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Evaluation of microbial diversity of different soil layers at a contaminated diesel site

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Abstract

In this study, we evaluated the hydrocarbon removal efficiency and microbial diversity of different soil layers. The soil layers with high counts of recoverable hydrocarbon degrading bacteria had the highest hydrocarbon removal rate compared with soil layers with low counts of hydrocarbon degrading bacteria. Removal efficiency was 48% in the topsoil, compared with 31% and 11% at depths of 1.5 and 1 m, respectively. In the 1 and 1.5 m soil layers, there was no significant difference between total petroleum hydrocarbon (TPH) removal in nutrient amended treatments and controls. The respiration rate reflected the difference in the number of bacteria in each soil layer and the availability of nutrients. High O_2 consumption corresponded positively with high TPH removal. Analysis of the microbial diversity in the different soil layers using functional diversity (community-level physiological profile, via Biolog) and genetic diversity using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rDNA revealed differences in, respectively, substrate utilisation patterns and DGGE profiles of 16S rDNA fragments. Microbial diversity as revealed by DNA fragments was lower in the highly contaminated soil layer (1.5 m) than in the topsoil and at 1 m.

1. Introduction

The effect of hydrocarbon contamination on soil microbial communities has been extensively studied, for example by Atlas et al. (1991), Wünsche et al. (1995), Lindstrom et al. (1999), MacNaughton et al. (1999), Stephen et al. (1999), Juck et al. (2000) and Bundy et al. (2002). However, these studies used mainly topsoil to investigate the effect of hydrocarbons on soil microbial communities. Information about the microbial diversity of different soil layers at the given sites is lacking. Because oil contamination normally penetrates deeper than the top layer, it is important to understand the distribution of degrading populations with soil depth and how the distribution patterns influence the efficiency of biodegradation.

The subsurface soil environment, though devoid of sufficient nutrients, oxygen and other factors, harbours an array of soil microorganisms that play an important role in decomposition and the recycling of nutrients (Krumholz, 1998). It is widely presumed that the number of heterotrophic bacteria changes with depth. This can be attributed to spatial and resource factors that can influence the microbial diversity of the soil (Zhou et al., 2002). The shallow subsurface microbiota appears to be predominantly prokaryotic and specially adapted for growth and survival in nutrient-poor conditions; it includes strains that can function over a wide range of nutrient concentrations; and may sometimes exert a significant effect on groundwater chemistry (Ghiorse and Balkwill, 1983; Balkwill and Ghiorse, 1985; Bone and Balkwill, 1988; Ghiorse and Wilson, 1988; Balkwill et al., 1989).

The availability of hydrocarbons in the vadose zone can alter the diversity of the heterotrophic community

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owing to an increase in the carbon substrate. According to Atlas (1981) and Leahy and Colwell (1990), the number and relative abundance of hydrocarbon-degrading bacteria in the bacterial communities increases significantly in the presence of readily available hydrocarbons. Also the changes in hydrocarbon content in soil result in characteristic shifts of substrate utilisation patterns by the microorganisms, and the altered pattern of substrate utilisation corresponds to similar changes in abundance of hydrocarbons in the soils (Wünsche et al., 1995). This is not surprising, and in accordance with theories of gene accumulation and selection pressures we can predict a lower abundance of hydrocarbon degraders with depth, as selection pressure and favourable growth conditions in general decline with depth.

In this study, we investigated the hydrocarbon removal capacity and the microbial diversity of different soil layers after diesel contamination. The capacity of soil layers to remove hydrocarbons was evaluated (using simple microbial assays), while microbial diversity was evaluated from functional diversity (community-level physiological profiles using Biolog microplates) and genetic diversity (polymerase chain reaction-denaturing gradient gel electrophoresis, PCR-DGGE, of 16S rRNA).

2. Materials and methods

Soil: The contaminated soil layers were collected in sterile bags from a diesel-contaminated site at Coalsbrook, in the Free State Province, South Africa. The soil layers were collected 1 month after contamination by a leaking diesel pipeline. Direct push drilling to 2 m was used to sample the contaminated soil (cs) layers at a depth of 1 m (cs1m) and 1.5 m (cs1.5m). The contaminated topsoil layer (cts) was collected within 10 cm of the surface of the soil. Uncontaminated topsoil (ucts) was also collected from the same site. Samples were kept at 4 °C until analysis, which was completed within 24 h.

Microbiological analysis: To each of a series of 250-ml Erlenmeyer flasks containing 10 g soil, a 100 ml portion of 0.2% tetra-sodium pyrophosphate was added, and the flasks were shaken orbitally (140 rpm) for 45 min. The mixtures were then allowed to settle for 5 min and serial dilutions prepared in physiological saline solution before inoculating agar and Biolog GN plates. Total recoverable heterotrophs (TRHs) were enumerated on nutrient agar (Biolab Diagnostics) spread plates. Hydrocarbon-degrading bacteria were isolated as described by Margesin and Schinner (1999a), with diesel being the only source of carbon and energy. Both types of agar plate were incubated in triplicate at 28 °C and counted after 24 h and 7 d, respectively.

Carbon source utilisation pattern determination: Sample dilutions were prepared as described above. Heterotrophic plate count data were used to adjust the samples to similar cell density for Gram-negative Biolog plate inoculation, and a 100-µl sample was added to each well. The Biolog plates were read at 600 nm using a Bio-Tek Elx800 microreader (Bio-Tek Instruments Inc.) before incubation at 28 °C, and again after 24, 48 and 72 h, all in triplicate. Statistical analyses were performed using STATISTICA for Windows release 5.1.

Respiration rate determination: The biological activity of the different samples was evaluated by monitoring oxygen consumption using a Micro-Oxymax Respirometer (Columbus Instruments). A 100-g sample of each soil layer was added to a 250-ml bottle containing 10 ml mineral salts medium without carbon or energy source. Nutrients were added to the soil layers to stimulate bioremediation in hydrocarbon-contaminated layers, treatments in which no nutrients were added serving as controls. O_2 consumption was measured over 5 days. The composition of the nutrient solution was ($g l^{-1}$ medium): 10 Na_2HPO_4, 10 KH_2PO_4, 2.5 $(NH_4)_2SO_4$, 0.4 $MgSO_4$, 0.05 $CaCl_2 \cdot 2H_2O$, 0.0086 EDTA, 0.01 $FeSO_4 \cdot 7H_2O$, 0.004 $ZnSO_4 \cdot 7H_2O$, 0.01 $MnSO_4 \cdot H_2O$, 0.0015 $CuSO_4 \cdot 5H_2O$, 0.0008 $Co(NO_3)_2 \cdot 6H_2O$ and 0.0001 $(NH_4)_6Mo_7O_{24}.4H_2O$.

Chemical analysis: The contaminated soil layers were analysed in triplicate using the total petroleum hydrocarbons (TPH) method described in Margesin and Schinner (1999a) on 10 g samples.

DNA extraction and purification: Total DNA was isolated from the soil using the Bio101 extraction kit (Bio Inc.)

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 µm of each primer, 100 mM of each deoxy-nucleoside triphosphate, 5 µl 10 × PCR buffer, 0.25 µl hot start polymerase (5 U/µl) (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′ATTA CCGCGGCTGCTGG-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 cycles of denaturation (1 min at 94 °C), annealing (30 s at 51 °C), 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.

DGGE: 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 developed by Svein Norland (Department of Microbiology, University of Bergen), where the presence/absence of bands was recorded. Clustering was based on the simple matching algorithm, while the dendrogram was drawn applying the group average method.

2.1. Diversity indices

The Shannon index, H' (Shannon, 1948), was calculated (log 2) on the basis of biotypes defined in the cluster analysis on data retrieved from PCR-DGGE. The equitability J index (Pielou, 1966) was also calculated (Watve and Gangal, 1996).

3. Results

3.1. Microbiological analysis

The number of TRHs decreased with soil depth (Fig. 1). Similar results were obtained for the hydrocarbon-degrading bacteria. There was little difference in the number of TRHs and hydrocarbon-degrading bacteria in the cs1m and cs1.5m samples.

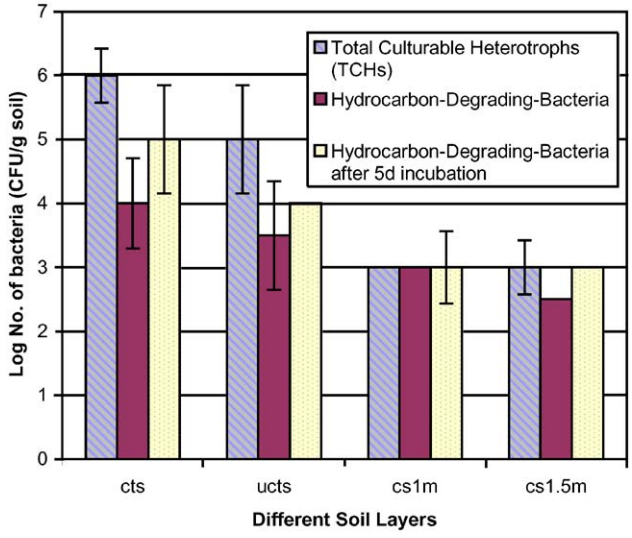

Fig. 1. Bacterial counts for different soil layers at a Coalsbrook diesel-contaminated site. Key: cts–contaminated topsoil; ucts–uncontaminated topsoil; cs1m–contaminated soil at 1 m; cs1.5m–contaminated soil at 1.5 m.

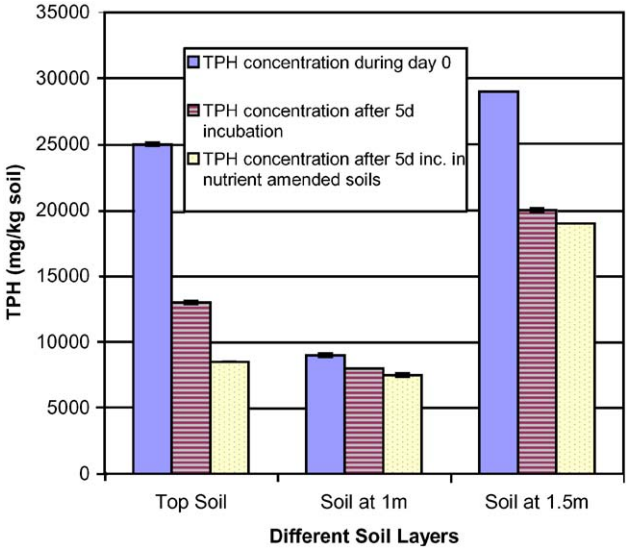

Fig. 2. Concentration of hydrocarbons in different soil layers at Coalsbrook.

The TPH concentration was highest in the cs1.5m layer, followed by the cts and then the cs1m layer (Fig. 2). However, TPH removal during the period of incubation reflected the difference in the number of bacteria in the samples, as it was higher in the topsoil than in the other soil layers.

There was no significant difference between TPH removal in the nutrient-amended treatments and the controls in the cs1m and cs1.5m layers (Fig. 2). Removal efficiency was 48% in the cts, compared with 31% and 11%, respectively, in the cs1.5 and cs1m layers. Soil layers with large numbers of recoverable hydrocarbon degrading bacteria had greater removal capacity than soil layers with low counts of such bacteria.

3.2. Respiration rate determination

The respiration rate reflected the difference in the number of bacteria in each soil layer and the availability of nutrients. The cts had the highest O_2 consumption rate (Fig. 3a). Where nutrients were added to the contaminated soil to stimulate the biological removal of hydrocarbons (Churchill et al., 1995; Braddock et al., 1997; Seklemova et al., 2001), the rate was also higher in the amended treatments than in unamended controls (Fig 3a, b). The respiration rate in ucts was low compared with the three contaminated soil layers. The respiration rate correlated positively with the high TPH removal rate (Fig. 2).

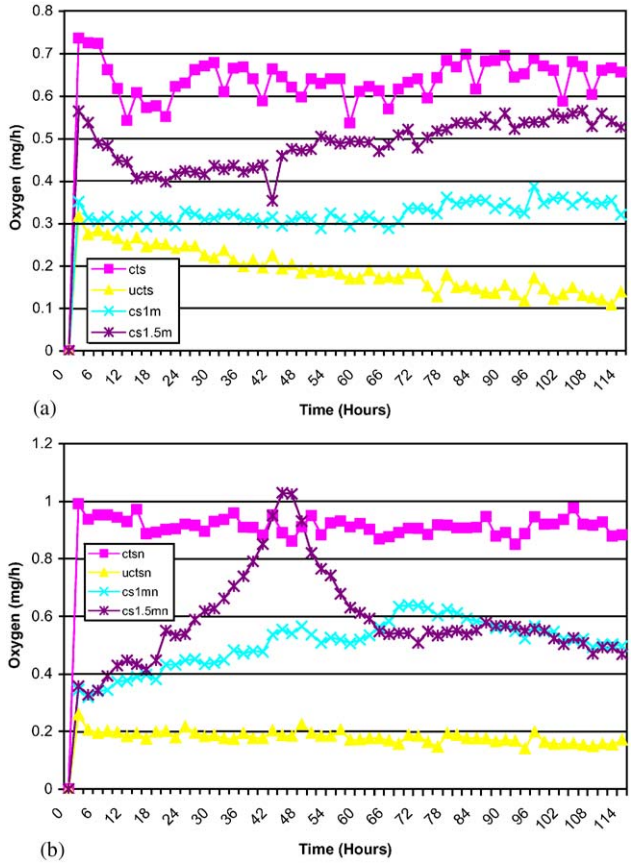

Fig. 3. Oxygen consumption by microorganisms in different layers of diesel-contaminated soil: (a) soil without added nutrients; (b) soil with added nutrients. Key: cts–contaminated topsoil, no nutrients added; ctsn–contaminated topsoil, nutrients added; ucts–uncontaminated topsoil, no nutrients added; uctsn–uncontaminated topsoil, nutrients added; cs1m–contaminated soil at 1 m, no nutrients added; cs1mn–contaminated soil at 1 m, nutrients added; cs1.5m–contaminated soil at 1.5 m, no nutrients added; cs1.5mn–contaminated soil at 1.5 m, nutrients added.

3.3. Biolog analysis

Principal component analysis (PCA) of the colour response data of the soil layers revealed different substrate utilisation patterns (Fig. 4).

PCA was performed to characterise the associations between samples, taking into account the absorbance values for all 96-response wells at different times of incubation. Two principal factors were isolated from the individual ucts, cts, cs1m and cs1.5m patterns that explained 58% of the variation. This low percentage variation, explained by the two factors, could be a result of the small number of samples used in the analysis. The use of more samples would probably improve the variation explained by the two factors. For the four samples, factor 1 was related to the absorbance values for the wells, while factor 2 was related to the incubation time.

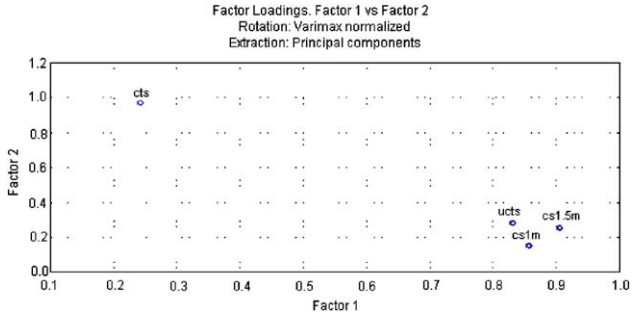

Fig. 4. Substrate utilisation pattern of soil layers as indicated by PCA. Key: as Fig. 1.

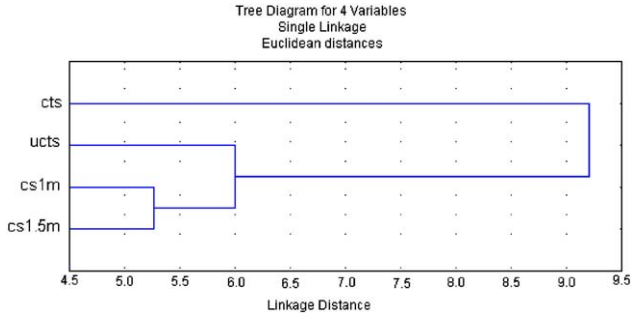

Fig. 5. Cluster analysis of different soil layers.

The relationship between the substrate utilisation patterns was further analysed using hierarchical clustering. In a dendrogram (Fig. 5), the results of cluster analysis also showed that the metabolic activities of cs1m were more 'closely' related to cs1.5m than to ucts and cts. The uncontaminated and contaminated soils differed in this respect.

Both the dendrogram and the PCA illustrate that the substrate utilisation pattern of the microbial communities in different soil layers differ.

3.4. Polymerase chain reaction-denaturing gradient gel electrophoresis

DGGE profiles of amplified 16S rDNA fragments from DNA extracted from the soil bacterial fractions revealed differences in the DNA fingerprint for the different soil layers. The profiles of the contaminated and uncontaminated samples were different (Fig. 6), with the profile of the cs1.5m being difficult to resolve.

Cluster analysis using a dendrogram revealed that cs1m was more closely related to cts than to ucts. The Shannon diversity index (H') revealed a higher diversity in topsoil compared with other soil layers (Table 1). The equitability (J') of all populations ranged from 0.73 to 0.95.

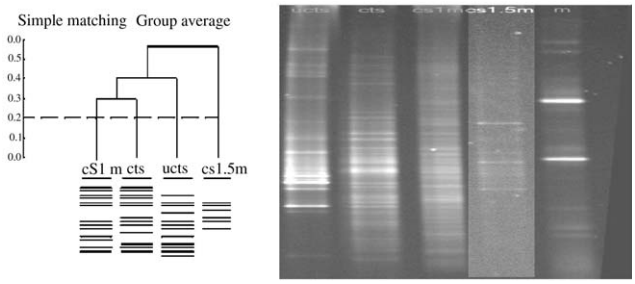

Fig. 6. (a) Cluster analysis of microbial communities at different soil layers and (b) DGGE fingerprints. Key: ucts–uncontaminated topsoil; cts–contaminated topsoil; cs1m—contaminated soil layer at 1 m; cs1.5m–contaminated soil layer at 1.5 m; m–marker. Lines beneath codes in (a) represent the bands detected.

Table 1
Diversity Indices

Soil type	*H′*	*J′*
ucts	2.120667	0.826787
cts	2.52069	0.909147
cs 1 m	1.981935	0.731868
cs 1.5 m	1.84471	0.947993

4. Discussion and conclusion

In this study, the number of TRHs and hydrocarbon-degrading bacteria decreased with soil depth. This can be attributed to nutrient and oxygen limitations to the biota of the soil subsurface. According to Zhou et al. (2002), spatial and resource factors influence microbial diversity in soil. Similar results were obtained with the hydrocarbon degrading bacteria after incubation for five days. However, there was little difference in the number of TRHs and hydrocarbon degrading bacteria in the 1- and 1.5-m samples, possibly owing largely to similarities of nutrient levels in the soil layers.

The high concentration of hydrocarbons at a depth of 1.5 m (Fig. 2) indicates the potential mobility of the pollutants to deeper soil layers. The removal of TPH reflected the number of bacteria in each soil layer, with removal being 48% in cts compared with 31% in cs1.5m and 11% in cs1m. Owing to the proximity of the 1.5 m layer to ground water at a depth of 2 m, microbial activity could consequently be higher at 1.5 m than at 1 m.

Since it is well known that different soil layers harbour different numbers of bacteria, it was expected that the soil layers would have a different pattern of substrate utilisation, especially as both PCA and cluster analysis revealed differences between the soil layers. The closer relationship of cs1m to cs1.5m than to ucts and cts (Fig. 5) can be attributed to the similarities in nutritional (organic matter, limiting nutrients) and environmental conditions (pH, temperature) of these 2 soil layers, but the closeness was not evident when clustering the DGGE profiles of the 16S rDNA fragments (Fig. 6).

The difference between ucts and cts evident in both substrate utilisation patterns and DGGE (Figs. 4 and 6) can be attributed to the changes in the composition of microbial populations brought about by changes in hydrocarbon content (Atlas et al., 1991; Wünsche et al., 1995). The higher diversity in the topsoil indicated by the Shannon diversity index (Table 1) can be ascribed to the relatively large amount of nutrients (organic matter and possibly limiting nutrients) in the topsoil than in the other soil layers. The diversity indices corroborated the observations on functional diversity; the high removal capacity of the topsoil can be attributed to a greater number of bacteria able to degrade the hydrocarbons.

As indicated earlier, the DGGE profiles of amplified 16S rDNA fragments for cs1.5m were difficult to resolve, however, the bands detected by the software program revealed few fragments relative to the other soil layers. The use of Archae primers instead of bacterial primers may enhance the chance of revealing the differences between the DGGE profiles of the different soil layers (Øvreås and Torsvik, 1998). The data on the functional and genetic diversity revealed that these two approaches to studying microbial diversity can complement each other, since the community-level physiological profiles (CLPP) measured the metabolic activities of different environmental samples while the PCR-DGGE provides information about the microbial structure. The data on CLPP and PCR-DGGE suggest that in combining the functional and genetic approaches there is the potential to provide a clear picture about the abundance of a variety of species in an ecosystem. Further studies are required in order to understand the effect of not only other pollutants but also the influence of soil components (pore volume, level of adsorbents and other environmental factors) on microbial diversity of different soil layers in both ‘shallow’ and deep aquifers. The results suggest that in hydrocarbon contaminated soil biological removal of hydrocarbons differs with soil layer, and also that the microbial diversity (as measured by CLPP and PCR-DGGE) varies with depth. Information about metabolic activities of different soil layers is critical when assessing the footprints of degradation processes during monitored natural attenuation (Smets et al., 2002).

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