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Original Article

Assessment of the bacterial community of soils contaminated with used lubricating oil by PCR-DGGE

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Abstract

The diversity of indigenous bacteria in three soils contaminated with used lubricating oil (ULO) was determined and compared using molecular analysis of bacteria cultured during the enrichment process. Sequencing analyses demonstrated that the majority of the DGGE bands in enrichment cultures were affiliated with four phyla of the domain, Bacteria: α, β, γ-Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria. Soil C had a higher ULO contamination level than soil A and B, which may explain why enrichment culture C had the greatest diversity of bacteria, but further studies would be needed to determine whether ULO concentration results in higher diversity of ULO-degraders in soils. The diversity of ULO-degraders detected in these three different soils suggests that biostimulation methods for increasing the activity of indigenous micro-organisms may be a viable approach to bioremediation, and that future studies to determine how to increase their activity *in situ* are warranted.

Keywords: enrichment cultures, microbial community, PCR-DGGE, used lubricating oil, soil bacteria

1. Introduction

Petroleum products, such as gasoline, lubricating oil, diesel and kerosene are used daily in various forms at mechanical workshops (Ekhaise and Nkwelle, 2011). The release of these products into the environment is a cause of concern for human and environmental health (Röling, 2002). Used lubricating oil (ULO) generated from mechanical workshops is becoming a major environmental problem in Thailand (Naladta and Milintawisamai, 2011; Pimda and Bunnag, 2012; Saimmai *et al*., 2012). Base fluid, the main fraction of ULO, is a complex mixture of hydrocarbons, linear and branched paraffins, cyclic alkanes and aromatic hydrocarbons (Kim *et al*., 2003). ULO also contains heavy metals (Aucélio *et al*., 2007) and heavy polycyclic aromatic hydrocarbons (PAHs) that contribute to chronic hazards including mutagenicity and carcinogenicity (Boonchan *et al*., 2000; Haritash and Kaushik, 2009).

Bioremediation is a potentially inexpensive technology for reducing hydrocarbon concentrations, and relies on multiple microorganisms to reduce, eliminate or transform hazardous substances into harmless substances through their metabolic processes (Vidali, 2001). Several methods of bioremediation have been developed to accelerate the natural biodegradation rates by supporting an increase in the population of indigenous bacteria in oil-contaminated soil (Berekaa, 2013). Biostimulation techniques can be used to increase the activity of the indigenous bacteria by adjusting

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environmental parameters (Perfumo *et al*., 2007; Kanissery and Sims, 2011; Zawierucha and Malina, 2011). Advancing knowledge and understanding of bacterial community structure in polluted sites is valuable for developing and optimizing bioremediation strategies (Zhang *et al*., 2011).

The primary goal of this study was to characterize and compare the diversity and identity of ULO-degrading bacteria present in three different contaminated soils. Enrichment cultures were used to establish ULO-degrading communities, and their community dynamics and diversity were compared among cultures from three different soils using PCR-DGGE analysis. The study aims to provide new insight into the biodegradation potential and diversity of contaminated soils that may be valuable for future biodegradation and bioremediation efforts.

2. Materials and Methods

2.1 Soil samples

Three ULO contaminated soils were obtained from three different motorcycle mechanical workshops around Prince of Songkla University, Hat Yai, Thailand. The soil samples were collected from different sites to a depth of 15 cm by hand digging. The soil was mixed thoroughly, sieved through screen with 4 mm mesh to remove gravel, stones and plant debris. The soil samples were stored in sterilized containers and placed in dark and dry area at room temperature (29±2°C) to prevent the occurrence of photooxidation reactions (Ramirez *et al*., 2001).

2.2 Enrichment of ULO-degrading cultures

Enrichment cultures of ULO-degrading bacteria were established using mineral salt medium (MSM) medium supplemented with 1% (w/v) ULO provided as the sole carbon source (Saimmai *et al*., 2012). The ULO used in this study consisted of aliphatic hydrocarbon with chain lengths of C_{13} to C_{36} and aromatic hydrocarbon (naphthalene and benzene). The pH of the medium was adjusted to 7.0 before autoclaving. A soil sample (10 g) was added to a 250-ml conical flask containing 90 ml MSM with the addition 1% of ULO. After one week of incubation at room temperature in an orbital shaker at 200 rpm, 5 ml of the culture were transferred to another 250-ml conical flask containing 45 ml MSM with the same amount of ULO for the second enrichment. Three consecutive enrichments were carried out to obtain a ULO-degrading microbial consortium (Yu *et al*., 2005). All treatments were carried out in triplicates. Samples from each individual enrichment culture step were taken on 3, 5, and 7 days of incubation.

2.3 Enumeration of heterotrophic and oil-degrading bacteria

Serial dilutions of bacterial cultures in 0.85% sterile saline solution were performed and dilutions used to inoculate plates for enumeration of total heterotrophic and oil-degrading bacteria using plate counts. Nutrient agar (NA) plates were incubated at room temperature overnight for heterotrophic total viable counts (Adebusoye *et al*., 2006). Appropriate dilutions were plated out onto the MSM agar containing ULO as the sole carbon source, and were incubated 5 to 7 days at room temperature for ULO-degrading bacterial counts (Jain *et al*., 2010). Results were calculated as the means of three determinations.

2.4 Cell harvesting for DNA extraction

For plate washes, all the colonies present on the surface of the plate (NA and MSM agar) were washed with 2 ml of 0.85% sterile saline solution, harvested with a sterile pipette and stored by freezing at -20°C (Ercolini *et al*., 2001). One milliliter of plate wash for each medium or 2 ml of cell culture directly obtained from enrichment cultures were used for the DNA extraction.

2.5 DNA extraction and PCR amplification of the 16S rRNA

In order to characterize the culturable bacteria community from enrichment cultures, DNA was extracted from enrichment cultures and also from agar plate washes (NA and MSM) after bacterial counts using a phenol-chloroform isoamyl alcohol DNA extraction method (Shahriar *et al*., 2011). The DNA extracts were stored at -20°C until used. The DNA extracts were used as templates for a PCR reaction to amplify bacterial 16S rRNA genes. PCR universal primers 341F (5′-CCT ACG GGA GGC AGC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Yamamoto *et al*., 2009) were used to amplify the V3 region of bacterial 16S rRNA connected to GC clamp attached to the forward primer (Muyzer and Smalla, 1998). PCR cycles were as follows: 95°C for 3 min; followed by 30 cycles of 94°C for 0.5 min; 55°C for 1 min and 72°C for 1 min; with a final elongation of 72°C for 10 min (Röling *et al*., 2002).

2.6 DGGE profiling and DNA sequencing

PCR products with equal concentration (40 µg) were loaded onto 1 mm thick vertical gels containing 10% polyacrylamide (w/v) (37.5:1 acrylamide:bisacrylamide). Each gel contained a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of gel to 60% at the bottom (100% denaturant is defined as 7 M urea and 40% (v/v) deionized formamide). The gels were prepared in 1×TAE buffer (40 mM Tris-acetate and 1 mM Na-EDTA, pH 8.0) at 60°C and 120V for 6h using an omniPAGE Maxi Electrophoresis Systems VS20-DGGE (Cleaver Scientific, England) (Röling *et al*., 2002). Gels were stained in 1×TAE buffer containing SYBR gold DNA gel stain and photographed. Prominent bands located in different positions in DGGE gel were cut out and diffuse into the water at 4°C overnight. Ten microliters of the solution was used as the DNA template in a PCR reaction

using the same primer without GC clamp. The reaction was carried out for 30 cycles of 95°C for 0.5 min, 50°C for 1 min and 72°C for 1 min. The PCR products were purified with HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taiwan) then sent for sequencing using the BigDye® Terminator v3.1 cycle sequencing kit (1st BASE, Malaysia). Nucleotide sequences were analyzed and compared with the Ribosomal Database Project (RDP) naïve Bayesian Classifier tool (Wang *et al*., 2007). The RDP classifier tool was used to classify all sequences from the phylum to genus levels. The RDP SeqMatch tool was used to compare reference sequences from individual operational taxonomic units (OTUs) to the RDP database. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AB927720 to AB927997.

2.7 Statistical analyses

The relationship among bacterial community structures at the OTUs level of the different samples was analyzed using detrended correspondence analysis (DCA) with the PAST open source software program, Version 3, August, 2013 (Hammer *et al*., 2001).

3. Results

3.1 Microbiological analyses

The microbial population shifts that occurred during the selective enrichment culture processes were monitored and are reported in Figure 1. All cultures showed substantial quantities of ULO-degraders from the beginning, and numbers increased rapidly during the first three days of the enrichment period. In enrichment culture A, heterotrophic bacteria and ULO-degrading bacteria ranged from 5.82 to 8.81 Log_{10} CFU/ml and 6.15 to 8.33 Log_{10} CFU/ml, respectively. In enrichment culture B, the heterotrophic bacterial population ranged between 6.62 and 8.85 Log_{10} CFU/ml and the ULO-degrading bacteria ranged between 6.56 and 7.85 Log_{10} CFU/ml. In culture C, the initial count of ranged between 4.92 and 8.86 Log_{10} CFU/ml and 5.93 and 8.49 Log_{10} CFU/ml, respectively.

3.2 Comparison of bacterial communities in ULO enrichment cultures

In order to better understand the microbial consortium present in different soils, PCR-DGGE and sequence analysis of selected bands were used to fingerprint and identify the soil bacteria present in enrichment cultures. Total DNA was extracted from samples from first week to third week of culture. The DGGE profiles of bacterial communities in all enrichment cultures are shown in Figure 2, 3, and 4. The prominent DGGE bands from different positions on the gel were excised,

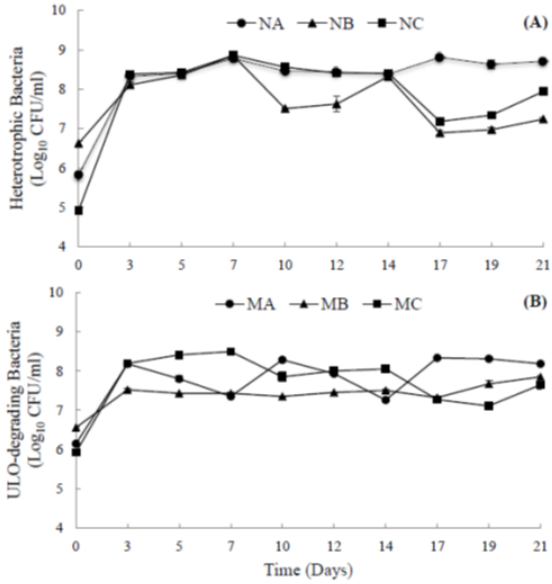

Figure 1. Heterotrophic total viable counts from nutrient agar (A) and ULO-degrading bacteria counts from MSM agar (B) in ULO enrichment cultures of soil A (NA, MA), soil B (NB, MB) and soil C (NC, MC).

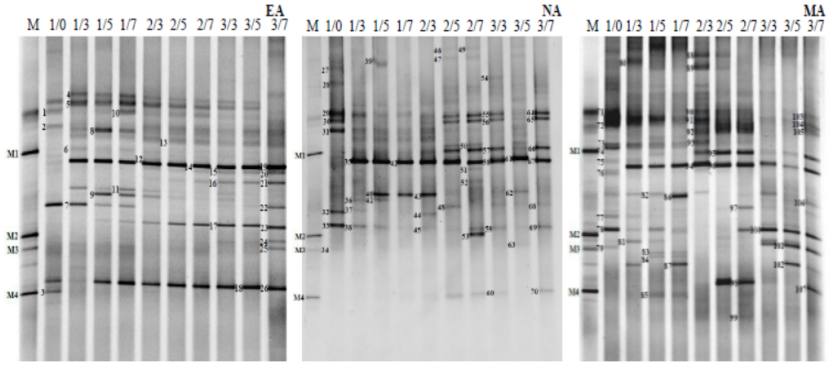

Figure 2. DGGE fingerprints of the bacterial 16S rRNA V3 region amplified from enrichment culture A in each individual enrichment culture step was taken on 3, 5 and 7 days for 3 weeks of incubation. The DNA extracted from enrichment cultures (EA), cells washed from NA (NA) and cells washed from MSM agar (MA)

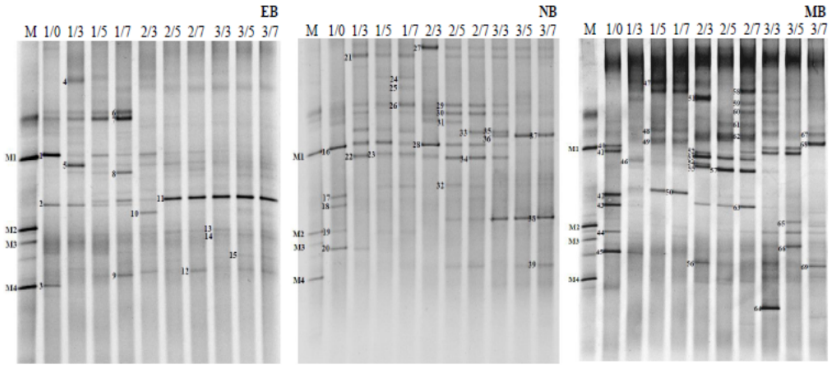

Figure 3. DGGE fingerprints of the bacterial 16S rRNA V3 region amplified from enrichment culture B in each individual enrichment culture step was taken on 3, 5 and 7 days for 3 weeks of incubation. The DNA extracted from enrichment cultures (EB), cells washed from NA (NB) and cells washed from MSM agar (MB)

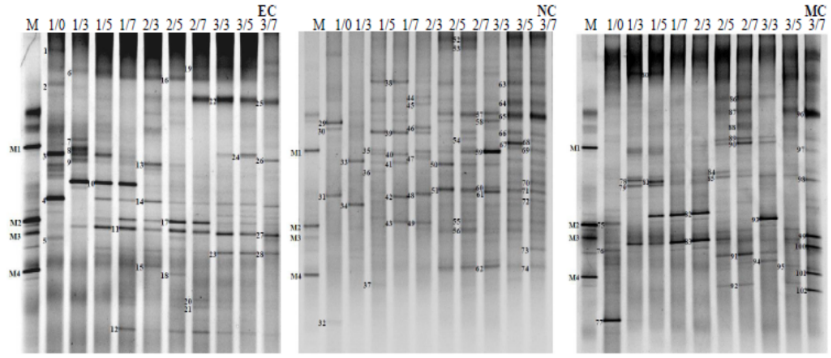

Figure 4. DGGE fingerprints of the bacterial 16S rRNA V3 region amplified from enrichment culture C in each individual enrichment culture step was taken on 3, 5 and 7 days for 3 weeks of incubation. The DNA extracted from enrichment cultures (EC), cells washed from NA (NC) and cells washed from MSM agar (MC)

re-amplified and sequenced. A total of 107 bands in the DGGE profiles were detected in enrichment culture A (Figure 2). Of these 107 bands, 26 bands were detected in enrichment cultures (EC), 44 bands were from NA plate washes (NA) and the remaining 37 bands were from plate washes of MSM agar with ULO as the sole carbon source (MSM). DGGE bands were identified using DNA sequence analysis, which showed that intense bands corresponding to *Citrobacter*, *Pseudomonas*, *Acinetobacter*, *Sphingobacterium*, *Achromobacter*, *Flavobacterium*, *Chryseobacterium*, *Ochrobactrum*, uncultured *Flavobacterium* and uncultured *Brachymonas* were present during three weeks of enrichment cultivation. *Enterobacter*, *Pentoea* and uncultured Betaproteobacterium were only observed in the first week, bands identified as *Sphingobium*, *Bordetella*, uncultured Alphaproteobacterium and uncultured bacterium were detected in the third week.

In enrichment culture B, 9 different bacterial genera dominated at all time points examined (Figure 3): *Pseudomonas*, *Acinetobacter*, *Brachymonas*, *Pseudoalteromonas*, *Rahnella*, *Achromobacter*, *Stenotrophomonas*, *Sphingobacterium* and uncultured bacterium. *Sphingobium*, *Microbulbifer*, *Kosakonia*, uncultured Alphaproteobacterium and uncultured Betaproteobacteria species were found only within the first week. *Rhodanobacter* was found only in the second week and *Moraxella* was present only in the third week. The bacterial community in enrichment culture C was characterized by the stable presence at all time points of *Sphingobacterium*, *Pseudomonas*, *Pseudoxanthomonas*, *Pantoea*, *Alcaligenes*, *Achromobacter*, *Clostridium*, *Enterobacter*, uncultured bacterium and uncultured Gammaproteobacterium (Figure 4). Other bacterial groups, including *Citrobacter*, *Acinetobacter*, *Brevibacterium*, *Leucobacter*, *Lactobacillus* and *Bacillus* were also identified at the beginning of the enrichment cultivation. Members of the genera *Kluyvera*, *Lysobacter* and *Chryseobacterium* were found only in second time point. *Xanthomonas* and *Thiorhodospira* were found only in third sampling time point.

The bacterial community structure of enrichment cultures in three different soils were compared in Figure 5, according to the ratio percent of different groups of bacteria in enrichment cultures and plate washes of each enrichment cultures. The sequencing analysis divided the 278 bacterial sequences into four main groups: Proteobacteria (total 219 sequences: 180 of γ-classes, 12 of α-classes and 26 of β-classes), Bacteroidetes (total 46 sequences: 14 of Flavobacteria classes and 33 of Sphingobacteria classes), Firmicutes (total

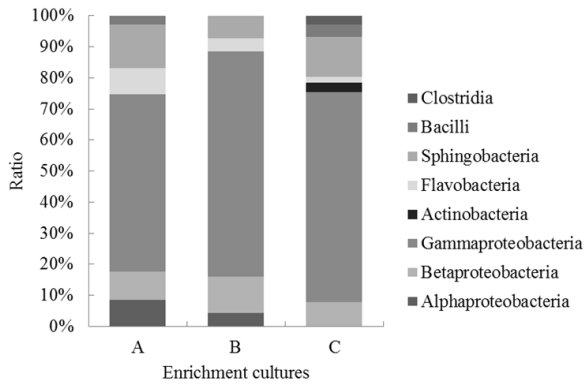

Figure 5. Comparison of predominant bacterial community among soils based on 16S rRNA-V3

10 sequences: 7 of Bacilli classes and 3 of Clostridia classes) and Actinobacteria (3 sequences of Actinobacteria classes). Proteobacteria and Bacteroidetes were the phyla most commonly found in the three enrichment cultures. It is clear that Gammaproteobacteria, Betaproteobacteria and Sphingobacteria are the most commonly detected culturable bacteria in the three enrichment cultures. It should be noted that Actinobacteria and Clostridia were only found in enrichment cultures C.

3.3 Comparison of enrichment cultures from different soils

The relative abundances of OTUs that became abundant in enrichment cultures was determined from DGGE data and then was compared among samples and treatments using detrended correspondence analysis (DCA). Bacterial samples from enrichment cultures directly (EA, EB, EC), cells washed from NA (NA, NB, NC) and cells washed from MSM agar (MA, MB, MC) were used for analysis program. The bacterial communities originating from the same soil sample generally grouped together (Figure 6). The statistical analysis by DCA showed that the different methods for detecting the bacterial community in each enrichment culture produced somewhat similar results, regardless of whether they were directly subjected to DGGE or first cultivated on agar media then subjected to DGGE of plate washes.

4. Discussion

In this study, enrichment culture approaches and molecular analysis were used to investigate the diversity of bacteria potentially involved in ULO biodegradation in contaminated soils. Each soil generated an enrichment culture with a distinct community structure, but all cultures contained members of the genera *Pseudomonas*, *Acinetobacter*, *Achromobacter* and *Sphingobacterium*. *Pseudomonas* species, in particular, have been very well characterized as having a wide range of hydrocarbon-degrading capabilities (Okoro *et al*., 2012). Silva *et al*. (2006) reported *Pseudomonas aeruginosa* strain AT18 able to grow on crude oil, lubricant oil, naphthalene, toluene and kerosene in hydrocarbons contaminated soils and degraded the mixture Mesa 30/Puerto Escondido (8:2) crude oil up to 81% in soil. Sihag *et al*. (2013) reported strain *Acinetobacter calcoaceticus* BD4 isolated from coastal area Mumbai was able to degrade 4T engine oil 84-86% in biometric flask containing minimal salt medium. Adelowo *et al*. (2006) reported that the genus *Achromobacter aerogenes* utilized up to 80.0% of used engine oil in minimal salts medium (MSM) supplemented with used engine oil with a degradation rate of 0.08 ml/day. Genus *Achromobacter* is also able to utilize naphthalene and phenanthrene as the sole carbon and energy source (Hanh *et al*., 2009). Saimmai *et al*. (2012) reported that *Sphingobacterium multivorum* SC9-2 showed the highest degradation of ULO (55.4%) in MSM supplemented with 1% of ULO and 1% of molasses in seven days of cultivation, and also degraded aromatics, resins as well as asphaltenes in ULO up to 96.55, 97.74, and 53.37%, respectively within five days of cultivation.

Phylogenetic analyses of 45 distinct groups by DGGE analysis revealed that the predominant bacterial taxa in this enrichment system were affiliated with four phyla: α, β, γ-Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria. Most corresponded to Proteobacteria in all soils and only a small fraction corresponded to Actinobacteria in soil C. Most of the predominant bacteria in all soils of this study are from the phylum Gammaproteobacteria. Gammaproteobacteria have previously been found to be the most dominant bacteria in soil, water and sediment contaminated with hydrocarbons in several tropical studies (Ghosh *et al*., 2010; Chikere and Ekwuabu, 2014).

It was not expected that at time zero, the heterotrophic bacteria (NA) and ULO-degrading bacteria (MSM) showed similar counts in enrichment culture B, and also that the ULO-degrading bacteria exhibited higher numbers than heterotrophic bacteria in enrichment cultures A and C (Figure 1). The ULO-degrading bacterial counts were also found to

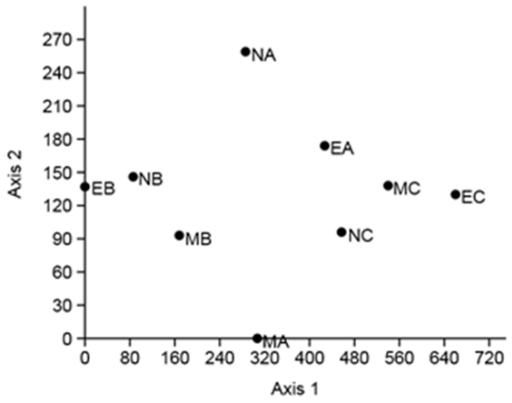

Figure 6. Detrended correspondence analysis (DCA) of DGGE of 16S rRNA data from three enrichment cultures (A, B, C) of ULO contaminated soils for three weeks. The DGGE data from enrichment cultures (EA, EB, EC), cells washed from NA (NA, NB, NC) and cells washed from MSM agar (MA, MB, MC)

be higher than heterotrophic bacterial counts in enrichment culture B at day 17 and 19. Usually there are many more heterotrophs than ULO-degraders in environmental samples (Lawson *et al*., 2012). This unusual pattern may be due to differential culture biases, or ULO-degraders may truly be more numerous than total heterotrophs. The latter has been reported in previous studies, which suggested that the toxicity of high levels of petroleum hydrocarbon contamination may reduce numbers of total heterotrophs in soil (Akoachere *et al*., 2008). At the same time, high levels of hydrocarbons can create selective conditions for ULO-degraders, increasing their numbers beyond those of total heterotrophs (Umanu *et al*., 2013).

Each of the three different ULO-contamianted soils had different soil properties. The organic matter content of soil A, B and C was 6%, 8% and 12%, respectively and moisture content were 4.6%, 11.9% and 4.8%, respectively, based on our previous studies, Meeboon *et al*. (2016). The bacterial communities in each soil may differ because of the different factors of soil such as oil type, age of oil, soil type, nutrients, organic matter and CEC. Soil A was sandy, and sandy soils tend to have a lower nutrient retention, organic matter and CEC. That might be explained by the fact that the ULO contamination in soil A was very old (more than ten years), and the latent population ULO-degraders increased rapidly when enriched with ULO as a sole carbon and energy source. Soil C showed high bacterial diversity based on DGGE in the original soil and during enrichment steps. The initially high diversity may have been due to the high nutrient retention, organic matter and cation exchange capacity (CEC) of this soil. The components of ULO in soil C showed higher amounts and variety of aliphatic hydrocarbon than another soils, indicating that the contamination was less weathered than the other soils. Soil B showed lower bacterial diversity than another soils, which may be due to the very high moisture content. High soil moisture may limit populations of ULO-degraders because of potentially low oxygen content in this soil. Additional studies would be needed to track the populations of ULO-degraders over time, and also to relate them to soil properties in a larger series of samples before firm conclusions can be drawn about the factors driving the differences in the microbial communities of these soils.

In this study we investigated ULO-degrading bacteria in contaminated soils in order to investigate the biodegradation and bioremediation potential of the soils. Many different taxa of putative ULO-degraders bacteria were present in the three contaminated soils investigated. Although they were not individually tested for their ULO-degrading ability, the organisms grew in enrichment cultures containing ULO, and many were members of taxa known to include hydrocarbon-degraders. These strains should be subjected to further testing to confirm their biodegradation abilities. Based on the evidence that ULO-degraders are present in soils, this study suggests that biostimulation methods, such as nutrient and biosurfactant additions, may accelerate the biodegradation rates in these soils.

5. Conclusions

The community structure of ULO-degrading bacteria was found to differ among in three different ULO-contaminated soils with different soil properties and age of contamination. Factors such as the age of oil, soil type, nutrients, moisture, pH and CEC may be important in determining the community structure, and warrant further investigation. The predominant ULO-degrading bacteria in all enrichment cultures were *Pseudomonas*, *Acinetobacter*, *Achromobacter*, and *Sphingobacterium*. The bacteria enriched from soil C, which had the most weathered ULO contamination and high soil nutrient, were the most diverse compare with other two soil enrichment cultures. The knowledge from this study revealed that biodegradation potential exists in these contaminated soils, and could be applied to find ways of enhancing biodegradation of ULO-contaminated soils. Additional research is underway to study ULO-degrader community structure and function in greater depth.

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