ELSEVIER

Soil Biology & Biochemistry 38 (2006) 303–310

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Soil microbial communities: Influence of geographic location and hydrocarbon pollutants

Mphekgo P. Maila[a,*], P. Randima[a], K. Drønen[b], Thomas E. Cloete[c]

[a]*Council for Scientific and Industrial Research, P O Box 395, Pretoria, 0001 South Africa*
[b]*Department of Microbiology, University of Bergen, Jahnebakken 5, N-5020 Bergen, Norway*
[c]*Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa*

Received 8 October 2004; received in revised form 4 May 2005; accepted 13 May 2005

Abstract

The importance and relevance of the geographical origin of the soil sample and the hydrocarbons in determining the functional or species diversity within different bacterial communities was evaluated using the community level physiological profiles (CLPP) and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE). Hydrocarbon contaminated and uncontaminated soils from different geographical locations were used in the study. In addition, the influence or relevance of the geographical location of the sample was further evaluated by artificially contaminating soils from different geographical locations with different petroleum products. The hydrocarbons rather than the geographical origin of the sample appear to be more important in determining functional or species diversity within the bacterial communities. Cluster analysis of the different community profiles using both functional and molecular responses revealed that the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils. The results of the soils from different locations artificially contaminated by different hydrocarbons also reached the same conclusion. The samples from different soils were as different as samples from the same soil contaminated by different petroleum products. In addition, the removal rate of the different hydrocarbons in the artificially contaminated soil was different. The results suggest that the pollutants rather than the geographical origin of the sample might be more important in determining the functional or species diversity within bacterial communities.

Keywords: Microbial communities; Geographical location; Hydrocarbons; Functional diversity; Species diversity; Biodegradation; DGGE

1. Introduction

The impact of petroleum hydrocarbons on soil microbial diversity has been the subject of investigation in recent years. The changes in hydrocarbon content in soils results in characteristics shifts in microbial populations and the abundance of hydrocarbon utilising bacteria (Atlas et al., 1991; Wünsche et al., 1995). Hydrocarbon contamination selects for a less diverse but catabolically versatile bacterial community (Atlas et al., 1991; Lindstrom et al., 1999). However, information about the importance of geographical origin of the soil and the hydrocarbons in determining the functional and species diversity within bacterial communities is not well documented. There is a need to understand the importance of geographical origin of the soil and the hydrocarbons when assessing the different soil environments contaminated by hydrocarbons. The improved knowledge of the influence of the geographical origin of the soil and the hydrocarbons on microbial diversity can help to improve microbial process used in the removal of hydrocarbons from the soil.

Bundy et al. (2002) used community level physiological profiles (CLPP) and phospholipids fatty acid (PLFA) to study the effect of diesel on microbial communities and reported that microbial communities in different soil types do not converge after diesel contamination. However, the soil used in the study was artificially contaminated and could therefore, not adequately reflect the contaminated field sites.

Juck et al. (2000) found that at two oil contaminated Arctic sites investigated by DGGE and Biolog analysis, absolute diversity was decreased at one site and remained the same or increased at the other. However, the study was conducted using the cold adapted microbial communities.

* Corresponding author. Tel.: +27 12 841 4279; fax: +27 12 841 2506.
E-mail address: pmaila@csir.co.za (M.P. Maila).

0038-0717/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2005.05.006

In this study, we evaluated soil microbial diversity of different geographic locations contaminated by similar hydrocarbons. We also investigated the biodegradation efficiency and microbial diversity of different soils artificially contaminated by different hydrocarbons. The aim of the study was to characterise the microbial diversity of different hydrocarbon contaminated soil environments to establish the importance and relevance of the geographic locations in relation to the stressor. The community level physiological profile and Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE) were used to characterise the microbial communities.

2. Material and methods

2.1. Soil and soil sampling

The soil samples were taken in sterile bags from a hydrocarbon-contaminated site in Secunda (Mpumalanga Province), Coalbrook (Free State Province), and Rosslyn (Gauteng Province), South Africa. The Secunda and Rosslyn soils were predominantly sandy loam while Coalbrook soil was a predominantly loam soil. The soils had a Total Petroleum Hydrocarbon (TPH) concentration during the day of sampling of 1.2 g kg soil^{-1}, 1 g kg soil^{-1} and 2.5 g kg soil^{-1}, respectively. Both contaminated and uncontaminated soils were collected. The uncontaminated soil was collected from the CSIR (Council for Scientific and Industrial Research) site in Gauteng Province, SA. The sites where the soil samples were collected is shown in Fig. 1. The samples were stored at 4 °C until further analysis.

2.2. Microbial community level physiological profiles

Microbial suspensions were prepared from soil as described by Wünsche et al. (1995). After appropriate dilutions in sterile saline solution, the cell suspensions were used to determine the number of culturable heterotrophs and to inoculate BIOLOG GN micro plates. The number of culturable heterotrophs, expressed as CFU, was determined by spreading 0.1 ml cell suspension on to a nutrient agar (Biolab Diagnostics) medium, amended with cycloheximide (200 μg ml^{-1}) to suppress fungal growth. Three replicates were spread on agar plates and incubated for 24 h at 28 °C. The results of the culturable heterotrophs for the different soil samples are shown in Fig. 2.

To obtain a substrate utilisation fingerprint of the microbial communities, three replicates of all the soil extracts were inoculated in BIOLOG GN microtiter plates (Biolog Inc., Hayward Calif) containing 95 different sole-carbon sources and a control without a carbon source. The dilutions yielding similar TRHs numbers were used to inoculate the plates. The BIOLOG GN plates were incubated at 28 °C and readings done using a Bio-Tek Elx800 (Bio-Tek Instruments Inc) micro plate reader at 600 nm after 24, 48 and 72 h. Once the raw data from the replicate Biolog plates was collected, an average of the three plates was calculated using MS Excel. Further statistical analyses were done using STATISTICA for Windows release 5.1.

2.3. Soil DNA extraction and purification

Total DNA was isolated from the soil using the Bio101 extraction kit (Bio Inc.). One gram of soil was used for DNA extraction. The extracted DNA was examined by horizontal electrophoresis in 1% agarose.

2.4. Community fingerprinting by PCR-DGGE

2.4.1. PCR conditions

A 1-μl volume of extracted DNA was amplified by PCR with a 9600 thermal cycler (Perkin-Elmer/Cetus). The PCR mixture used contained 100 μmol of each primer, 100 mM each deoxy-nucleoside triphosphate, 5 μl 10× PCR buffer, 0.25 μl hot start polymerase (5 U μl^{-1}), (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ), 2.5 μl 2% bovine serum albumin and 40 μl sterile water, to a final volume of 50 μl. The 16S rRNA genes from soil microbial communities were amplified by PCR using the primers, pA8f-GC (5′-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3′) and KPRUN518r (5′ATTACC GCGGCTGCTGG-3′), which have been found to be useful for 16S rRNA gene amplification in ecological and systematic studies (Øvreås and Torsvik, 1998). Samples were amplified as follows: 95 °C for 10 min, 30 s cycles of denaturation (1 min at 94 °C), annealing (30 s at 51 °C), and extension (1 min at 72 °C), and a final extension at 72 °C for 10 min. Amplified DNA was examined by horizontal electrophoresis in 1% agarose with 5-μl aliquots of PCR product.

2.4.2. Denaturing gradient gel electrophoresis

DGGE was performed using a Hoefer SE600 vertical dual cooler system (Hoefer Scientific, San Francisco, CA). PCR samples were loaded onto 8% (w/v) polyacrylamide gels in 0.5× TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na-EDTA, pH 7.4). The polyacrylamide gels (bisacrylamide gel stock solution, 37.55:1; BioRad Laboratories) were prepared with a 20–55% gradient of denaturant (urea and formamide) and allowed to polymerise. Electrophoresis was run at 60 °C, first for 10 min at 20 V and then overnight at 70 V. After electrophoresis, the gels were stained for 15 min in SYBR Green I nucleic acid gel stain, rinsed in distilled water for 1 min and photographed with a Polaroid MP4 Land camera. The gels were analysed using a software program Gel2K (www.im.uib.no) developed by Svein Norland (Department of Microbiology, University of Bergen, Norway), where presence/absence of bands was recorded. Clustering was based on the simple matching

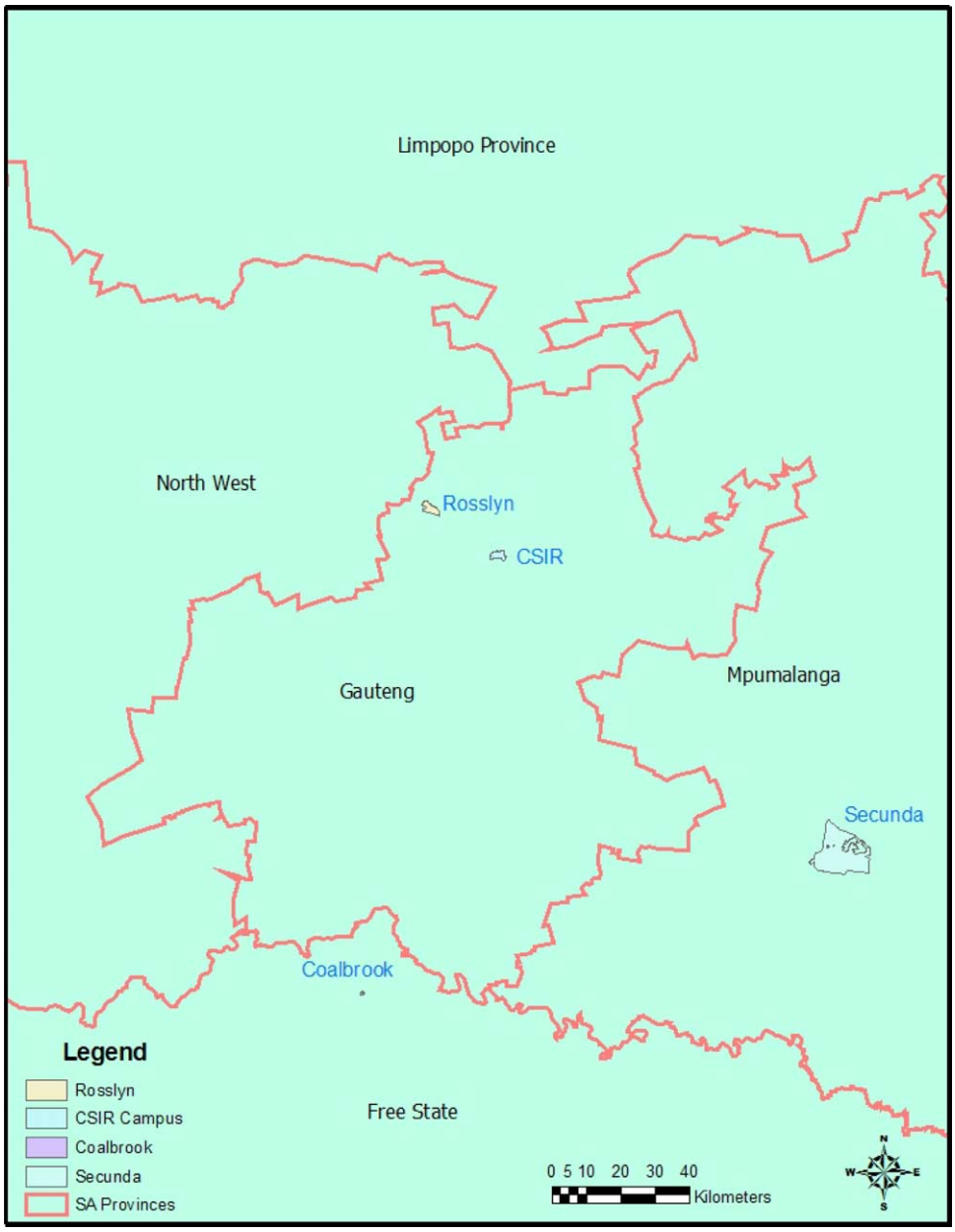

Fig. 1. A map showing the locations were the soil samples were collected.

algorithm, while the dendrogram was drawn applying the group average method.

2.5. Influence of different hydrocarbons

Crude oil (obtained from Petronet Pty Ltd, SA), mineral oil and diesel (both from Exel Pty Ltd, SA) were used to artificially contaminate both the CSIR and Coalbrook uncontaminated soils. These two soils were chosen as they had contrasting characteristics. Coalbrook soil is a loam soil while CSIR soil is a predominantly sandy loam soil. This experiment was done to further evaluate the importance or relevance of the geographical origin of the sample in relation to the stressor and also to evaluate the removal of different petroleum products (mineral oil, crude oil and diesel) in the same soil. The different treatments used for the experiment are shown in Table 1. Each hydrocarbon was added to the soil to make the initial concentration of the artificially contaminated soil 40 g kg soil^{-1}. The hydrocarbons and the soil were thoroughly mixed before adding the soil mixture to different pots. The replicates, containing 500 g of the contaminated soil of each treatment were prepared in 10 cm pots. All the pots were incubated at room temperature in the greenhouse with normal day–night cycle. The pots were watered three times a week with 200 ml of water to maintain the ideal soil moisture for microbial activity. In cases where leachates were produced, the leachate was used to water the same pots. Every 2 weeks

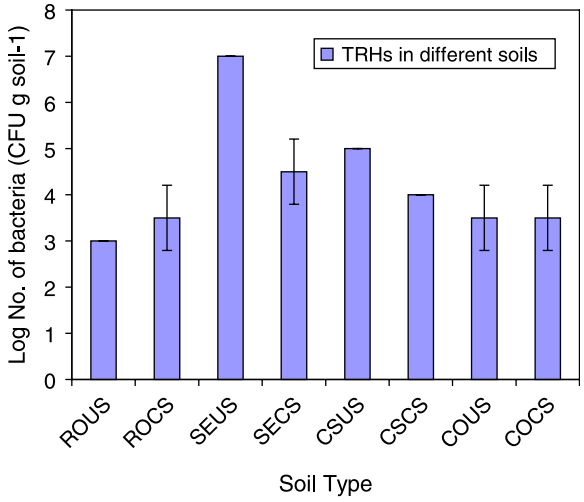

Fig. 2. Total recoverable heterotrophs in soils sampled. Key: COCS, hydrocarbon-contaminated Coalbrook soil; COUS, Coalbrook uncontaminated soil; CSCS, hydrocarbon-contaminated CSIR soil; CSUS, CSIR uncontaminated soil; ROCS, hydrocarbon-contaminated Rosslyn soil; ROUS, Rosslyn uncontaminated soil; SECS, hydrocarbon-contaminated Secunda soil; SEUS, Secunda uncontaminated soil.

two replicates were removed from the experiment to determine the level of hydrocarbons in the soil. The soil from each treatment (after 9 weeks) was used for determining the CLPP. The 9-week incubation was deemed to be sufficient for the exposure of the soil to the different petroleum products. The CLPP was determined as described above.

2.6. Chemical analysis

The contaminated soils were analysed using the TPH method described in Margesin et al. (1999). Ten grams of the polluted soil was used for the analysis. The analyses were done in triplicate.

3. Results

3.1. Microbial community level physiological profiles

The Principle Component Analysis (PCA) revealed differences in the substrate utilisation patterns of both the contaminated and uncontaminated soil from each geographic location (Fig. 3). PCA was performed to characterise the associations amongst samples, taking in to account the absorbance values for all 96-response wells at different incubation times. However, dendrogram analysis only clustered the Rosslyn samples based on geographic location (Fig. 4). The contaminated and uncontaminated soils from the other three sites were not clustered together based on geographic location. The samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils.

Table 1
Treatments used during bioremediation

Treatments	Additions/preparations
COCOIL	Coalbrook soil + Crude oil
CSCOIL	CSIR soil + crude oil
CSD	CSIR soil + diesel
COD	Coalbrook soil + diesel
CSMO	CSIR soil + mineral oil
COMO	Coalbrook soil + mineral oil
COUN	Uncontaminated coalbrook soil
CSUN	Uncontaminated CSIR soil

3.2. Community fingerprinting by PCR-DGGE

The results of the DGGE profiles corroborated the CLPP results. Cluster analysis of DGGE profiles revealed that the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils (Fig. 5). There was no clustering between the samples from the same geographical location (Fig. 5(a)).

Because of soil usage and heterogeneity, which can influence microbial diversity, it was expected that the geographical origin of the sample rather than the hydrocarbons was more important in determining functional or species diversity within the bacterial communities. However, the results appear not to reinforce the hypothesis that geographical origin of the soil samples, rather than the hydrocarbons, is important in determining functional or species diversity within the bacterial communities, as contaminated and uncontaminated samples from the majority of the sites were not clustered together (Figs. 3–5).

3.3. Influence of different carbon substrates

The importance of geographical origin of the soil samples and the hydrocarbons in determining functional diversity within bacterial communities were further evaluated using two different soils artificially contaminated by different petroleum products. The community level physiological profiles of the different contaminated and uncontaminated soils were analysed after incubation using the Principle Component Analysis and cluster analysis.

The PCA revealed differences in the substrate utilisation pattern of the contaminated and the uncontaminated soils (Fig. 6). The uncontaminated soils from the CSIR and Coalbrook (CSUN and COUN, respectively) did not demonstrate a similar substrate utilisation profile as their respective hydrocarbon contaminated soils. The crude oil contaminated CSIR soil (CSCOIL) also did not demonstrate a similar substrate utilisation profile as the crude oil contaminated Coalbrook soil (COCOIL). However, the mineral oil contaminated CSIR (CSMO) and Coalbrook soils (COMO) demonstrated similar substrate utilisation profiles. Similar results were also found for the diesel contaminated CSIR (CSD) and Coalbrook soil (COD).

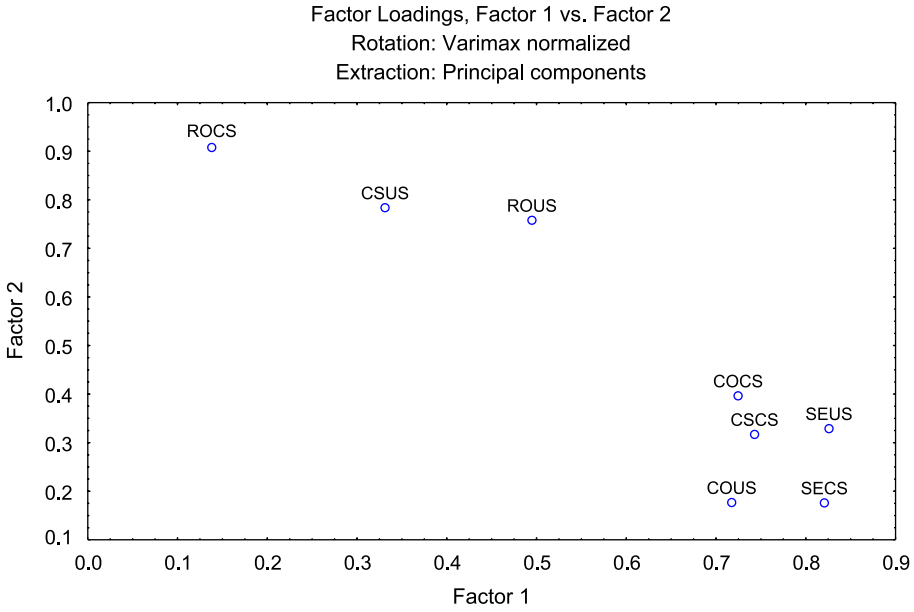

Fig. 3. Principle component analysis of the different soil samples. COCS, hydrocarbon-contaminated Coalbrook soil; COUS, Coalbrook uncontaminated soil; CSCS, hydrocarbon-contaminated CSIR soil; CSUS, CSIR uncontaminated soil; ROCS, hydrocarbon-contaminated Rosslyn soil; ROUS, Rosslyn uncontaminated soil; SECS, hydrocarbon-contaminated Secunda soil; SEUS, Secunda uncontaminated soil.

The difference in the community level physiological profiles of the different hydrocarbon contaminated soils were further analysed using hierarchical clustering (Fig. 7). The results of cluster analysis corroborated the PCA results. The mineral oil contaminated CSIR soil was clustered together with the mineral oil contaminated Coalbrook soil. Similar results were obtained with the diesel contaminated CSIR and Coalbrook soil. No clustering was evident in the case of the crude oil contaminated CSIR and Coalbrook soil. However, all of the hydrocarbon-contaminated soils were clustered together (Fig. 7).

The removal of different petroleum hydrocarbons in the 'same soil' is different (Table 2). Diesel was removed much faster than the crude oil and the mineral oil. There was not much difference in the overall amount of hydrocarbons removed in the CSIR and Coalbrook soil after 9 weeks of incubation. In addition, similar diesel removal rate was found in both soils. The CSIR soil had a higher number of both the total recoverable heterotrophs (TRHs) and the culturable hydrocarbon-utilisation bacteria than the Coalbrook soil. The TRHs results are shown in Fig. 2, while the hydrocarbon-utilisation bacteria for the CSIR soil and

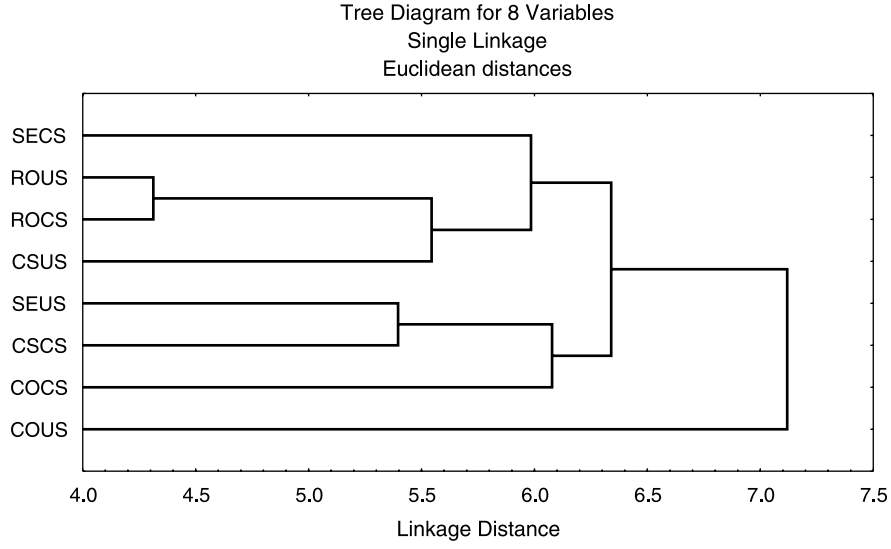

Fig. 4. Cluster analysis of the different soil samples from the different geographic locations.

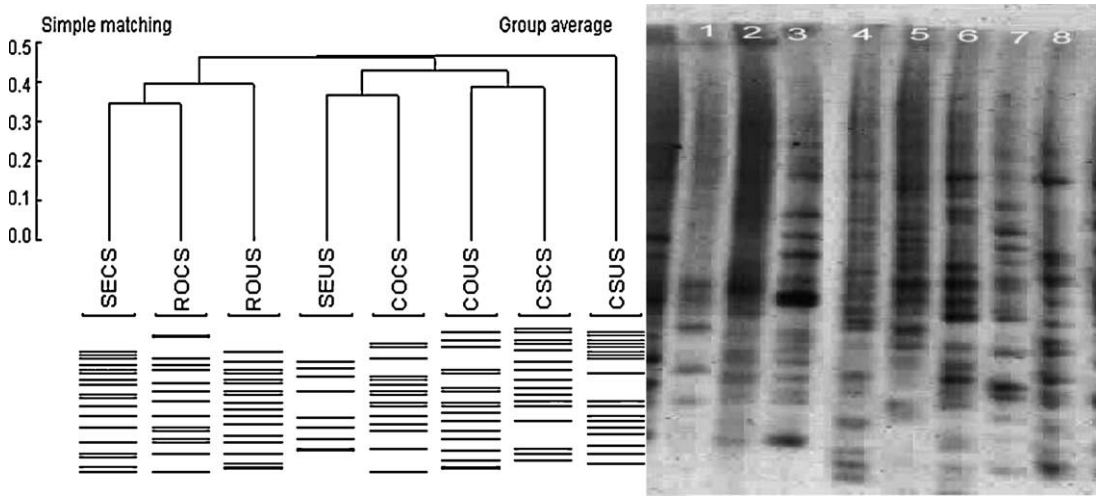

Fig. 5. (a) Cluster analysis of microbial communities in different soil layers and (b) DGGE fingerprints: Key: SECS (1), Secunda Contaminated Soil; SEUS (2), Secunda Uncontaminated Soil; ROCS (3), Rosslyn Contaminated Soil; ROUS (4), Rosslyn Uncontaminated Soil; COCS (5), Coalbrook Contaminated Soil; COUS (6), Coalbrook Uncontaminated Soil; CSCS (7), CSIR Contaminated Soil; CSUS (8), CSIR Uncontaminated Soil.

Coalbrook soil were 10^5 and 10^4 CFU g soil^{-1}, respectively. The removal of hydrocarbons from the soils was highest in the first 2 weeks of the treatment.

4. Discussion

4.1. Microbial community level physiological profiles

The results indicate that different locations contaminated by different hydrocarbons have different microbial communities. The Principle Component Analysis revealed differences in the substrate utilisation patterns of both the contaminated and uncontaminated soil from each geographic location. This is inline with similar findings that the increase in hydrocarbon content in soil results in significant changes in the microbial communities of the affected soil environments (Atlas et al., 1991; Wünsche et al., 1995).

The dendrogram analysis clustered only the Rosslyn samples based on geographic location. The contaminated and uncontaminated soils from the other three sites were not clustered together. In addition, the contaminated and uncontaminated soils from each geographical location did not demonstrate a similar substrate utilisation profiles.

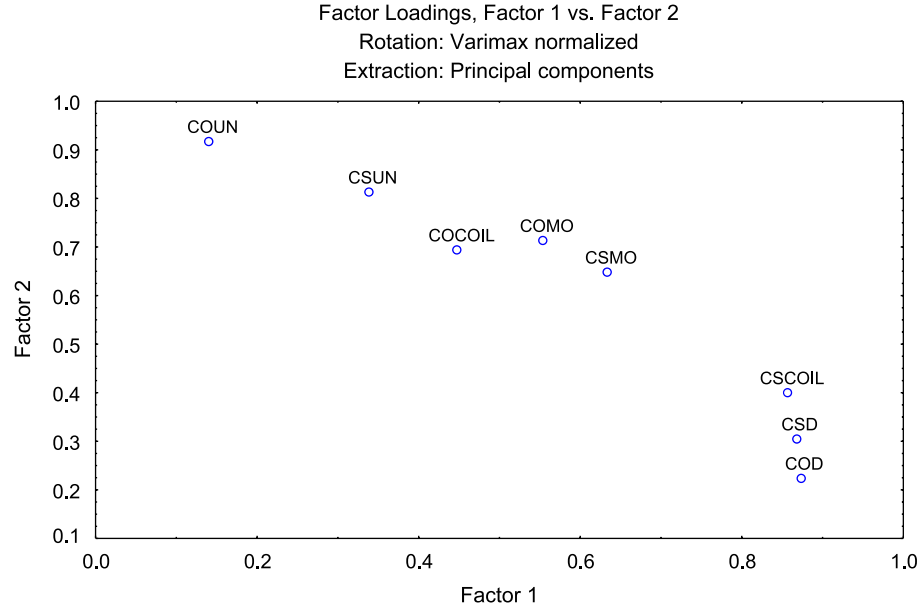

Fig. 6. PCA of the different soils contaminated by different hydrocarbons. Key: COD, Diesel contaminated Coalbrook soil; COUN, uncontaminated Coalbrook soil; COCOIL, Crude oil contaminated Coalbrook soil; COMO, mineral oil contaminated Coalbrook soil; CSMO, mineral oil contaminated CSIR soil; CSD, diesel contaminated CSIR soil; CSUN, uncontaminated CSIR soil; CSCOIL, crude oil contaminated CSIR soil.

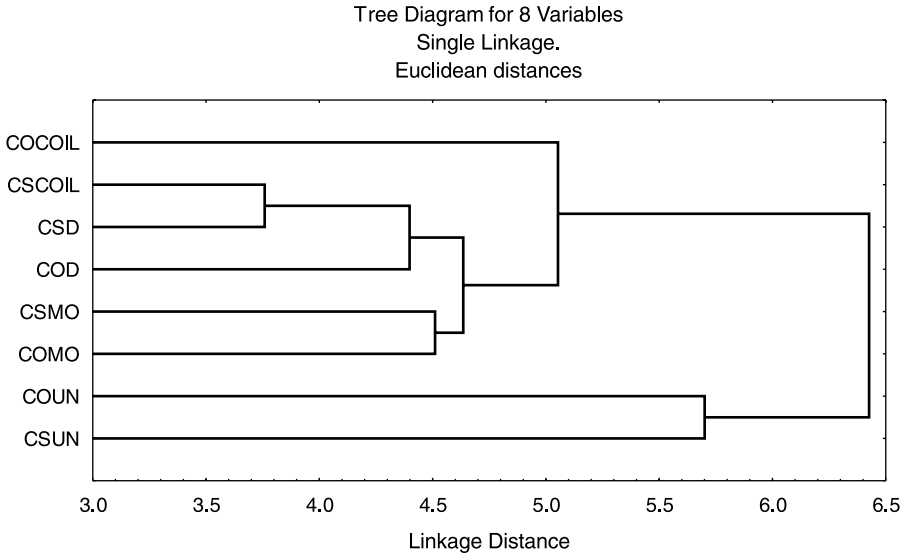

Fig. 7. Cluster analysis of the soils contaminated by different hydrocarbons.

These results contrast those reported by Juck et al. (2000), who reported clustering of samples based on the geographic origin of the samples. However, Juck et al. (2000) examined cold-adapted bacterial communities while the current study examined mesophillic bacterial communities.

4.2. Community fingerprinting by PCR-DGGE

The results of the DGGE profiles corroborated the CLPP results. The DGGE profiles revealed that the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils. There was no clustering of the samples based on geographical origin of the sample. This difference in both the functional and molecular responses of the samples can be attributed to soil heterogeneity and usage that can influence microbial diversity.

It was expected that the geographical origin of the sample rather than the hydrocarbons was more important in determining functional or species diversity within the bacterial communities. However, the results did not support this hypothesis as the samples from different locations were as different as samples from the same location but from contaminated versus uncontaminated soils.

4.3. Influence of different carbon substrates

The importance of geographical origin of the samples and the hydrocarbons in determining functional diversity in bacterial communities were further evaluated using two different soils contaminated by different hydrocarbons. As with the soil samples from the different contaminated sites, the uncontaminated soils from each of the CSIR and Coalbrook sites did not demonstrate similar substrate utilisation profiles as their respective artificially hydrocarbon contaminated soils. However, the substrate utilisation profile of the mineral oil-contaminated CSIR soil was similar to that of the mineral oil contaminated Coalbrook soil. The same result was obtained for the soils contaminated with diesel. There was no clustering between the soils contaminated with crude oil. However, all the hydrocarbon-contaminated soils were clustered together. This similarity in substrate utilisation profile of the different soils contaminated by similar hydrocarbons can be ascribed to the selective enrichment of microbial population capable of using these petroleum products as carbon and energy source. The results suggest that hydrocarbons rather than the geographical origin of the sample are more important in determining the functional or species diversity within the bacterial communities.

Table 2
Biodegradation of different hydrocarbons in different soils (Key, as in Table 1. Average ± SD)

Incubation time (weeks)	TPH concentration (g kg soil^{-1})					
	COCOIL	CSCOIL	COMO	CSMO	COD	CSD
0	40 ± 1.02	40 ± 0.565	40 ± 0.424	40 ± 0.565	40 ± 0.565	40 ± 0.424
2	36 ± 2.83	16 ± 0.424	31 ± 0.707	28 ± 0.283	12 ± 0.707	10 ± 0.283
4	11 ± 0.707	5 ± 0.141	15 ± 0.424	11 ± 0.989	5 ± 0.565	6 ± 0.848
7	11 ± 0.848	4 ± 0.283	11 ± 0.848	9 ± 1.131	5 ± 0.282	6 ± 0.424
9	4 ± 0.283	3 ± 0.424	10 ± 0.141	7 ± 0.141	3 ± 0.566	2 ± 0.283

Different soil environments may harbor different microbial diversity. However, with the availability of the hydrocarbons, the functional diversity of the soil, as revealed by the substrate utilisation patterns appears to be similar. There was no clustering of either the CSIR or Coalbrook uncontaminated soil with their respective contaminated soil to suggest the influence or importance of geographic location in relation to the 'stressor' in determining functional or species diversity within the bacterial communities. Even though the uncontaminated CSIR and the uncontaminated Coalbrook soil were clustered together, the linkage distances illustrated their differences. These results corroborated the results obtained using field samples.

The removal of petroleum hydrocarbons from the soils was highest in the first 2 weeks of the 'treatment'. According to Harmsen et al. (1994) and Hejazi et al. (2003), the dominant removal mechanism of hydrocarbon during the initial phase involves the volatilisation of the low molecular weight volatile compounds. The principal removal mechanism at a later stage of the treatment appears to be biodegradation.

The removal of different petroleum products in the 'same soil' is different. Diesel was removed faster followed by crude oil and mineral oil. This was not surprising as the different substrates have different compositions of hydrocarbons or different aliphatic chains which can influence biodegradation (Dias and Alexander, 1971). In addition, the biodegradation of mineral oil and crude oil was much higher in the CSIR soil than in the Coalbrook soil. However, similar biodegradation of diesel was found in both soils. This can be attributed to the significant role played by other removal mechanisms other than biodegradation. According to Morgan and Watkinson (1989), up to 40% of the hydrocarbons may evaporate in hotter climates. The differences in the biodegradation efficiency in the two different soils can be attributed to the higher number of both the total recoverable heterotrophs and the culturable hydrocarbon-utilisation bacteria in the CSIR soil than the Coalbrook soil.

In conclusion, the study did not support the hypothesis that the geographical origin of the sample rather than the hydrocarbons is important in determining functional or species diversity within bacterial communities. Further work is required to investigate the importance of soil heterogeneity in community studies of soil environments contaminated by other pollutants (e.g. halogenated aromatics or aliphatics). In addition more studies (using many field samples with different soil characteristics) are required to corroborate these findings.

Acknowledgements

The authors would like to thank the National Research Foundation and the Norwegian Research Council for providing us with the funds to carry out this work.

References

Atlas, R.M., Horowitz, A., Krichevsky, M., Bej, A.K., 1991. Response of microbial populations to environmental disturbance. Microbial Ecology 22, 249–256.

Bundy, J.G., Paton, G.I., Campbell, C.D., 2002. Microbial communities in different soil types do not converge after diesel contamination. Journal of Applied Microbiology 92, 276–288.

Dias, F.F., Alexander, M., 1971. Effect of chemical structure on biodegradability of aliphatic acids and alcohols. Applied and Environmental Microbiology 22, 1114–1118.

Harmsen, J., Velthorst, H.J., Bennehey, I.P.A.M., 1994. Cleaning of residual concentrations with an extensive form of landfarming. In: R.E., Hinchee, D.B., Anderson, F.B., Blaine, G.D., Sayles (Eds.), Applied Biotechnology for Site Remediation. Lewis Publishers, Boca Raton, pp. 84–91.

Hejazi, R.F., Husain, T., Khan, F.I., 2003. Landfarming operation of oily sludge in arid region-human health risk assessment. Journal of Hazardous Materials B99, 287–302.

Juck, D., Charles, T., Whyte, L.G., Greer, C.W., 2000. Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. FEMS Microbiology Ecology 33, 241–249.

Lindstrom, J.E., Barry, R.P., Braddock, J.F., 1999. Long-term effects on microbial communities after a subarctic oil spill. Soil Biology & Biochemistry 31, 1677–1689.

Margesin, R., Zimmerbauer, A., Schinner, F., 1999. Soil lipase activity-a useful indicator of oil biodegradation. Biotechnology Techniques 13, 859–863.

Morgan, P., Watkinson, R.J., 1989. Hydrocarbon degradation in soils and methods for soil biotreatment. CRC Critical Reviews in Biotechnology 8, 305–332.

Wünsche, L., Bruggemann, L., Babel, W., 1995. Determination of substrate utilisation patterns of soil microbial communities: An approach to assess population changes after hydrocarbon pollution. FEMS Microbiology Ecology 17, 295–306.