including uncultured microorganisms). Denaturing gradient gel electrophoresis (DGGE) was used to determine the influence of plant rhizosphere on microbial community structure in diesel-contaminated soil.[11] We expected that the presence of plant rhizosphere would increase the bacterial population density and diversity and alter the bacterial community structure, resulting in enhanced bioremediation. This research was designed to understand how plants influenced microbial activity, population density and diversity in hydrocarbon-contaminated soils, especially those having no previous history of oil pollution.

MATERIALS AND METHODS

Properties of Soil and Preparation of Pots

Soil was obtained from a hillock at Ewha Womans University in Seoul, Korea. The soil texture can be described as loamy sand and the physico-chemical properties of the soil are listed in Table 1. Soil pH was determined by adding soil to water at a ratio of 1:2.5 w/v and measuring the pH after 1h. Total organic carbon (TOC) was calculated as 58% of organic matter, which was analyzed by loss on ignition in a furnace (MAS 7000, CEM). The cation-exchange capacity (CEC) of soils was measured by the EPA method 9081. The concentrations of anions were analyzed through an ion chromatography (WATERS Co., USA). Diesel- contaminated soils were prepared by thorough mixing with 2.82 ml diesel yielding initial concentrations of 6000 ppm (mg kg^{-1}-soil).

Table 1. Physicochemical properties of the original soil

Soil properties	Values
Sand (%)	80.64
Silt (%)	13.74
Clay (%)	5.62
TOC[1] (%)	3.05
CEC[2] (mmol kg^{-1})	34.32
pH	8.60
Cl^- (μg/g)	6.72
NO^{3-} (μg/g)	170.98
PO_4^{3-} (μg/g)	272.27
SO_4^{2-} (μg/g)	0.00

[1]total organic carbon
[2]cation exchange capacity

Four pot treatments were established: (1) unplanted control without diesel, (2) unplanted control with diesel, (3) planted control without diesel, and (4) planted treatment with diesel. The unplanted control was used to assess contaminant biodegradation in the absence of plant rhizosphere treatment. All treatments were performed in at least triplicate. The pots were allowed to stabilize for 3 weeks before planting.

Planting and Cultivation

Approximately ten barnyard grass seeds were sowed in each planted pot. The plants were grown in a growth chamber for 8 weeks at a fixed temperature (25°C) and humidity (60%). The periods of day and night were 16 h and 8 h, respectively. To prevent water or Hoagland's nutrient solution passing through the bottom of pots, 30 mL of it was provided alternately three times a week. Once the barnyard grass seedlings reached around 4 cm in height, all but three randomly selected seedlings were removed.

Sampling

The rhizosphere soil was defined in this study

as the soil particles that closely adhere to the root surface, and was obtained by gently shaking and rubbing the roots by hand. There were six types of samples for soil analysis in addition to the original soil (OS): (1) planted and diesel-contaminated rhizosphere soil (PDR), (2) planted and diesel-contaminated bulk soil (PDB), (3) planted and uncontaminated rhizosphere soil (PUR), (4) planted and uncontaminated bulk soil (PUB), (5) unplanted and diesel-contaminated bulk soil (UDB), and (6) unplanted and uncontaminated bulk soil (UUB). All rhizosphere soils and planted and unplanted bulk soils were refrigerated until they were used for bacterial enumeration, microbial activity and diesel concentration assessments (within 1 week). To determine the dry weight, 1 g of each sample was placed in an oven at 110°C for 24 hours.

Plate count Method

The plate count method was used for the enumeration of bacteria in soil samples. Although this technique enumerates only culturable species, many researchers favor its use because it is simple, cost-effective, and adequate for the relative comparison of total microbial activity or population density. Of each sample, 1 g was added to 9 mL of sterilized water and mixed vigorously with a vortex mixer. After settling, 1 mL of supernatant from the mixture was transferred to another test tube containing 9 mL of sterilized water to achieve a dilution of 10^{-2}. Other samples were treated in the same manner. A serial dilution technique subsequently yielded five additional test tubes with dilutions from 10^{-3} to 10^{-7}, each with three replicate samples. For dilutions of 10^{-5} - 10^{-7}, 0.1 mL was spread on Tryptic soy agar plates and incubated at room temperature (25°C) for 10 days. All colonies were counted and an average was calculated for use in analyses.

Most Probable Number (MPN) Method

To compare the numbers of diesel-degrading microorganisms, microbial counts were performed by the MPN method using a diesel (1%, v/v)-mixed BH medium containing 0.409 g $MgSO_4 \cdot 7H_2O$, 0.0265 g $CaCl_2 \cdot 2H_2O$, 1 g KH_2PO_4, 1 g NH_4NO_3, 6 g $Na_2HPO_4 \cdot 12H_2O$, and 0.0833 g $FeCl_3 \cdot 6H_2O$ in 1 L distilled water. Every sample was serially diluted from 10^{-1} to 10^{-5}, with three replicate samples as for the plate count method. The 10^{-3} to 10^{-5} dilutions were then cultured in a shaking incubator for two weeks at room temperature (25°C) until any color appeared as an indicator of growth. The resulting MPN score was translated to cells per g dry soil using a standard statistical algorithm.[12]

Dehydrogenase Activity (DHA)

Dehydrogenase activity was assayed for measuring the microbial activity of soil samples. Dehydrogenase activity is normally evaluated with respect to the original soil (OS) because the experimental conditions vary in each lab. 1 g of each sample was added to a solution of 2 mL 0.5% INT (Indonitrotetrazolium violet) plus 1.5 mL sterilized water, and thoroughly mixed with a vortex mixer. For the control, soil was sterilized by autoclaving. The mixtures were placed in a shaking incubator at 37°C for 24 hours. A 50 mL aliquot of methanol was then added to each tube, followed by thorough mixing. After centrifuging the mixture or a settling period of one hour, the soil suspension was filtered through a Whitman filter paper. The filtrate was diluted with methanol to double its volume, and its absorbance was measured at 490 nm using a spectrometer (Spectronic 20, Milton-Roy Co., USA). The concentrations of formazan for all the samples were determined from a previously prepared INT formazan (INTF) standard curve, and converted to μg/g oven dry soil describing dehydrogenase activity.

Gas Chromatography Analysis

Gas Chromatography (GC) analysis was performed to determine the amount of diesel remaining in the soil. Mixtures of 1.5 g of each sample and 1.5 mL of n-hexane were placed in an ultrasonic bath for 10 min for diesel extraction. After centrifuging for 5 minutes, the

samples were filtered into vials through PTFE syringe filters (0.45 μm) for GC analysis. Using an HP-5 capillary column (30 m length, 0.25 mm internal diameter and 0.25 μm film thickness), the total petroleum hydrocarbon (TPH) concentrations in the extracts were measured with a gas chromatograph (HP 5890 II plus, Hewlett-Packard, Florida, USA) equipped with a flame ionization detector,. The oven temperature was set at 45°C for the first 1 min and increased by 5°C per min to a temperature of 100°C, and then increased by 8°C per min to a final temperature of 320°C at which it was held for 5 min. The carrier gas (N_2) flow rate was set at 1.5 mL/min, and the temperatures of the injector and detector were 280 and 250°C, respectively. The sample volume injected was 1 μL. All measurements were conducted in triplicate.

Microbial Community Structure Analysis

16S rDNA polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were employed to analyze the bacteria community structure in the consortium of biofilter. The genomic DNA was extracted from about 0.5 g soil sample using BIO101 FastDNA SPIN KIT for soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer's manual. PCR was used to amplify a 177 base pair portion. Bacterial primers 341FGC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GCC-TAC-GGG-AGG-CAG-CAG-3') and 518R (5'-ATT-ACC-GCG-GCT-GCT-GG-3') were used. All reactions were carried out in 25 μL volumes containing 20 pmol of each primer, 10 mM of each deoxynucleoside triphosphate mixture, 12.5 mg/mL of bovine serum albumin, 2.5 μL of 10× PCR buffer (20 mM Tris-HCl, 100 mM KCl, pH 8.0), 2 μl of 25 mM $MgCl_2$, and 0.5 U of Taq DNA polymerase (Promega, WI, USA) made up to 25 μL with sterile water. 1 μL of the DNA extraction was used as template DNA. PCR was performed with the following regime: 93°C (2 min, denaturing), 35 cycles of 92°C (1 min), 55°C (1 min), 68°C (45 s), followed by 72°C (2 min, extension).[13]

DGGE was performed with a 16 × 16 cm 8% (w/v) polyacrylamide gel using a Dcode™-System (Bio-Rad, Hercules, CA, USA) maintained in 7 L of 0.5× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA, pH 7.8). Gradient gels were prepared with 40% and 60% denaturant (100% denaturant contains 7 M urea and 40% (v/v) formamide). Gels were run at 60°C with 50 V for 11.5 h. After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with a Mupid-21 (Cosmo Bio Co., Tokyo, Japan).[13] DGGE images were assessed for microbial community structure comparisons with GelCompar II software (version 3.5, Applied Maths, Belgium), using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and a Jaccard coefficient based on band position.

RESULTS

Removal of Diesel in Rhizosphere and Bulk Soil

To assess the effect of the rhizosphere treatment, the concentration of diesel of each soil sample was determined (Fig. 1). As expected, the concentration of TPH (a measure of the diesel concentration) was lowest in the rhizosphere (PDR) soil (0 mg/kg), showing 100% removal from the initial concentration (6,000 mg/kg) after 11 weeks (3 weeks stabilization plus 8 weeks growth). In contrast, the concentrations of TPH in the two bulk soils (PDB and UDB) were 1,411 and 2,253 mg/kg, respectively, showing 76% and 62% removal from the initial concentrations. The concentration of the control (CON: no microbial activity) was 4,088 mg/kg, which equated to the rhizosphere effect showing at least 1.5~2.2 times faster diesel elimination than the bulk soils (planted or unplanted) after 2 months.

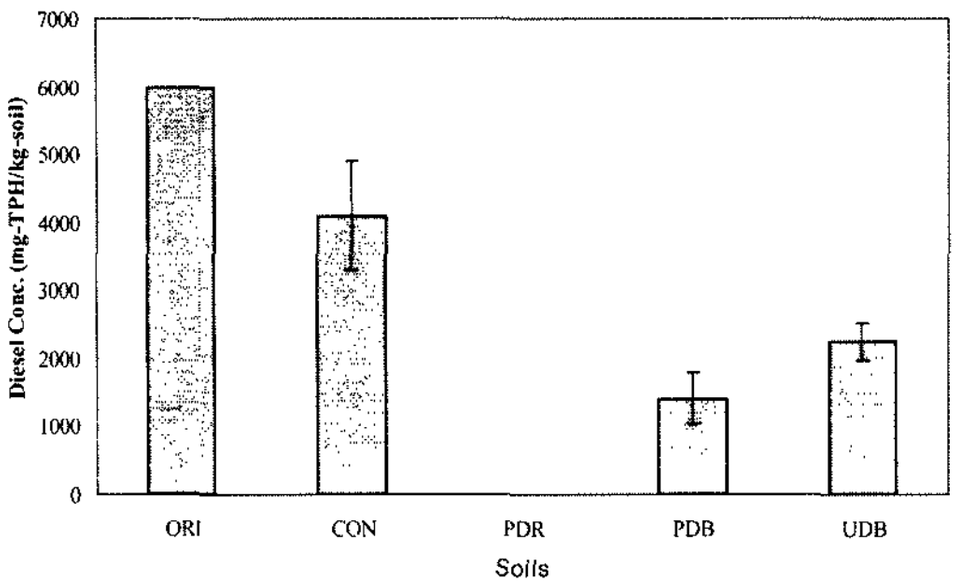

Figure 1. The concentration of total petroleum hydrocarbon (TPH) (n = 3, ± 1 S.E.). Abbreviations: ORI (originally spiked soil), CON (no microbial activity), PDR (planted and diesel-contaminated rhizosphere soil), PDB (planted and diesel- contaminated bulk soil), and UDB (unplanted and diesel-contaminated bulk soil).

Enumeration of Soil Bacteria

The total bacterial numbers in soil samples as estimated from plate counts are shown in Figure 2. The bacterial numbers ranged between approximately 10^7 and 10^9 per g soil, with the highest count being 5.89×10^8 per g soil in the diesel-contaminated rhizosphere soil (PDR) and the lowest count being 3.21×10^7 per g soil in the original soil (OS). Other values were 5.12×10^8, 3.62×10^8, 2.13×10^8, 1.85×10^8 and 1.04×10^8 per g soil for the PUR, UUB, UDB, PUB and PDB treatments, respectively.

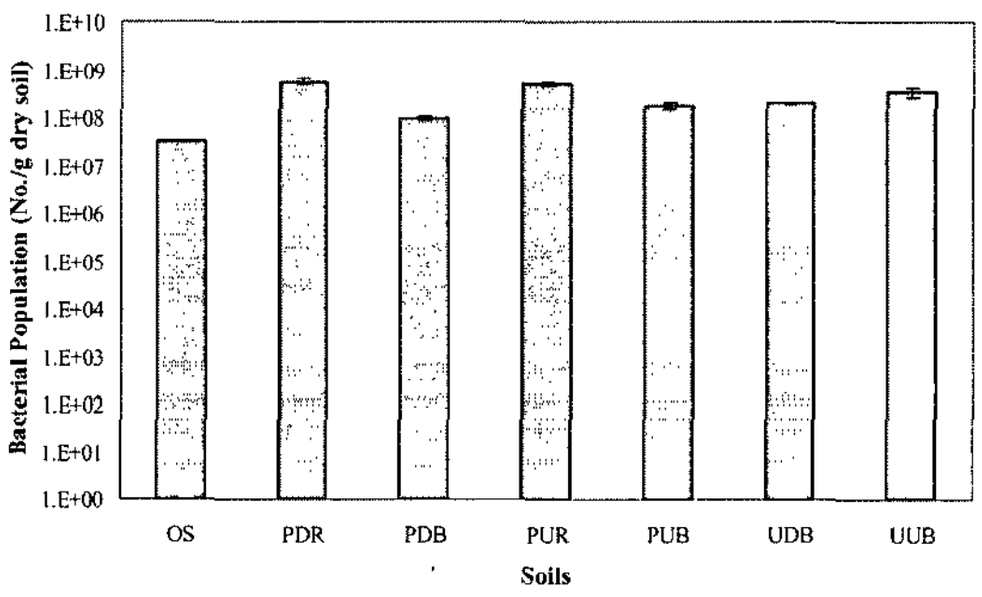

Figure 2. The number of soil bacteria (n = 3, ± 1 S.E.). Additional abbreviations (see Fig 1): OS (original soil), PUR (planted and uncontaminated rhizosphere soil), PUB (planted and uncontaminated bulk soil), and UUB (unplanted and uncontaminated bulk soil).

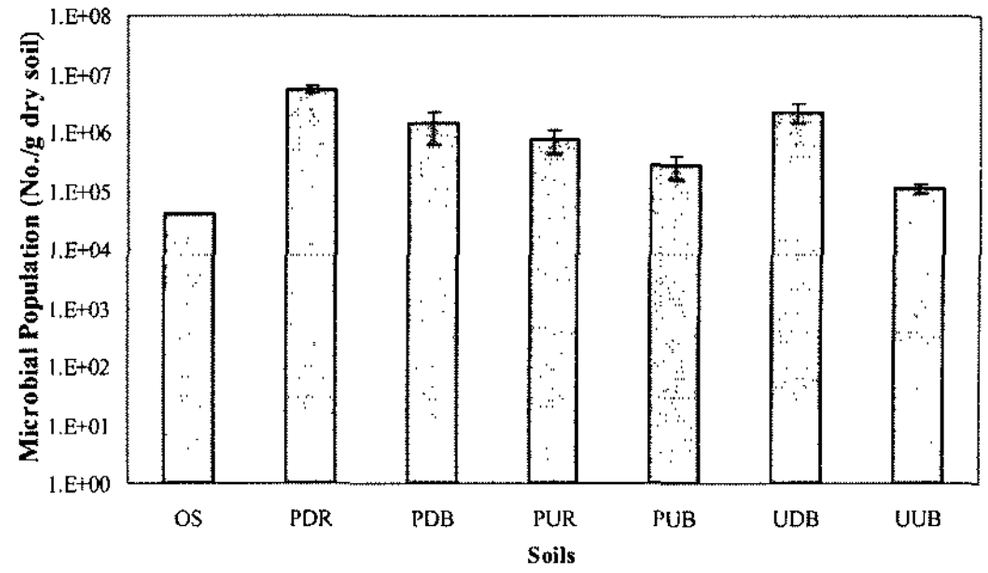

Figure 3. The number of hydrocarbon-degraders (n = 3, ± 1 S.E.). Abbreviations: see Figs. 1 and 2.

As for the MDN method, this technique also provides only the number of culturable microbes. The numbers of microbes in samples ranged from approximately 10^3-10^6, which was around 10^3-10^4 times lower than the results of plate counts (Fig. 3). The largest number of microbes was found in the contaminated rhizosphere soil (PDR) with 5.65×10^6 per g dry soil, followed by the contaminated bulk soil (UDB) with 2.24×10^6 per g dry soil. The population sizes for other soil samples were 1.40×10^6, 7.40×10^5, 2.79×10^5, 1.11×10^5 and 4.13×10^4 per g dry soil in the PDB, PUR, PUB, UUB and OS treatments, respectively. In brief, the results showed that a larger number of diesel- degraders existed in diesel-contaminated rhizosphere and bulk soils than in uncontaminated soils.

Microbial Activity

A measurement of dehydrogenase activity reflects the metabolic level of soil microorganisms during a defined period, and may therefore be related to the elimination of organic pollutants that can be used as carbon and energy sources. Although this technique is not directly associated with the number of soil microbes, an estimate of all the active microorganisms, including uncultivable ones, can be obtained. Dehydrogenase activities for soil samples are shown in Figure 4. The highest value was 202.2 μg/g in contaminated rhizosphere soil (PDR), which was 3.46 times higher than the OS (58.5 μg/g). The second highest value was 170.9 μg/g in uncon--taminated rhizosphere soil (PUR), which was 2.92

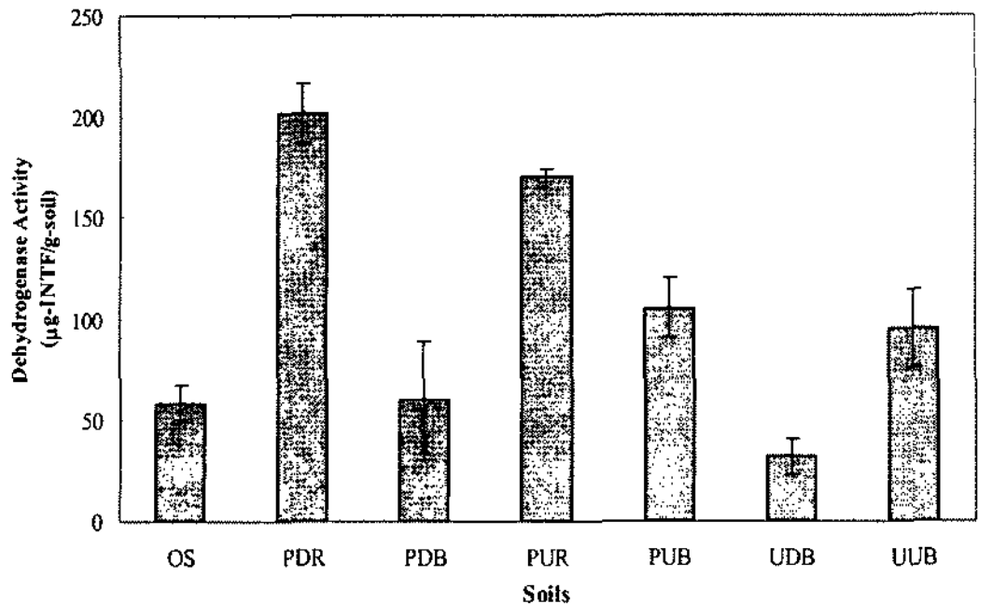

Figure 4. Hydrogenase activity of soil microorganisms (n = 3, ± 1 S.E.). Abbreviations: INTF (Indonitrotetrazolium Formazan) and others (see Figs. 1 and 2.).

times greater than the OS value. Unexpectedly, the activity within diesel-contaminated bulk soils (PDB and PUB) was lower in planted soil than in unplanted soil. In a comparison between the two unplanted bulk soils (UDB and UUB), the diesel-contaminated soil was less active than the uncontaminated soil. Microbial activity was therefore increased by the presence of a rhizosphere, but was decreased by diesel contamination.

Analysis of Bacterial Community Structures

DGGE profiles differed among the soil samples (Fig. 5). The numbers of distinct DNA bands ranged from 11 in unplanted diesel-contaminated bulk soil (UDB) to 22 in diesel-contaminated rhizosphere soil (PDR) (Table 2). If the number of DNA bands is a reliable index of bacterial diversity, these results suggest that diesel contamination considerably decreased the number of dominant bacterial species in unplanted pots only. As shown in Table 3, the largest difference (77.42% dissimilarity) in banding patterns was between original soil (OS) and the unplanted uncontaminated bulk soil (UUB), while the greatest similarity (81.82% similarity) was between the planted contaminated rhizosphere soil (PDR) and the planted contaminated bulk soil (PDB). Cluster analysis showed that the banding patterns fell broadly into two groups: group 1 included PUR, PUB and UUB treatments, and group 2 included the remainder (Fig. 5). With respect to the control (UUB), the

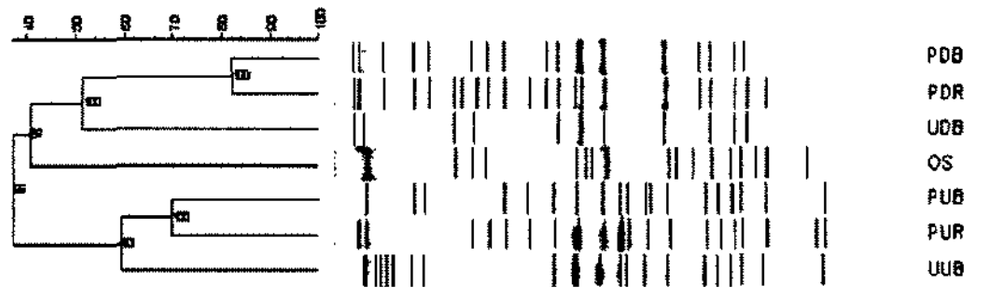

Figure 5. Genetic similarity of microbial community profiles based on DGGE band patterns (band based: Jaccard; dendrogram type: UPGMA). Note: metric scale indicates % similarity and numbers at branches describe cophenetic correlations.

Table 2. The number of DGGE bands, implying bacterial diversity

Soil Samples	Number of DGGE bands
OS	17
PDR	22
PDB	18
PUR	20
PUB	19
UDB	11
UUB	21

greatest shift in bacterial community structure occurred in UDB with 23.00% similarity. Other similarity values were 34.38% in PDR, 44.44% in PDB, 51.85% in PUR and 66.67% in PUB. This result strongly indicates that diesel contamination influenced bacterial community structure in soil more than the rhizosphere treatment.

DISCUSSION

We conducted a microbial characterization of the rhizosphere of barnyard grass in a diesel-contaminated soil. We assessed bacterial population density and community structure using several numerical methods and a DNA fingerprinting method. The primary objective was to determine how the rhizosphere exerts a positive effect on the number, activity and diversity of indigenous bacterial populations in contaminated and uncontaminated rhizosphere and bulk soils. In agreement with previous studies,[14-17] this study revealed that greater degradation occurred in rhizosphere than in bulk soils (planted and unplanted) (Fig. 1). Greater degradation in rhizosphere soil corresponded to higher population

densities of general soil bacteria (Fig. 2) and diesel-degraders (Fig. 3) than in other soil treatments. Although the two numerical methods used in this study (plate count and MPN) allow only culturable microbes to be evaluated, we suggest they may be sufficient for determining the relative population profiles for all soil microbes. Results from the two enumeration techniques suggest that the culturable general soil bacteria and the diesel-degraders increased similarly by 2.77 and 2.52 times, respectively, based on the comparison between PDR and UDB. The rhizosphere effect thus appears to have equally influenced the general bacteria and the hydrocarbon degraders. In contrast, Kirk et al.[18] found that there was five times more culturable heterotrophic bacteria and nine times more petroleum degrading bacteria in rhizosphere soil than in bulk soil. However, these workers obtained MPN results indicating a relatively large number of aerobic, heterotrophic bacteria capable of utilizing F2 diesel (10^9-10^{10} per g dry soil) in comparison to our study (10^3-10^6 per g dry soil). This difference may be due to the much higher concentration of petroleum oil (31,000 ppm compared to 6000 ppm in this study) or to the soil history, as the soil in our study had no previous history of hydrocarbon contamination. Evans et al.[19] obtained a similar range in density of hydrocarbon degraders (10^2-10^5 per g dry soil) as in our study.

We could not determine the absolute size of bacterial populations, although measurement of dehydrogenase activity is expected to reflect real live populations of both cultivable and uncultivable soil microbes. Using this measure, the rhizosphere effect increased microbial activity (between PDR and PDB, and between PUR and PUB), but the addition of diesel (between PDB and PUB, and between UDB and UUB) was inhibitory (Fig. 4). The rhizosphere effect (between PDR and PUR) was more influential than the diesel effect (between UDB and UUB). In addition, the rhizosphere effect increased hydrocarbon-degraders relative to general bacteria in comparisons between PDR and PUR treatments (Figs. 2, 3 and 4). This may be because plants could selectively increase a degrading microorganism population in the rhizosphere by altering exudation,[18,20] or there may be some other factor(s) that stimulate the growth of hydrocarbon-degraders only.

In a comparison between bulk soils, the addition of diesel significantly decreased general soil bacteria (Fig. 2) and bacterial activity (Fig. 4) (between PDB and PUB, and between UDB and UUB). This result implies a strong rhizosphere effect, although there were some significant diesel effects for hydrocarbon-degraders (Fig. 3). Overall, the rhizosphere treatment increased populations of general bacteria and hydrocarbon-degrading microbes in soil, but the diesel treatment increased only the hydrocarbon degraders.

DGGE banding profiles (Fig. 5) showed that the rhizosphere effect did not alter diversity of dominant bacteria. In contrast, the presence of diesel greatly decreased diversity, with the number of distinct bands falling from 21 (UUB) to 11 (UDB) (Table 2). These results corresponded well to the MPN results, but not to the plate count and dehydrogenase activity results, although they have different aspects between diversity and population, indicating the number of dominant bacterial species and the total number of bacteria, respectively. The rhizosphere treatment may therefore stimulate the growth of the same species as in the control regardless of the presence or absence of diesel. On the other hand, only the presence of diesel appears to inhibit the diversity of non-hydrocarbon degraders, as indicated by the drop in number of DGGE bands (Table 2). Such an effect may occur because hydrophobic contaminants, such as diesel, can change water/soil interactions affecting oxygen transfer, available water uptake, and nutrient mobility.[18]

The difference in banding patterns between PDR and PUR (44.83% similarity) occurred as some bands disappeared in PDR and appeared in PUR or vice versa. The former case might be due to the toxicity of diesel and the latter case might be due to some bacterial species utilizing

Table 3. Similarity analysis (%) of DGGE banding patterns

	OS	PDR	PDB	PUR	PUB	UDB	UUB
OS	100.00						
PDR	34.48	100.00					
PDB	40.00	81.82	100.00				
PUR	37.04	44.83	46.15	100.00			
PUB	24.14	41.38	42.31	69.56	100.00		
UDB	47.37	50.00	52.63	47.62	36.36	100.00	
UUB	22.58	34.38	44.44	51.85	66.67	23.00	100.00

diesel as a carbon and energy source or caused by rhizosphere factor, which might stimulate the growth of other species and protect them form the toxicity of diesel. The greatest shift in bacterial community structure from the control (UUB) occurred in UDB (23.00% similarity), followed by PDR (34.38%), PDB (44.44%), PUR (51.85%) and PUB (66.67%). This indicates that diesel contamination most influenced the diversity of soil microbial community, followed by the combination of contamination and rhizosphere, and was least influenced by rhizosphere treatment in isolation. In contrast, the change of banding pattern of UUB from OS may be due to the different physicochemical conditions of the soils, such as temperature, moisture content, or additional nutrition.

CONCLUSIONS

In conclusion, whereas the population of general soil bacteria was increased to a greater extent by the rhizosphere treatment than the diesel treatment, the hydrocarbon-degraders were increased individually and synergistically by both. In addition, only the diesel treatment decreased the diversity of bacterial community structure, and the dominant members were more influenced by the presence of diesel than a rhizosphere.

ACKNOWLEDGEMENTS

This work was supported by a Korea Research Foundation Grant from the Korean Government (HOEHRD, Basic Research Promotion Fund) (KRF-2005-050-D00007).

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