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Bacterial community dynamics during *in-situ* bioremediation of petroleum waste sludge in landfarming sites

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Abstract

In-situ bioremediation of petroleum waste sludge in landfarming sites of Motor Oil Hellas (petroleum refinery) was studied by monitoring the changes of the petroleum composition of the waste sludge, as well as the changes in the structure of the microbial community, for a time period of 14 months. The analyses indicated an enhanced degradation of the petroleum hydrocarbons in the landfarming areas. A depletion of n-alkanes of approximately 75–100% was obtained. Marked changes of the microbial communities of the landfarms occurred concomitantly with the degradation of the petroleum hydrocarbons. The results obtained from terminal restriction fragment length polymorphism (T-RFLP) analysis of polymerase chain reaction (PCR) amplified 16S rRNA genes demonstrated that bacteria originating from the refinery waste sludge and newly selected bacteria dominated the soil bacterial community during the period of the highest degradation activity. However, the diversity of the microbial community was decreased with increased degradation of the petroleum hydrocarbons contained in the landfarms. T-RFLP fingerprints of bacteria of the genera *Enterobacter* and *Ochrobactrum* were detected in the landfarmed soil over the entire treatment period of 14 months. In contrast, the genus *Alcaligenes* appeared in significant numbers only within the 10 month old landfarmed soil. Genes encoding catechol 2,3-dioxygenase (subfamily I.2.A) were detected only in DNA of the untreated refinery waste sludge. However, none of the genes known to encode the enzymes alkane hydroxylase *AlkB,* catechol 2,3-dioxygenase (subfamily I.2.A) and naphthalene dioxygenase *nahAc* could be detected in DNA of the landfarmed soils.

Abbreviations: OTUs – Operational Taxonomic Units; PCR – Polymerase chain reaction; T-RFLP – Terminal restriction fragment length polymorphism fingerprinting

Introduction

The current knowledge about the degradation capabilities of pollutants by bacteria is limited to those demonstrated by cultivated microorganisms that are only a minor fraction of bacterial diversity and accounts for less than 1% of the naturally occurring diversity (Amann et al. 1995; Brock

1987). In order to gain additional information about the bacterial communities that may play important roles in the *in-situ* degradation of petroleum hydrocarbons in contaminated soils, we have applied a cultivation-independent strategy for examining the community diversity and its temporal changes in soils highly polluted with refinery waste sludge. Cultivation-independent methods, based mainly on molecular retrieval of 16S rRNA (rDNA) sequences, have become the most important tools for investigating of microbial diversity of environmental samples (Head et al. 1998; Nogales et al. 2001; Pace 1997). Nucleic acid fingerprinting analysis of the total bacterial diversity, by 16S rDNA T-RFLP fingerprinting has been performed in this study to monitor resulted changes in the community structure in on-site field experiments (Liu et al. 1997; Osborn et al. 2000). The advantage of 16S rDNA T-RFLP is that it is a relatively rapid profiling method and is probably more reproducible than most other fingerprinting methods. When the T-RFLP analyses are carried out using PCR-amplified ribosomal DNA with frequent cutting (i.e., four base cutting) restriction enzymes, the level of resolution is at, approximately, the genus level (i.e., sequence types of organisms from different genera are able to be differentiated) with confidence. Furthermore, rapid profiling procedures, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993), temperature gradient gel electrophoresis (TGGE) (Felske et al. 1997; Heuer et al. 1997) and single-strand conformation polymorphism (SSCP) analysis (Lee et al. 1996; Schwieger & Tebbe 1998), all allow the analysis of multiple samples, but the community fingerprints they generate do not translate into taxonomic information.

Additionally, polymerase chain reaction (PCR) with highly degenerate primers, based on conserved sequence elements, was applied in the present study as a method for the *in-situ* detection of degradation genes in the soil samples of the landfarms (Mesarch et al. 2000).

In previous studies, bacterial strains isolated from a 4-month old landfarm and identified as *Alcaligenes* sp. EK5, *Enterobacter* spp. EK3.1 and EK4 and *Ochrobactrum* sp. EK6, were observed to degrade several classes of substrates of petroleum hydrocarbons, such as monoaromatic, polycyclic aromatic hydrocarbons (PAHs), n- and branched alkanes (Katsivela et al. 2003a, b). In parallel, the changes in the structure of the microbial community were assessed in parallel with the changes of the contaminant composition of the polluted soil for a time period of 4 months, which was the period of the highest degradation activity (Katsivela et al. 2003a; 2004). Marked changes in the microbial community of non-polluted soil were observed after its pollution with petroleum waste sludge. Operational Taxonomic Units (OTUs), based upon 16S rDNA sequence types and originating from the waste sludge, dominated the bacterial community of the constructed landfarm within the first 4 months treatment, suggesting their metabolic superiority with respect to the degradation of petroleum hydrocarbons. On the other hand, a number of OTUs that were not present in the control soil neither in the waste sludge were obtained after 4 months of landfarm treatment, reflecting the enrichment of bacteria with degradation capabilities under the selective pressure of petroleum pollution. The relative abundance of the soil bacteria that previously dominated the non-polluted control soil after 4 months landfarming treatment was quite low (Katsivela et al. 2004).

In the present study, the changes in the structure of the microbial community have been compared with the changes of the contaminant composition of the polluted soil *in-situ* and on-site for a period of 14 months, in which the diversity of the microbial community was decreased with increased degradation of the petroleum hydrocarbons contained in the landfarms. Additionally, the abundances of the indigenous genera *Alcaligenes, Enterobacter* and *Ochrobactrum* were also monitored in the landfarmed soil using T-RFLP analysis of PCR-amplfied 16S rDNA during the whole treatment period.

In parallel, PCR with highly degenerate primers was used for the amplification of degradation genes in the extracted genomic DNA of the soil samples as well as in the isolated bacteria. In particular, the PCR amplification targeted conserved sequence elements of the degradation enzymes alkane hydroxylase B, catechol 2,3-dioxygenase (subfamily I.2.A) and naphthalene dioxygenase (Hamamura et al. 2001; Hedlund et al. 1999; Mesarch et al. 2000; Simon et al. 1993; Smits et al. 1999).

Materials and methods

Sampling

Refinery sludge, before and after landfarming treatment for 14 months, was used in this study. The samples were collected from a landfarming site of the petroleum refinery Motor Oil Hellas (Korinth Refineries, Greece), specifically designed for this project. Soil samples were collected from the surface (0–15 cm) from: (a) an untreated, non-polluted control soil; (b) a newly produced refinery waste sludge; and (c) a landfarmed site treated with refinery waste sludge, at the beginning of the experiment (October 2000) and after 4 (February 2001), 10 (August 2001) and 14 months treatment (December 2001). Soil samples from the non-polluted control soil were collected in every sampling time and compared with the samples of the landfarmed soil. Duplicates of all samples were analysed directly after collection.

Hydrocarbon extraction from soil samples

The hydrocarbons present in the fresh refinery waste sludge, as well as in the landfarming soil samples, were separated by extraction, as described previously (Katsivela et al. 2004). Specifically, 2 g of soil was extracted with 140 ml of n-hexane in a Soxhlet apparatus for 2 h. The organic phase was concentrated by evaporation of the organic solvent after drying over Na_2SO_4. The hydrocarbon fractions were analysed, without further purification, by gas chromatography coupled with mass spectrometry (GC–MS) after drying and concentration.

Analysis by GC–MS

GC–MS analyses of the petroleum hydrocarbons were carried out on the collected samples, using a GC 17A gas chromatograph (Shimadzu, Kyoto, Japan), equipped with an XTI 5 column (Resteck, Bellefonte, Pa.), as described previously (Katsivela et al. 2003a, 2004). The QP 5000 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was operated in the electron impact mode at 70 eV with an ion source temperature of 320 °C. Helium was used as a carrier gas with a flow rate of 1.0 ml min^{-1}. The oven temperature was maintained at 60 °C for 2 min and then increased to 150 °C at a rate of 20 °C min^{-1}, followed by an increase to 320 °C at a rate of 6 °C min^{-1}. Samples of 1.0 μl were injected into the GC, operating in the splitless mode with an injector temperature of 270 °C. MS analyses were performed in the full scan mode (50–400 m z^{-1}).

Isolation of DNA

DNA was extracted from soil samples (0.5 g), directly after sampling, using the Fast DNA Spin Kit for Soil and the protocol of the manufacturer (Bio 101, La Jolla, CA, USA) (Borneman et al. 1996). DNA was obtained from isolated strains as follows. Single colonies were picked from agar medium, resuspended in 100 μl TE buffer (10 mmol l^{-1} Tris, 0.1 mmol l^{-1} EDTA, pH 8.0) and 'cooked' at 95 °C for 15 min. The suspension was centrifuged briefly, to pellet cell debris, and 1 μl of the supernatant was used for PCR amplification (Moore et al. 1999).

16S rDNA T-RFLP analyses

The terminal-restriction fragment length polymorphism (T-RFLP) analysis was performed as described previously by Osborn et al. (2000). The 16S rDNA was amplified, using 2.5 U of *Taq* DNA polymerase (Ampli-Taq, Perkin-Elmer) and standard reaction conditions (Moore et al. 1996, 1999), with the primers 16F63 [5′-CAG-GCCTAACACATGCAAGTC-3′] and 16R1389 [5′-ACGGGCGGTGTGTACAAG-3′]. The primer 16F63 was labelled at the 5′ end with the phosphoramidite dye 6 FAM. Reactions (50 μl final volume containing 1 μl of the extracted community DNA, 20 pmol primer and 50 μM dNTP) were denatured initially for 2 min at 94 °C, followed by 30 cycles of: 94 °C for 1 min; 55 °C for 1 min; and 72 °C for 2 min; with a final extension step of 72 °C for 10 min. The PCR products were purified using QIA-quick columns (Qiagen, Germany) and digested with 20 U of *Alu*I in a total volume of 15 μl at 37 °C for 3 h. This restriction digestion (2 μl) was mixed with 2 μl of deionised formamide, 0.5 μl of ROX-labelled GS500 internal size standard (Applied Biosystemsl, Inc.) and 0.5 μl of loading buffer. Samples (1.5 μl of the denatured digest) were denaturation at 95 °C for 5 min and electrophoresed in a 5% polyacrylamide gel, containing 7 M urea, for 6 h

at 3000 V on an ABI 377 genetic analyser with filter set A and a well-to read length of 36 cm. T-RFLP profiles were analysed using the Genescan software (version 2.1) (Applied Biosystems). The terminal restriction fragment (T-RF) sizes, in basepairs, were estimated by reference to the internal standard, using the Local Southern method. T-RFs with a peak height of less than 100 fluorescence units were excluded from analyses.

Bacterial strains

The 16S rDNA sequences of the bacterial strains used in this study have been deposited in the EMBL database under the following accession number: *Alcaligenes* sp. strain EK5; accession number AJ 440003; *Enterobacter* sp. strain EK3.1; accession number Z76673; *Enterobacter* sp. strain EK4; accession number Z76674; and *Ochrobactrum* sp. strain EK6; accession number Z76675 (Katsivela et al. 2003a, b). The two *Enterobacter* spp. strain EK3.1 and EK4 have been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) as *Enterobacter* sp. strain EK3.1 DSM 15156 and *Enterobacter* sp. strain EK4 DSM 15213.

Detection of degradation genes by PCR with degenerate primers

Purified DNA was amplified, using 5 U of *Taq* DNA polymerase (Qiagen Taq PCR core kit) and standard reaction conditions, with degenerate primers:

1. Amplification of the *alcB* gene was performed by the following PCR program: initial denaturation step consisting of 4 min at 95 °C; 25 cycles consisting of denaturation at 95 °C for 45 s, primer annealing at 40 °C for 1 min; primer extension at 72 °C for 1 min; and elongation at 72 °C for 5 min using primers **TS2S** (5′-AAYAGAGCTCAYGARYTRGGTCAY-AAG-3′) and **deg1RE** (5′-GTGGAATTCGC-RTGRTGRTCIGARTG-3′). The expected length of the PCR fragment is 557 bp (Hamamura et al. 2001; Smits et al. 1999).
2. Amplification the catechol 2,3-dioxygenase gene (subfamily I.2.A.) was performed by the following PCR program: initial denaturation step consisting of 4 min at 94 °C; 10 cycles consisting of denaturation at 94 °C for 45 s, primer annealing at 60 °C for 45 s and primer extension at 72 °C for 2 min; and 20 cycles consisting of denaturation at 94 °C for 45 s, primer annealing at 53 °C for 45 s and an extension at 72 °C for 2 min; and a final primer elongation at 72 °C for 10 min, using primers **C23DO_R** (5′-TCAG-GTCAGCACGGTCA-3′) and **C23DO_F** (5′-CGACCTGATCTCCATGACCGA-3′). The expected length of the PCR fragment is 238 bp (Mesarch et al. 2000).
3. Amplification of the *nahAc* gene was performed by the following PCR program: initial denaturation step consisting of 4 min at 96 °C; 35 cycles consisting of denaturation at minutes; and a final primer elongation at 72 °C for 7 min, using primers **pPAH-F** (5′-GGYA AYGCNAAAGAATTCGTNTGYWSHTAY CAYGGITGGG-3′) and **pPAH-NR700** (5′-CCAGAATTCNGTNGTRTTHGCATCRAT SGGRTKCCA-3′). The expected length of the PCR fragment is 630 bp (Hedlung et al. 1999; Simon et al. 1993).

Amplifications were performed in a total volume of 25.5 μl in 0.2 ml reaction tubes with a DNA thermal cycler (mastercycler gradient, Eppendorf). Aliquots of the PCR products were supplemented with the dye ethidium bromide and checked by electrophoresis in a 1% agarose gel and comparison to a 1 kb DNA Ladder (MBI Fermentas).

Results and discussion

Changes in the petroleum composition of the soil samples

The changes in the petroleum composition of the landfarmed site over time were examined after extraction of the soil samples and analysis of petroleum contaminants by GC–MS. Figure 1 shows a typical trend for hydrocarbon degradation during landfarming. During the 14-month period, all n-alkanes were reduced 75 to 100% of the original levels. Even though the percentage depletion of each individual petroleum hydrocarbon varied between 5 and 10% between duplicates of samples and samples collected from different areas of the landfarming sites. We have calculated the remaining hydrocarbon concentration in our analysis using the mean value of two duplicates for every single sample.

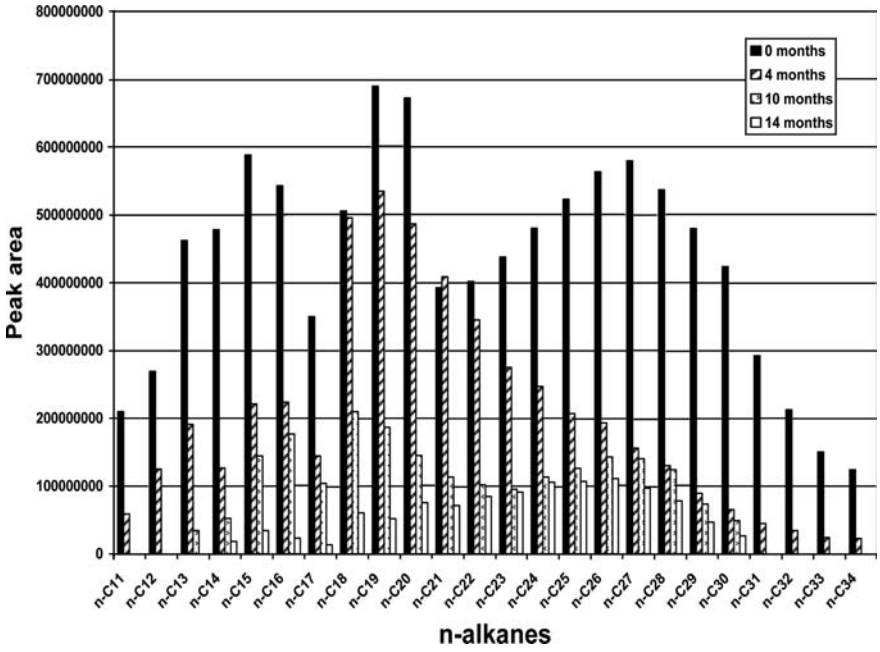

Figure 1. Changes in the composition of n-alkanes in the landfarming soil. Samples were collected from soil from the landfarming site treated with refinery waste sludge, at the beginning of the experiment and after 4, 10 and 14 months treatment.

Shifts in the relative abundances of the different components were observed during landfarming. During the first 4 months of landfarming, a rapid depletion of long chain length (C23–C34) and short chain length (C11–C13) compounds was observed (depletion by more than 50%), whereas depletion of n-alkanes with chain lengths of C18–C22 was much less pronounced (depletion by less than 20%).

After 10 months of landfarming treatment, a further reduction in the concentration of all n-alkanes was observed, specifically those of medium chain lengths, whereas residual concentrations of longer chain length n-alkanes was only slightly reduced further. A decrease of the n-alkanes with chain lengths of C13–C14 and C18–C24 of more than 50% was obtained. In parallel, a 100% reduction of the n-alkanes with chain lengths of C11–C12 and C31–C34 was obtained.

The n-alkane spectrum after the 14 months landfarming treatment showed yet a further reduction of all n-alkanes from the levels observed after 10 months. Again, the concentrations of low- and medium-chain length n-alkanes were reduced. In this case, n-alkanes with chain lengths of C14–C21 and C30 were depleted more than 50%, whereas the n-alkane tridecane was completely reduced. However, the amounts of n-alkanes with chain lengths of C22–C29 were 5–35% lower than the levels measured after the 10-month treatment (Figure 1).

As shown in Figure 1, the highest level of degradation of n-alkanes occurred during the first 4 months of treatment, suggesting the existence of an active bacterial community in this period. Kaplan & Kitts (2004) reported also a rapid degradation of petroleum hydrocarbons in a land treatment unit during the first period of the soil pollution. The degradation rate is decreased during the later phase.

Bacterial community analyses using 16S rDNA T-RFLP fingerprinting

T-RFLP fingerprinting of PCR-amplified 16S rDNA was used to assess the relative diversity of the microbial communities existing in the six investigated soil communities. DNA was extracted from the different soil samples: (a) the non-polluted control soil; (b) the fresh refinery waste sludge; and (c) the landfarming soil (at the time of its construction, after 4 months, after 10 months

and after 14 months treatment). The T-RFLP analyses were used to compare the compositions of the soil bacterial communities.

The similarities between the six different complex bacterial communities were analysed, and the origins of predominant bacterial groups were elucidated by comparing the communities qualitatively. Thus, the evaluation of the T-RFLP profiles was carried out by observation of the presence or absence of specific T-RFs. The measured community profiles were labelled only with the 'blue' FAM dye at the 5′-end of the T-RFs. The T-RFLP fingerprints of all six soil samples were complex, each demonstrating different profiles (Figure 2). The overall 16S rDNA T-RFLP fingerprint patterns of the bacterial communities in the soil of the

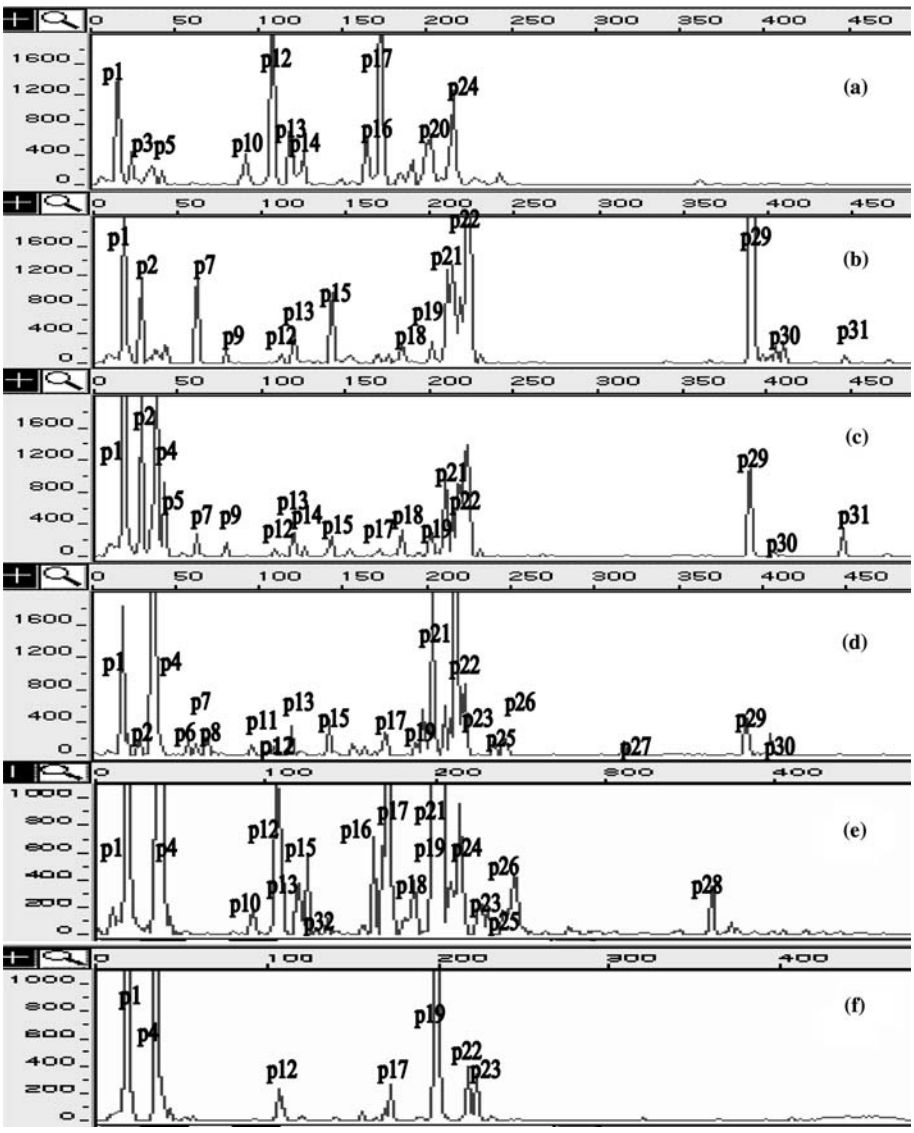

Figure 2. T-RFLP assessment of diversity of (a) untreated control site; (b) fresh refinery waste sludge; (c) landfarming site directly after augmentation with waste sludge; (d) 4-month old landfarming site; (e) 10-month old landfarming site; and (f) 14-month old landfarming site. Fragment sizes, in base pair lengths, are shown at the top of each profile, while peak heights are shown as fluorescent units. Only the blue (shown as grey) 5′-FAM-labelled T-RFs are shown. The peaks with the same fragment size in base pairs are labelled with the same peak number (p1–p31) and indicate characteristic T-RFs of OTUs detected in T-RFLP profiles.

non-polluted control site, over the whole treatment period, were distinct from those of the contaminated landfarming site and were relatively unchanged from each other. Each T-RF peak in the community profiles indicates a different sequence type and was used as an OTU, with an approximate resolution level able to differentiate bacterial genera (Osborn et al. 2000). 'TU' is a term, derived from numerical taxonomy and generally applied to strain, species or genus or other taxonomic entity which may not necessarily be defined. With the growing application of nucleic acid sequence data for assessing microbial diversity, etc., the term is used to define a sequence type that is distinct. The exact taxonomic classification of the sequence type may not be able to be defined with certainty.

The peaks with the same fragment sizes in the different samples are labelled with the same respective peak numbers (p1–p31 in Figure 2) and indicate characteristic OTUs. The total number of peaks (i.e., the total number of OTUs), as well as the common peaks possessing the same fragment sizes (i.e., the same OTU), in each T-RFLP profile of the six different soil samples were recorded and compared.

The T-RFLP profile from the landfarmed soil after the 4-month treatment, which was the point when the bacterial community exhibited the highest degradation activity (compare with Figure 1), was used as a reference.

The T-RFLP profiles obtained from the landfarmed soil after 4 and 10 months of treatment contained the highest number of OTUs among the samples (26 and 24 different OTUs, respectively). The T-RFLP profiles generated from the refinery waste sludge, as well as from the landfarmed soils directly after augmentation with waste sludge, contained the same total number of OTUs, (22 different OTUs), although their compositions were different. The soil from the non-polluted control site, as well as from the landfarmed soil after 14 months of treatment, contained 16 and 15 different OTUs, respectively, indicating lower levels of diversity and complexity, at the detection limits for the methods that were used. Thus, it may be assumed that the lowest bacterial diversity was indicative of soil samples poor in carbon sources. In contrast, the microbial communities demonstrating the highest degradation activities after 4 and 10 months of landfarming treatment (as shown in Figure 1) were also the communities comprised of the highest number of OTUs. Kaplan & Kitts (2004) reported similar results of their investigations in a petroleum land treatment unit at Guadalupe. The bacterial cell counts decreased whereas the total petroleum hydrocarbons degradation rate slowed.

Comparison of the T-RFLP profiles from the four landfarmed soils showed six common OTUs existing in all landfarmed soil samples, i.e., at the beginning of the experiment, after 4, 10 and 14 months of incubation (p1, p4, p12, p17, p19 and p23). OTU number p17 of the common OTUs was the same as that originating within the control soil, whereas OTU number p19 was the same as that originating from the waste sludge. Two OTUs represented newly selected bacterial OTUs (p4 and p23). However, only two OTUs were common in all six community profiles (p1 and p12).

Four types of OTUs, with respect to their origins, could be recognised in the landfarmed soils: OTUs originating from the control soil; OTUs originating from the waste sludge; newly selected OTUs of low abundance in the original soil or waste sludge, but becoming dominant in the mixture; and OTUs existing in all T-RFLP profiles.

As described previously, the addition of waste sludge to pristine soil resulted in dramatic changes in the microbial community. OTUs originating from the non-polluted control soil were reduced dramatically, whereas OTUs originating from the refinery waste sludge dominated the bacterial community of the constructed landfarm after 4-month treatment (Katsivela et al. 2004). In the present work, data from the monitoring of these four kinds of OTUs for a treatment period of 14 months suggests a distinct influence derived from the refinery waste sludge, an enhanced bacterial diversity at 4 and 10 months, with a progressive and marked selection, with decreased diversity, at 14 months. These data suggest that specific OTUs were associated with the different phases of petroleum degradation in the landfarmed soil. Figure 3 shows the relative abundances of the four kinds of OTUs observed in the four landfarmed soil samples with different treatment histories. As shown in Figure 3, the relative abundance of OTUs from the control soil did not exhibit any significant changes over the whole treatment time period of 14 months although the relative intensities of some peaks suggest quantitative changes over the landfarming treatment.

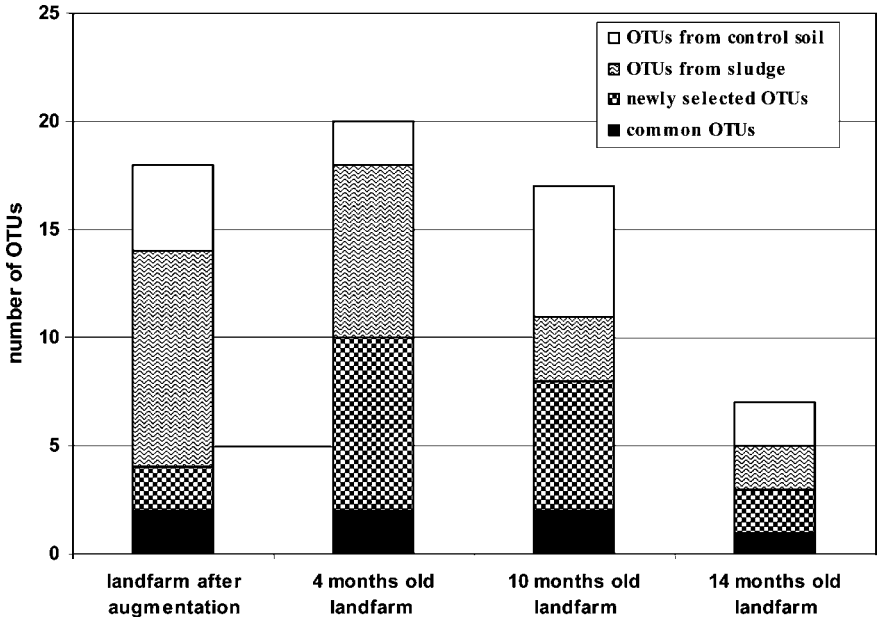

Figure 3. Relative abundance of the number of OTUs with different origin found in the four soil samples with different treatment history using T-RFLP analysis.

Surprisingly, some indigenous bacteria (i.e., the OTUs originating from the non-polluted control soil which survived after the pollution with refinery waste sludge) turned out to be stable over the whole treatment period. On the other hand, newly selected OTUs, as well as OTUs originating from the refinery waste sludge, dominated the bacterial community of the constructed landfarm after 4 and 10 months treatment, which was the period with the highest metabolic activity.

Although the relative abundance of the bacteria originating from the refinery waste sludge increased after augmentation, a decrease in OTU abundance was observed over the treatment time period of 14 months. The highest numbers of newly selected OTUs were present in the T-RFLP profiles at the time with the highest degradation activity (in 4 and 10 months old landfarms), suggesting their metabolic superiority in this period (Figures 1 and 2).

The isolation of four bacterial strains with degradation capabilities towards petroleum hydrocarbons from landfarming soils was reported previously, i.e., *Alcaligenes* sp. EK5, *Enterobacter* spp. EK3.1 and EK4 and *Ochrobactrum* sp. EK6 (Katsivela et al. 2003a, b). In order to assess the importance of members of the genera *Alcaligenes, Enterobacter* and *Ochrobactrum* the abundances of the characteristic T-RFs of these genera was monitored in the T-RFLP profiles of the six soil samples. Figure 4 shows the T-RFLPs of *Alu*I restriction digested 16S rDNA of: (a) the non-polluted control soil; (b) the fresh refinery waste sludge; and (c) the landfarming soil after 4 months; (d) after 10 months: and (e) after 14 months treatment; as well as (f) *Alcaligenes* sp. EK5. For this T-RFLP analysis we used both the 5′ as well as the 3′ T-RFs that are shown as blue and green peaks, respectively.

The characteristic T-RFs of the genus *Alcaligenes* were present in the community T-RFLP fingerprint of the non-polluted control soil, as well as in that of the 10-month old treated soil (Figure 4). On the other hand, the characteristic T-RFs of the genera *Enterobacter* and *Ochrobactrum* were abundant in the T-RFLP fingerprints of the landfarmed soil over the whole treatment period of 14 months (compare T-RFLP profiles of the soil samples in Figure 4 with the T-RFLP profiles of *Enterobacter* spp. EK3.1 and EK4 and *Ochrobactrum* sp. EK6 in Figure 3 of the published data by Katsivela et al. 2003a). The T-RFs from the genus

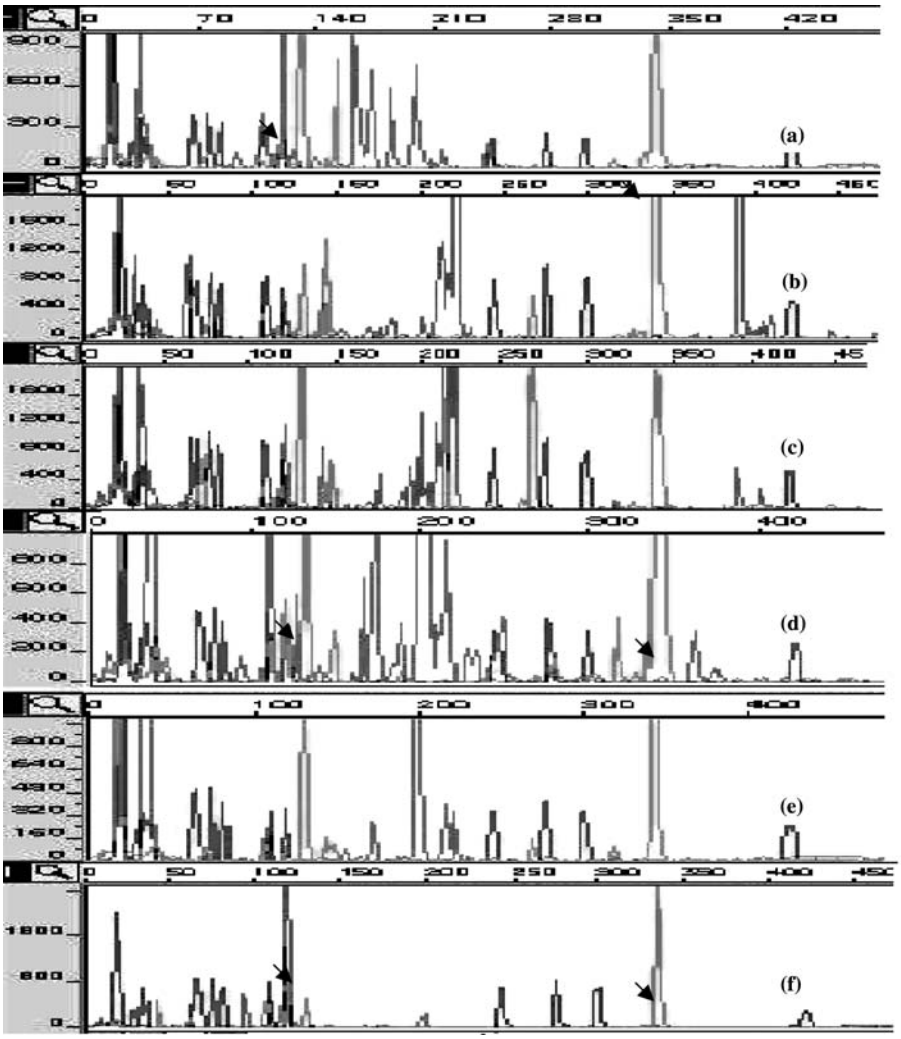

Figure 4. T-RFLPs of *Alu*I restriction digested 16S rDNA, to assess the bacterial diversity of (a) untreated control soil; (b) refinery waste sludge; (c) 4-month old landfarmed soil; (d) 10-month old landfarmed soil; (e) 14-month old landfarmed soil; and (f) *Alcaligenes* sp. EK5. The arrows indicate the characteristic T-RFs of the genus *Alcaligenes*. Fragment sizes, in base pairs, are shown at the top of each profile, while peak heights are shown as fluorescent units. The red dye-labelled fragments indicate the GS500 DNA internal size standard, while 5′ and 3′ T-RFs are shown as blue and green peaks, respectively.

Ochrobactrum were also present in the T-RFLP fingerprint of the non-polluted control soil.

The relative abundances of the genera *Alcaligenes* and *Ochrobactrum* in the non-polluted soil indicates their indigenous origin. In contrast, as described previously (Katsivela et al. 2004), *Enterobacter* belongs to the bacterial genera that were present only in the landfarmed soil samples. *Alcaligenes* appears in significant numbers only within the 10-month old landfarmed soil, whereas *Ochrobactrum* and *Enterobacter* were present in the landfarmed soil over the whole treatment period of 14 months. These differences in the relative abundances of these three bacterial genera may be related to their metabolic capabilities of utilisation of specific carbon sources as well as the chemical composition of the existing petroleum hydrocarbons in the landfarmed soils in the different sampling periods. Kaplan & Kitts (2004) reported also that specific phylotypes of bacteria were associated with the different phases of petroleum degradation in a land treatment unit.

The authors showed also that the relative abundance of a phylotype associated with *Alcaligenes* increased only temporally during the bioremediation process.

Detection of catabolic gene sequences by PCR

DNA extracted from the soil samples: (a) an untreated, non-polluted control soil; (b) a newly produced refinery waste sludge; and (c) a landfarming site treated with refinery waste sludge, after 4 and 14 months treatment, were analysed by PCR with degenerate primers for the presence of genes encoding key enzymes in the degradation of petroleum hydrocarbons. Alkane hydroxylase *AlkB*, catechol 2,3-dioxygenase (subfamily I.2.A) and naphthalene dioxygenase *nahAc* were used as marker genes. Control experiments using *P. oleovorans* GPo1, *P. putida* mt-2 (pWWO) and *P. putida* G7 as strains reported to harbour alkane hydroxylase *AlkB*, catechol 2,3-dioxygenase (subfamily I.2.A) and naphthalene dioxygenase *nahAc* yielded PCR fragments of the expected length of 557, 238, and 630 bp, respectively.

DNA extracted from the refinery waste sludge showed a PCR product using catechol 2,3-dioxygenase specific primers with a size of 238 bp (Figure 5, lane 3). This result suggests the presence of bacteria containing catechol 2,3-dioxygenase (subfamily I.2.A), which is an enzyme involved in the degradation of a large group of aromatic compounds. Also, a PCR product generated using the primers for alkane hydroxylase *AlkB* was obtained from the DNA extracted from the refinery waste sludge. However, the size of the PCR product (485 bp) differed significantly from the one expected (630 bp), indicating the absence of alkane hydroxylase genes in the tested soils. Surprisingly, none of the other analysed soil samples showed any positive PCR products. The lack of PCR products of the alkane hydroxylase *AlkB* gene, contrasts the active degradation of n-alkanes in the landfarmed soils, and may suggest that the degrading bacteria contain alkane oxidation enzyme systems that are unrelated or only distantly related to *AlkB*. Alternatively, potential PCR inhibitors coextracted with DNA, non-amplifying DNA, soil factors (humics), and soil pollutants (petroleum hydrocarbons) could have disturbed the PCR detection of the reported genes.

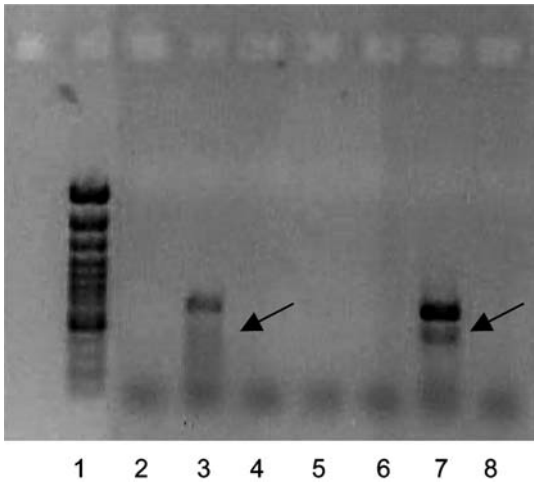

Figure 5. Agarose gel with PCR products from the amplification of degradation genes for catechol 2,3-dioxygenase (subfamily I.2.A). Lane 1: 1 kb DNA ladder. Lanes 2–5: PCR products of the DNA extracted from a 4-month old landfarmed soil, a refinery waste sludge, a 14-month old landfarmed soil and an untreated control soil. Lane 6: negative control without genomic DNA. Lane 7: positive control (PCR product from *P. putida* mt-2 (pWWO)).

Besides DNA extracted from the soil samples, four bacterial strains with degradation capabilities, *Alcaligenes* sp. EK5, *Enterobacter* spp. EK3.1 and EK4 and *Ochrobactrum* sp. EK6, previously isolated from the landfarms (Katsivela et al. 2003a, b), were also analysed by the same PCR approach. None of the four analysed bacterial strains showed any PCR product for the genes encoding alkane hydroxylase *AlkB*, catechol 2,3-dioxygenase (subfamily I.2.A) and naphthalene dioxygenase *nahAc*. The lack of the expected PCR products of the three tested degradation genes in genomic DNA of the four isolated bacteria may be due to existence of gene sequences completely different from those characterised from bacteria of the genus *Pseudomonas*. Alternatively, these bacteria may contain genes homologues in which the regions that were used to develop the primers were not so well conserved.

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