Biodegradation (2007) 18:769–782
DOI 10.1007/s10532-007-9105-y

ORIGINAL PAPER

Metabolic and phylogenetic analysis of microbial communities during phytoremediation of soil contaminated with weathered hydrocarbons and heavy metals

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Received: 26 June 2006 / Accepted: 22 January 2007 / Published online: 20 March 2007

Abstract In the current study, the microbial ecology of weathered hydrocarbon and heavy metal contaminated soil undergoing phytoremediation was studied. The relationship of functional diversity, measured as carbon source utilisation in Biolog plates and extracellular enzymatic activities, and genetic diversity of bacteria was evaluated. Denaturing gradient gel electrophoresis was used for community analyses at the species level. Bulk soil and rhizosphere soil from pine and poplar plantations were analysed separately to determine if the plant rhizosphere impacted hydrocarbon degradation. Prevailing microbial communities in the field site were both genetically and metabolically diverse. Furthermore, both tree rhizosphere and fertilisation affected the compositions of these communities and increased activities of extracellular aminopeptidases. In addition, the abundance of alkane hydroxylase and naphthalene dioxygenase genes in the communities was low, but the prevalence of these genes was increased by the addition of bioavailable hydrocarbons. Tree rhizosphere communities had greater hydrocarbon degradation potential than those of bulk soil. Hydrocarbon utilising communities were dominated generally by the species *Ralstonia eutropha* and bacteria belonging to the genus *Burkholderia*. Despite the presence of viable hydrocarbon-degrading microbiota, decomposition of hydrocarbons from weathered hydrocarbon contaminated soil over four years, regardless of the presence of vegetation, was low in unfertilised soil. Compost addition enhanced the removal of hydrocarbons.

Keywords Denaturing gradient gel electrophoresis · Functional genes · Heavy metals · Hydrocarbons · Metabolic activity · Phytoremediation

Abbreviations

AMC	7-amino-4-methylcoumarin
HC	Hydrocarbon
MUF	4-methylumbelliferone (MUF)
UPGMA	unweighed pair group average clustering

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Introduction

Phytoremediation research has been intensive over the past two decades, driven by the need

for a low-cost, in situ alternative to the more expensive engineering-based remediation technologies. Phytoremediation uses green plants to remove, contain or render harmless environmental contaminants. Phytostabilisation refers to the in situ immobilisation of contaminants by use of vegetation (Vangronsveld et al. 1995; Salt et al. 1998). Plants native to the contaminated site are often used in phytostabilisation (Kremer 2005). Plants or plant-associated microflora can convert certain hydrocarbons (HC) to non-toxic forms (Cunningham and Berti 1993; Cunningham et al. 1995). During normal metabolism plant roots exude to the rhizosphere organic and inorganic substances (Anderson et al. 1993), which act as substrates for soil microorganisms thereby enhancing the degradation of toxic organic chemicals. The rhizosphere of trees may affect both the genetic composition (Tesar et al. 2002) and the functional abilities (Heinonsalo et al. 2000) of microbial communities of HC-contaminated soil.

Phytoremediation has been shown to remove aged petroleum hydrocarbons from soil (Porta et al. 1999; Hosler and Drake 1999; Hutchinson et al. 2001a, b). Soils polluted with multiple contaminants, organic as well as metals, may hold extreme challenges to the maintenance of phylogenetically and functionally diverse microbial communities (Shi et al. 2002) and metals may be inhibitory to organic-contaminant degrading microorganisms (for review see Sandrin and Maier 2003).

In the current study, weathered hydrocarbon- and heavy metal -contaminated soil was treated with phytoremediation. Microbial ecology of the soil was studied to determine the microbial role in hydrocarbon degradation and the microbes's heavy metal tolerance. Bulk soil and soil from the rhizospheres of pine and poplar trees were analysed separately to determine if the plant rhizosphere impacted on hydrocarbon degradation.

Materials and methods

Site description

Soil from a bus depot was transported to a municipal landfill and subsequently found to be contaminated with hydrocarbons and heavy metals originating from decades of bus maintenance. The 50 cm layer of contaminated soil was transferred to a field plot. The field plot base was prepared from layers of cement-stabilised peat ash, filter fabric, gravel and bentonite liner. Composted sieved biowaste ($0.11\ m^3/m^{2)}$ was added to two treatments while one treatment was left unfertilised. Scots pine (*Pinus sylvestris*) and poplar (*Populus deltoides x Wettsteinii*) were planted on two of the treatments, unfertilised soil and compost amended soil. A grass mixture (60% red fescue, *Festuca rubra*; 30% smooth meadow-grass, *Poa pratensis;* and 10% perennial ryegrass, *Lolium perenne*), and white clover (*Trifolium repens*) were sown over the entire plot. The field experiment lasted 39 months i.e four growing seasons.

Soil chemical analyses

Soil hydrocarbon concentration was determined according to Palmroth et al. (2006). Briefly, the method was modified from standard methods (ISO, 2000) and involved soil extraction with heptane and acetone. Soil dry matter was determined by oven drying at 105°C overnight and hydrocarbon concentrations were reported as mg hydrocarbons (kg dry soil)$^{-1}$. Total metal concentrations in soil were determined with nitric acid digestion in microwave oven (EPA, 1994) at the end of the first, second and fourth growing season.

Metabolic activities of microbial communities

The effects of green plants, applied soil amendment and contaminants, i.e., weathered and diesel fuel hydrocarbons as well as heavy metals, on the metabolic activity of soil microorganisms was studied by measuring carbon source utilisation and extracellular enzymatic activities. Soil samples were diluted 1:100 (mass/weight) with sterile deionised water (Milli-Q®) and shaken on a rotary shaker overnight. The soil solution was not buffered to better simulate enzyme activity under field conditions. Carbon source utilisation of soil microbial communities and diesel fuel degradation potential of soil microbial communities were assessed using the Biolog system according to Palmroth et al. (2005). Naphthalene and decane

utilisation were determined using similar procedures as described for diesel utilisation (Palmroth et al. 2005). Briefly, Biolog® ECO and MT2 plates (Biolog Inc., Hayward, CA) were inoculated with a soil suspension. The suitability of the assay for decane and naphthalene was tested with two known hydrocarbon degrading strains (*Pseudomonas oleovorans* possessing alkane monooxygenase gene and *Pseudomonas putida* G7 possessing naphthalene dioxygenase gene) and decane and naphthalene as carbon sources. Colonies of these strains were suspended in autoclaved 0.8% NaCl solution and the cell counts were determined with DAPI staining and epifluorescence microscopy in order to standardise the inoculation density added to MT2 plates. The utilisation of the volatile fraction of diesel fuel was used as an indicator of diesel fuel degradation. Carbon source (diesel fuel, naphthalene or decane) was allowed to evaporate into a desiccator atmosphere and Biolog MT2 plates without lids were incubated in the closed desiccator. Absorbance was measured at 590 nm with either a Victor™ Multilabel reader or Thermo Labsystems Multiskan Ascent reader. Data analyses, principal component analysis and calculation of parameters of diesel fuel, decane and naphthalene utilisation were conducted according to Palmroth et al. (2005).

The activity of extracellular enzymes was assessed in soil samples with a fluorometric method (Palmroth et al. 2005). The method uses fluorogenic model substrates, which upon hydrolysis release fluorogenic molecules, 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC). Enzymatic activity of 10 enzymes, listed in Table 1, involved in the hydrolysis of C, N and P compounds was determined. Usually alkaline and acidic phosphomonoesterase are determined separately, but since the pH in soil suspensions was near-neutral and both types could be active, no differentiation was made i.e pH of soil suspensions was not adjusted. Samples were excited at 355 nm prior to measurements. Fluorescence in wells was measured at 460 nm. The rates of hydrolysed enzyme substrate $\left[\frac{\mu\text{mol MUF}}{\text{l}\times\text{g soil}\times\text{h}}\right]$ or $\left[\frac{\mu\text{mol AMC}}{\text{l}\times\text{g soil}\times\text{h}}\right]$ were calculated for suspensions of dry soil and water (1 *g* of dry soil per 0.1 l water) to enable comparison of soil samples with different moisture content. The potential maximum rate of extracellular enzyme hydrolysis is referred in text as v^*_{max}. The term potential is used in this context due to the interference of the soil matrix to the assay. The v^*_{max} values were calculated from Michaelis–Menten plots (SigmaPlot 2001™ Enzyme Kinetics Module 1.1, SPSS Inc.).

Molecular analyses

Phylogenetic analyses of microbial communities in the rhizosphere soil of pine and poplar trees and bulk soil of the field plot as well as field soil suspensions grown in MT2 plates with volatile HCs were performed using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA genes. The occurrence of hydrocarbon degrading genes including naphthalene dioxygenase (*nah*Ac) (EC 1.14.12.12) and alkane hydroxylase (*alk*B) (monooxygenase) (EC 1.14.15.3) was assessed from soil DNA extracts and communities grown on MT2 plates with diesel, decane or naphthalene.

DNA extraction

Prior to the extraction of DNA from microbial communities in tree rhizospheres, loose soil was shaken from the roots and the roots were cut with sterile scissors on to a sterile petri dish. Extraction was performed with an Ultraclean™ soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA, US) using duplicates of approx. 0.25 g of roots or soil.

Cells were separated from Biolog MT2 plates as follows: 50 µl of cell suspension was transferred from the microplate wells to Eppendorf tubes. The tubes were centrifuged for 15 min at 14,000 × *g* to pelletise the cells. The pellets were subsequently washed with 50 µl of sterile 1 × PBS and centrifuged for 5 min at 14,000 × *g*. The washing step was repeated three times. Washed pellets were resuspended into 30 µl of sterile DNase- and RNase-free water and cells lysed with three boiling (95°C) and freezing (–20°C) cycles.

PCR

The phylogenetic analyses of field site and MT2 plate communities were conducted using

Table 1 Enzymes, substrates and the concentrations used in the extracellular enzyme (EE) activity experiments

Enzyme	EE-substrate	Substrate concentration (μM)
Phosphomonoesterase	4-MUF phosphate, free acid	0.5–200
Butyrate-Esterase	4-MUF butyrate	1–500
Acetate-Esterase	4-MUF acetate	2–1,000
α-Glucosidase	4-MUF-α-glucoside	1–500
β-Glucosidase	4-MUF-β-glucoside	1–500
Leucine aminopeptidase	L-leucine–7-AMC	1–500
Alanine aminopeptidase	L-alanine–7-AMC	1–500
Serine aminopeptidase	L-Serine-AMC	1–500
Galactosidase	4-MUF-β-galactose	1–500
Cellulase	4-MUF-cellobiose	0.5–200

PCR-DGGE with duplicate samples. Partial bacterial 16S rRNA genes were amplified using primer pair GC-BacV3f (Muyzer et al. 1993) and 907r (Muyzer et al. 1996). Forward primer included a GC-clamp (Muyzer et al. 1993). PCR mix contained 1 × reaction buffer IV, 1.5 mM $MgCl_2$, 100 μM dNTP, 2.5 U RedHot® DNA polymerase, 500 nM primers and 400 ng μl^{-1} of bovine serum albumin (Kreader 1996). PCR was performed using the following program: 95°C for 5 min, 31 cycles of 94°C for 0.5 min, 50°C for 1 min and 72°C for 2 min followed by 72°C for 10 min.

Amplification of a fragment of the *nah*Ac gene was performed with the primer pair Ac114f and Ac596R according to Wilson et al. (1999), except that the denaturation temperature was 95°C and final extension after the last cycle lasted 10 min. 20 μl reaction volumes were used and the PCR mixture per 100 μl was as follows: 1 × reaction buffer IV, 1.5 mM $MgCl_2$, 50 μM dNTP, 2.5 U RedHot® DNA polymerase (Abgene, Epson, U.K.) and 1 μM primers. *Pseudomonas putida* G7 possessing the naphthalene dioxygenase gene was used as the positive control. Amplification of a fragment of a the *alk*B gene was performed with a primer pair AlkB703f and AlkB1572R according to Whyte et al. (1996). The constituents of PCR mixture were as described above. *Pseudomonas oleovorans* possessing alkane hydroxylase gene was used as the positive control.

Denaturing gradient gel electrophoresis and sequencing

Denaturing Gradient Gel Electrophoresis was performed with Dcode System (Biorad laboratories, Hercules, CA, U.S.) using 8% polyacrylamide gels (acrylamide/bisacrylamide stock solution 37.5:1) with a denaturing gradient from 30% to 65% (100% denaturing solution contains 7 M of urea and 40% formamide). Gels were run 16 h in 1 × TAE at 60°C and stained with ethidium bromide (0.5 mg l^{-1}) in 1 × TAE. The dominant bands were excised from the gels, eluted in sterile H_2O and re-amplified for sequencing as described above with the exception that forward primer had no clamp. 16S rRNA genes were sequenced at the Institute of Biotechnology, University of Helsinki using BigDye (v.3.1) Terminator chemistry. Sequence data was analysed with Bioedit-software (version 7.0.5.2; Hall 1999) and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/). The existence of chimeras was analysed using CHIMERA_CHECK–software (version 2.7; Center for Microbial Ecology, Michigan State University [http://www.rdp.cme.msu.edu/cgis/chimera.cgi?su = SSU]). The DGGE profiles were compared using GelCompar II–software (Applied Maths, Gent, Belgium). Similarity matrixes of the banding patterns were made with the Dice equation and dendrograms were constructed using an unweighed pair group, average clustering (UPGMA).

Results and Discussion

Chemical analyses

The soil was originally contaminated by maintenance of public transportation buses. The initial soil hydrocarbon concentration was 11,400 ± 4,300 mg kg^{-1} of dry soil. Comparison

of the GC/MS chromatogram obtained from soil sample extract (Fig. 1) by GC/MS to chromatograms of fresh diesel fuel and lubricating oil indicated that about one third of mineral oil in soil was diesel fuel and two thirds were lubricating oils. However, the chromatogram indicated that the most easily degradable HCs had been removed before the phytoremediation study and the remaining HCs consisted mainly of unresolved complex matter (UCM). UCM compounds are considered recalcitrant (Chaineau et al. 1996) and they consist of polycyclic alkylated, saturated and aromatic HCs (Oudot 1984) as well as T-shaped molecules (Gough and Rowland 1990). HC concentrations were followed monthly during the four growing seasons. Hydrocarbon concentrations were reduced by approximately 60% in compost-amended soil and by the fourth growing season measured 3300 ± 2500 mg kg^{-1} of dry soil. On the other hand, in unfertilised soil the removal of hydrocarbons was not statistically significant ($P < 0.05$). However, hydrocarbon concentrations were reduced slightly, to 8,800 ± 5,500 mg kg^{-1} of dry soil, after the fourth growing season.

The soil contained relatively high concentrations of copper, lead, zinc, cadmium and barium (2,040 ± 5,860 mg kg^{-1}, 500 ± 700 mg kg^{-1}, 520 ± 270 mg kg^{-1}, 1.5 ± 0.6 mg kg^{-1} and 220 ± 40 mg kg^{-1}, respectively). The metal concentrations did not change significantly ($P < 0.05$) during the study and heavy metals did not accumulate into plant tissue (Palmroth et al. 2006).

Heavy metals can inhibit pollutant biodegradation (reviewed by Sandrin and Maier 2003) and addition of lead nitrate has been shown to decrease hydrocarbon degradation (Al-Saleh and Obuekwe 2005). The soil toxicity to *Vibrio fischeri* (bacterium) and *Enchytraeid albidus* (enchytraeid worm) was low despite the presence of both heavy metals and HCs, which might indicate low bioavailability of the contaminants (Palmroth et al. 2006) and low bioavailability of metals decreases inhibition to pollutant biodegration (Sandrin and Maier 2003).

Bacterial phylogeny

Prevailing field site communities and those utilising HCs in the MT2 plates were characterised

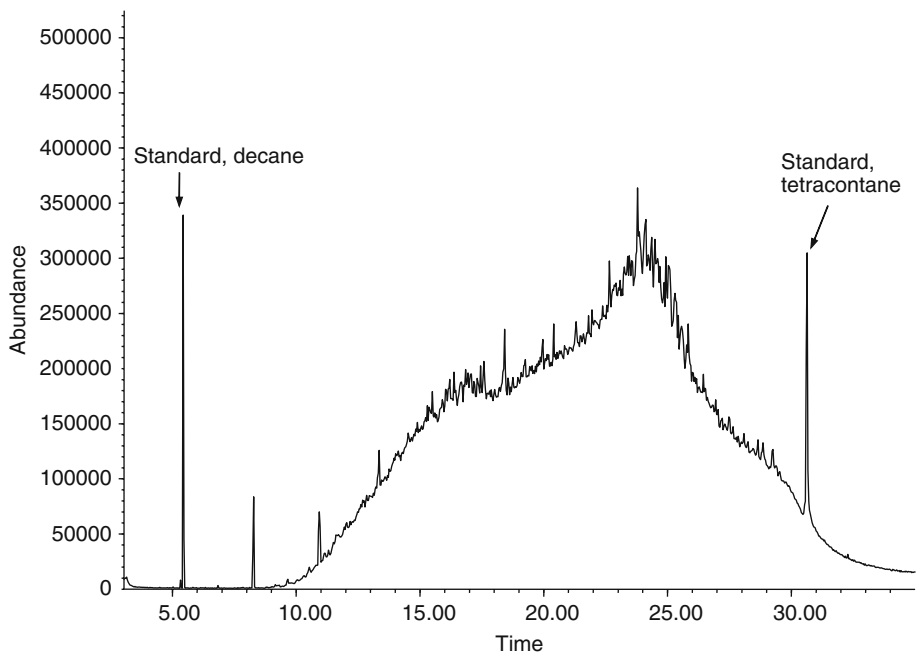

Fig. 1 Chromatogram (GC/MS) of soil extract of weathered hydrocarbon contaminated soil prior to field-scale phytoremediation study

phylogenetically using DGGE followed by band sequencing. DGGE analyses revealed a high diversity of bacterial communities prevailing in the field site (Fig. 2A, although diversity of species in a soil microbial communities may decrease as a result of heavy metal contamination stress (review by Bååth 1989). Due to the high diversity, species were not properly separated and, therefore, the majority (10 of 13) of DNA sequences in the DGGE bands were heterogenic disabling the characterisation of species. Three bands characterised, were closely related to *Burkholderia, Oxalobacter* and unidentified gamma proteobacterium (Table 2).

The diversity of bacterial communities was reduced when grown in MT2 plates with volatile hydrocarbons (Fig. 2B and C). Only a limited number of DGGE bands analysed contained sequence heterogeneity (17 of 19), and thus, most of the species involved in utilisation of volatile HCs were characterised (Table 2). With diesel utilising rhizosphere communities, bands 4 and 10 had 100% sequence similarity with *Burkholderia* sp. (AY367011), originally detected in a PAH-contaminated environment (Vacca et al. 2005). (Fig. 2B). Band 5 had 100% sequence similarity with the species *Ralstonia eutropha* (AF501365) originally detected from the rhizosphere of *Galega orientalis* in oil-contaminated soil. This species was present likely in all rhizosphere communities grown in MT2 plates with diesel. Band 8 was closely related to nitrogen fixing bacteria *Herbaspirillum lusitanum*. Additionally, unknown bacteria (bands 6 and 9) originally detected in the heavy metal contaminated environments and species distanty related to group Bacteroides (band 7) originating from waste-gas degrading biofilter were detected in rhizosphere communities grown in MT2 plates with diesel.

Species involved in the degradation of alkanes, small aromatic compounds and a mixture of HCs were studied with bulk and rhizosphere soil communities using MT2 plates and decane, naphthalene or diesel fuel as the carbon source. Bulk soil communities grown with diesel were less diverse as compared to those from rhizosphere,

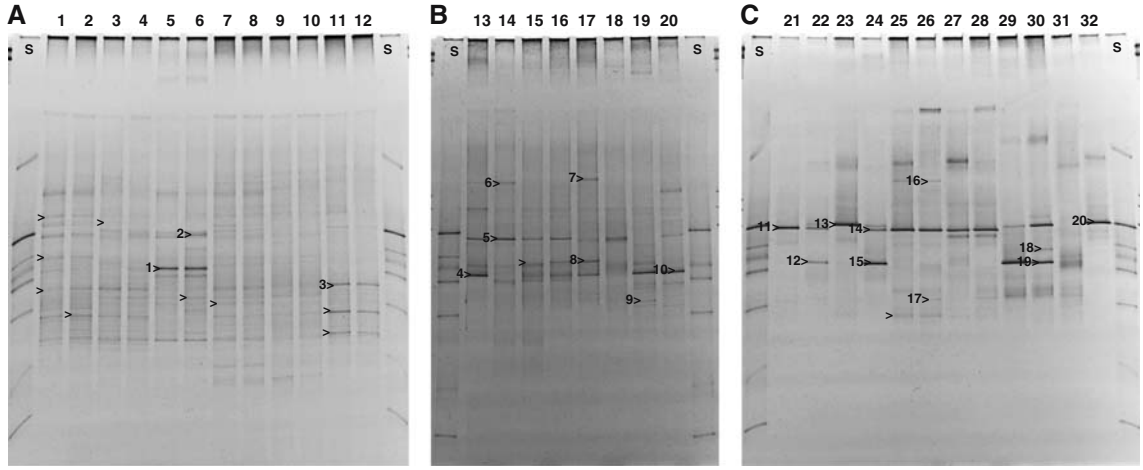

Fig. 2 DGGE profiles of bacterial communities from field site (**A**) and grown in MT2 plates with diesel, decane or naphthalene (**B** and **C**). Lanes 1–12: Communities prevailing in the field site. Lane numbers for unfertilised soil: 1 and 2 bulk soil, 3 and 4 rhizosphere of pine, 5 and 6 rhizosphere of pine, and for compost amended soil: 7 and 8 rhizosphere of pine, 9 and 10 rhizosphere of poplar, 11 and 12 bulk soil. Lanes 13–20 rhizosphere communities grown with diesel fuel as carbon source. Lane numbers for unfertilised soil: 13 and 14 rhizosphere of pine, 15 and 16 rhizosphere of poplar, and for compost amended soil: 17 and 18 rhizosphere of pine, 19 and 20 rhizosphere of poplar. Lanes 21–32 bulk soil communities grown with diesel, decane and naphthalene as carbon source. Lane number for diesel as carbon source: 21 and 22 unfertilised soil, 23 and 24 compost amended soil. Decane as carbon source: 25 and 26 unfertilised soil, 27 and 28 compost amended soil. Naphthalene as carbon source: 29 and 30 unfertilised soil, 31 and 32 compost amended soil. Lanes S: standard mix. > Bands sequenced with sequence heterogeneity

Table 2 Bacterial species identified from bands in denaturing gradient gel electrophoresis (DGGE) gels

Band[a]	Soil origin	Fertilisation	Enrichment	Identity[b]	Taxonomic group	Sim[c]	Source/description
1	Poplar rhizosphere	None	None	*Burkholderia* sp. (AY307923)	Betaproteobacteria	0.97 (537)	Carbon monoxide oxidizer
2	Poplar rhizosphere	None	None	*Oxalobacter* sp. (AJ496038)	Betaproteobacteria	0.99 (537)	Antarctic soil
3	Bulk soil	Compost	None	Uncultured gamma proteobacterium (AJ581598)	Gammaproteobacteria	0.99 (560)	Heavy metal contaminated environments
4	Pine rhizosphere	None	MT2 diesel	*Burkholderia* sp. (AY367011)	Betaproteobacteria	1.00 (535)	PAH contaminated environment
5	Pine rhizosphere	None	MT2 diesel	*Ralstonia eutropha* (AF501365)	Betaproteobacteria	1.00 (532)	Oil-contaminated rhizosphere of *Galega orientalis*
6	Pine rhizosphere	None	MT2 diesel	Uncultured gold mine bacterium (AF337873)	Unknown	0.95 (514)	Heavy metal contaminated environment
7	Pine rhizosphere	Compost	MT2 diesel	Uncultured *Bacteroidetes* bacterium (AJ318191)	Bacteroidetes	0.93 (543)	Waste gas-degrading biofilter community
8	Pine rhizosphere	Compost	MT2 diesel	*Herbaspirillum lusitanum* (AF543312)	Betaproteobacteria	0.99 (540)	Nitrogen fixing root nodule bacteria
9	Poplar rhizosphere	Compost	MT2 diesel	uncultured beta proteobacterium (AJ58219)	Betaproteobacteria	0.99 (540)	Heavy metal contaminated environment
10	Poplar rhizosphere	Compost	MT2 diesel	*Burkholderia* sp (AY367011)	Betaproteobacteria	1.00 (535)	PAH contaminated environment
11	Bulk soil	None	MT2 diesel	*Ralstonia eutropha* (AF501365)	Betaproteobacteria	1.00 (532)	Oil-contaminated rhizosphere of *Galega orientalis*
12	Bulk soil	None	MT2 diesel	*Burkholderia* sp (AY367011)	Betaproteobacteria	1.00 (535)	PAH contaminated environment
13	Bulk soil	Compost	MT2 diesel	*Burkholderia* sp. (AF452132)	Betaproteobacteria	1.00 (535)	Agricultural soil
14	Bulk soil	Compost	MT2 diesel	*Ralstonia eutropha* (AF501365)	Betaproteobacteria	1.00 (532)	Oil-contaminated rhizosphere of *Galega orientalis*
15	Bulk soil	Compost	MT2 diesel	*Burkholderia* sp. (AF247495)	Betaproteobacteria	0.99 (529)	Petroleum HC and PAH contaminated soil, phenanthrene degradation

Table 2 continued

Band[a]	Soil origin	Fertilisation	Enrichment	Identity[b]	Taxonomic group	Sim[c]	Source/description
16	Bulk soil	None	MT2 decane	uncultured gold mine bacterium (AF337873)	Unknown	0.95 (522)	Heavy metal contaminated environment
17	Bulk soil	None	MT2 decane	*Stenotrophomonas maltophilia* strain (X95924)	Gammaproteobacteria	0.98 (542)	Laboratory strain
18	Bulk soil	None	MT2 naphthalene	*Burkholderia* sp. (AF068011)	Betaproteobacteria	0.99 (536)	PAH degrading bacteria
19	Bulk soil	None	MT2 naphthalene	*Burkholderia* sp. (AF068011)	Betaproteobacteria	1.00 (542)	PAH degrading bacteria
20	Bulk soil	Compost	MT2 naphthalene	*Burkholderia* sp. (AF452132)	Betaproteobacteria	1.00 (529)	Agricultural soil

[a] Band number in Fig. 2

[b] Closest match of DGGE band sequence obtained with BLAST-search with accession number in parenthesis

[c] Sequence similarity with number of matching basepairs to the closest match of DGGE band sequence obtained with BLAST-search in parenthesis

based on the DGGE patterns. *R. eutropha* (bands 11 and 14) and species belonging to the genus *Burkholderia* (bands 12, 13, 15) dominated the diesel grown bulk soil communities. Bands 11 and 14 had similar 16S rRNA gene sequence with band 5 and band 12 with those of bands 4 and 10, respectively, detected from rhizosphere communities grown with diesel. Bands 13 and 15 had a 100% sequence similarity with bacteria belonging to the genus *Burkholderia* from agricultural soil (Goris et al. 2002) and petroleum HC and PAH contaminated soil (Friedrich et al. 2000), respectively.

When decane was used as a volatile carbon source, *R. eutropha* was likely the dominant species extracted from the MT2 plate suspensions, of both unfertilised and compost amended soil. Additionally, bacteria related to *Stenotrophomonas maltophilia* (band 17) and unknown bacteria originating from metal contaminated environments (band 16) were detected. *S. maltophilia* has been detected earlier in HC contaminated soil (Vacca et al. 2005). In contrast to communities grown with decane, *Burkholderia* related strains (bands 18–20) dominated the naphthalene utilising bulk soil communities while *R. eutropha* was not seen in the DGGE profiles. These results indicate that *R. eutropha* utilised decane and *Burkholderia* related strains utilised naphthalene. Genus *Burkholderia* is known to include PAH degrading species (Juhasz et al. 1997). Diesel, a mixture of HCs, induced the growth of *R. eutropha* and *Burkholderia* strains, indicating the presence of both alkane and aromatic HC degrading species. In summary, microbial communities in field site were diverse. Potential decane, naphthalene and diesel degrading organisms were identified from field site communities by growing them in MT2 plates with volatile HCs. The species detected in MT2 plates were typical of both HC and heavy metal contaminated environments.

The differences in bacterial community structures between different rhizosphere and bulk soil communities of the field site and MT2 plates were analysed using cluster analysis of DGGE profiles. Fig. 3 shows the UPGMA dendrogram of the prevailing field site communities. Field communities were heterogeneous: with the exception of

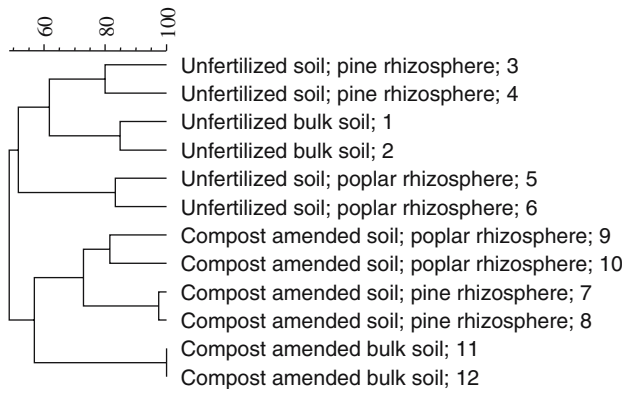

Fig. 3 Cluster analysis of field site bacterial communities using Dice algorithm and unweighed pair group, average clustering (UPGMA) of DGGE profiles. Numbers in sample titles refer to lane numbers in Fig. 2. Scale bar represents similarity percentages of the profiles

communities derived from compost-amended bulk soil, parallel communities had different compositions. However, parallel communities clustered together in every case. Therefore, both tree rhizosphere and compost amendment selectively enriched the microbial communities. Soil type seemed to be the main selective factor in this experiment, because communities from compost-amended soil clustered away from those in soil with no amendments. In addition, in compost-amended soil, rhizosphere communities clustered away from those in bulk soil. Also Gremion et al. (2004) reported that vegetation changed DGGE patterns of bacterial 16S rRNA during phytoremediation of heavy metal contaminated soil. Rhizosphere communities grown with diesel in MT2 plates clustered away from bulk soil communities grown with diesel, decane and naphthalene (Fig. 4). This indicates the differences in the volatile HC utilising community from rhizosphere and bulk soil. As with field communities, the diesel utilising rhizosphere communities in MT2 plates clustered according to soil type. Bulk soil communities grown with decane clustered

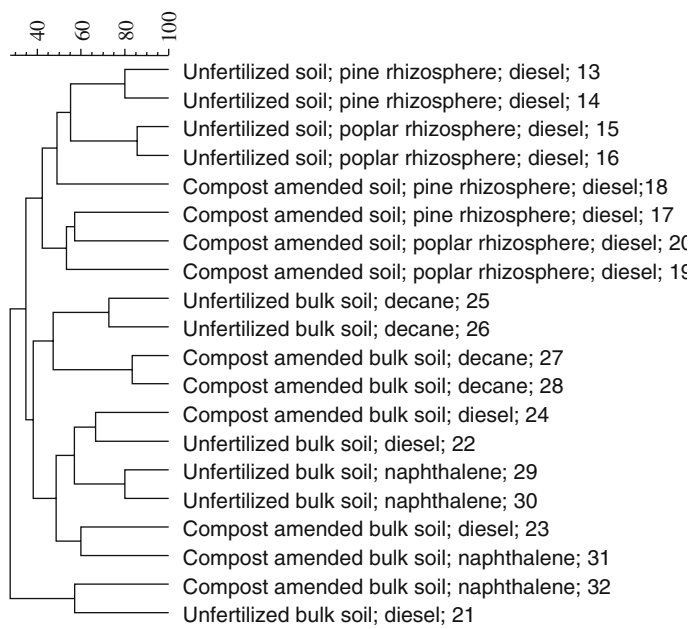

Fig. 4 Cluster analysis of rhizosphere and bulk soil bacterial communities grown in MT2 plates with HCs using dice algortihm and unweighed pair group, average clustering (UPGMA) of DGGE profiles. Soil and rhizophere suspensions were grown in MT2 plates with diesel, decane or naphthalene. Numbers in sample titles refer to lane numbers in Fig. 2. Scale bar represents similarity percentages of the profiles

together and according to soil fertilisation regime. Diesel and naphthalene grown communities were mixed and neither clustered according to carbon source nor to soil type; however, the species identification indicated that the selection of carbon source clearly favored the growth of certain species.

Presence of alkane hydroxylase and naphthalene dioxygenase genes

The prevalence of two genes involved in hydrocarbon degradation, alkane hydroxylase (*alk*B) and naphthalene dioxygenase (*nah*Ac), was studied to estimate the degradation efficiency and potential of soil microbial communities (Whyte et al. 1996; Siciliano et al. 2003). *NahAc* genes were in low abundance in rhizosphere soil and the *alkB* gene was not detected in field site microbial communities (Table 3). When soil suspensions were incubated in the MT2 plates with volatile diesel HCs, *alk*B and *nah*Ac genes were enriched (Table 3). On the other hand, *alk*B and *nah*Ac genes were not detected in every sample, even though the communities were utilising volatile HCs based on the MT2 plate color development. This suggests that the some of the HC utilising bacteria in the MT2 plates did not carry classical *alk*B or *nah*Ac genes (Whyte et al. 1997; Lloyd-Jones et al. 1999; Zocca et al. 2004) or that alkane and naphthalene degradation pathways were not active in the field site communities. Lack of these functional genes is likely due to the low concentrations or low bioavailability of compounds degraded via these pathways due to diesel weathering in soil. E.g. a*lk*B primers mainly target the genes involved in the degradation alkanes ranging from C5 to C12 (Whyte et al. 1997), which are volatile and easily degradable. However, the enrichment of *alk*B and *nah*Ac genes by fresh HCs indicates the HC degradation potential of the field site bacteria.

Metabolic activity of soil microbial communities

Substrate utilisation patterns obtained in Biolog® microbial community analysis can be used to classify microbial communities in environmental samples and characterise their community level physiological profiles, and have been used to monitor effects of soil contamination with hydrocarbons (Dobler et al. 2000; Wünsche et al. 1995), pesticides (El Fantroussi et al. 1999), and heavy metals (Dobler et al. 2000) as well as effects of soil vegetation (Baudoin et al. 2001; Palmroth et al. 2005). Soil microbial communities utilised most of the 31 carbon sources present in Biolog ECO plates, thus indicating a metabolically diverse soil microbial community (results not shown). Colour developed rapidly, i.e., within 48 h in inoculated Biolog plates, indicating a high density of active microbial populations (Konopka et al. 1998). Generally, the carbon source utilisation patterns of principal component analysis of

Table 3 Screening of *alk*B and *nah*Ac genes from bacterial communities in field site (2 parallel samples). Functional genes were detected either directly in soil DNA extracts (3 replicates) or in field site soil suspensions grown on Biolog MT2 plates (2 replicates)

Soil origin and treatment	Soil fertilisation	*Alk*B detection			*Nah*Ac detection		
		Pine rhizosphere	Poplar rhizosphere	Bulk soil	Pine rhizosphere	Poplar rhizosphere	Bulk soil
Soil DNA extract	None	–	–	–	–	+	–
Soil DNA extract	Compost	–	–	–	–	–	–
MT2 plate with diesel	None	+	+	+	+	–	–
MT2 plate with naphthalene	None	NS	NS	NS	NS	NS	+
MT2 plate with decane	None	NS	NS	+	NS	NS	NS
MT2 plate with diesel	Compost	+	–	–	–	+	+
MT2 plate with naphthalene	Compost	NS	NS	NS	NS	NS	+
MT2 plate with decane	Compost	NS	NS	+	NS	NS	NS

+ = positive signal,– = negative signal, NS = not studied

Biolog ECO plates were unable to detect differences within treatments; however, when the results of individual sampling times were analysed, compost-amended soil was clustered apart from unfertilised soil (results not shown), similar to DGGE patterns (Fig. 3).

Plants and microorganisms release extracellular enzymes to soil during normal metabolic activities. Soil enzymes are involved in the decomposition of organic compounds and the detoxification of xenobiotics (Margesin et al 1999). Land management (Kandeler et al 1999; Naseby and Lynch 2002, Marcote et al. 2001; Crecchio et al. 2004), contamination (Brohon et al. 2001, Margesin et al. 1999; Margesin et al. 2000) and vegetation (Broughton and Gross 2000) may influence soil enzymatic activities. In the present study extracellular enzymatic activities were determined to assess the impacts of vegetation and fertilisation on the biological quality of soil and to determine if aged hydrocarbon contaminated soil contained metabolically active microorganisms. The potential maximum rates of extracellular enzyme hydrolysis of serine, leucine and alanine aminopeptidase, measured as v^*_{max}, were higher in compost-amended soil than in unfertilised soil (Table 4). Marcote et al. (2001) and Crecchio et al. (2004) also found that municipal solid waste compost addition enhanced microbial enzymatic activities. However, in the present study aminopeptidase activities were impacted by sampling time in addition to fertilisation. Aminopeptidase activities of soil samples collected in October, when grasses and legumes had become dormant, were higher, i.e., 4,400–1,7000 μmol AMC l^{-1} g^{-1} h^{-1}, than samples of May to September (100–2,000 μmol AMC l^{-1} g^{-1} h^{-1}). Acetate esterase and butyrate esterase activities were slightly higher in soil amended with compost (20,000–35,000 μmol MUF l^{-1} g^{-1} h^{-1} and 12,000–47,000 μmol MUF l^{-1} g^{-1} h^{-1}, respectively) than in non-fertilised treatments (17,000–32,000 μmol MUF l^{-1} g^{-1} h^{-1} and 4,900–12,000 μmol MUF l^{-1} g^{-1} h^{-1}, respectively). Fertilisation impacted activities of cellulase, phosphomonoesterase, β-galactosidase, α- and β-glucosidase activities, measured as v^*_{max}, at some sampling times, but their activities were more impacted by season than by fertilisation (results not shown).

Of the two bacterial strains tested, *Pseudomonas putida* G7 grew well in the MT2 plates and its maximum specific growth rate was $2.37 \cdot 10^{-2}$ h^{-1}. *Pseudomonas oleovorans* did not grow in the MT2 plates on decane, although similar inoculation density was used as with *Pseudomonas putida* G7. The colour development of soil suspension grown on decane was at same magnitude as with diesel fuel and naphthalene indicating that some bacteria were able to utilise decane when grown in MT2 plates. Diesel fuel utilisation potential in Biolog MT2 plates inoculated with a soil suspension was enhanced by compost addition compared to non-amended soil (Table 5). Furthermore, of the unfertilised soil samples, diesel fuel utilisation was higher in the rhizosphere soil suspensions. However, in compost-amended soil, diesel fuel utilisation was at the same scale in rhizosphere and bulk soils. Naphthalene utilisation was enhanced by the presence of vegetation, but fertilisation did not impact its utilisation. Decane utilisation was higher in the soil suspensions from poplar rhizosphere than in soil suspensions originating from bulk and pine rhizosphere soils. These results suggest that the presence of

Table 4 Extracellular enzymatic activities of serine, leucine and alanine aminopeptidases, measured as v^*_{max} in soil suspensions from soil undergoing phytoremediation at field-scale

Field plot quarter	Aminopeptidase of (μmol AMC l^{-1} g^{-1} h^{-1})								
	serine			leucine			alanine		
	Min	Max	Average	Min	Max	Average	Min	Max	Average
no additions + trees, grasses and clover	100	4,800	1,700	120	6,300	2,100	200	11,000	3,600
compost + grasses and clover	80	10,000	2,900	140	11,000	3,800	100	17,000	5,600
compost + trees, grasses and clover	90	8,100	2,600	150	7,800	3,200	110	11,000	4,400

Table 5 Maximum specific growth rates ($\mu_{m,}$ 1/h) of diesel fuel utilisation in Biolog MT2 plates inoculated with soil suspension

Type of soil	Maximum specific growth rate (h^{-1}) on carbon source		
	Diesel fuel	Decane	Naphthalene
Unfertilised unvegetated soil	$5.0 \cdot 10^{-4}$	$1.2 \cdot 10^{-3}$	$2.0 \cdot 10^{-4}$
Unfertilised bulk soil	$4.3 \cdot 10^{-4}$	$3.7 \cdot 10^{-3}$	$3.7 \cdot 10^{-2}$
Unfertilised poplar rhizosphere	$2.7 \cdot 10^{-3}$	$4.5 \cdot 10^{-3}$	$5.3 \cdot 10^{-2}$
Unfertilised pine rhizosphere	$3.9 \cdot 10^{-3}$	$3.7 \cdot 10^{-3}$	$3.7 \cdot 10^{-3}$
Compost-amended bulk soil	$4.2 \cdot 10^{-3}$	$2.6 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$
Compost-amended poplar rhizosphere	$4.7 \cdot 10^{-3}$	$7.7 \cdot 10^{-3}$	$6.7 \cdot 10^{-3}$
Compost-amended pine rhizosphere	$3.9 \cdot 10^{-3}$	$3.0 \cdot 10^{-3}$	$1.6 \cdot 10^{-2}$

vegetation enhances degradation of these hydrocarbons. Palmroth et al. (2005) found that the utilisation of diesel fuel by soil bacteria in the Biolog MT2 plate was higher in soil contaminated with diesel fuel than utilisation of diesel fuel in uncontaminated soil, especially in the presence of pine or poplar.

Conclusion

Prevailing microbial communities in the contaminated field site were both genetically and metabolically diverse. Furthermore, both vegetation and compost addition affected the compositions of these communities. In addition, *alk*B and *nah*Ac genes were in low abundance in the communities, but the abundances of these genes were enriched by the addition of bioavailable HCs. Further, compost addition increased microbial metabolic activities.

Tree rhizosphere communities had greater hydrocarbon degradation potential than those of bulk soil. Bacterial species found in soil were typical of hydrocarbon and/or metal polluted soil. Furthermore, the compositions of volatile hydrocarbon utilising communities in MT2 plates were dependent on the carbon source used. The diesel degrading communities consisted of both alkane- and aromatic- degrading species. Despite the presence of viable hydrocarbon-degrading microbiota, confirmed with Biolog MT2 plates, bacterial strains with known HC degradation capabilities and vegetation in weathered hydrocarbon contaminated soil, decomposition of hydrocarbons was low in unfertilised soil over four years. Compost addition enhanced the decomposition and removal of hydrocarbons.

Acknowledgements Neste Foundation, Finland, funded the study and Tampere Regional Solid Waste Management Ltd. is thanked for the construction of the field plot. We thank Chiara Truccolo for technical assistance.

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