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# Impact of bacterial and fungal processes on $^{14}$C-hexadecane mineralisation in weathered hydrocarbon contaminated soil

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ABSTRACT

In this study, the impact of bacterial and fungal processes on $^{14}$C-hexadecane mineralisation was investigated in weathered hydrocarbon contaminated soil. The extent of $^{14}$C-hexadecane mineralisation varied depending on the bioremediation strategy employed. Under enhanced natural attenuation conditions, $^{14}$C-hexadecane mineralisation after 98 days was 8.5 ± 3.7% compared to <1.2% without nitrogen and phosphorus additions. $^{14}$C-hexadecane mineralisation was further enhanced through Tween 80 amendments (28.9 ± 2.4%) which also promoted the growth of a *Phanerochaete chyrsosporium* fungal mat. Although fungal growth in weathered hydrocarbon contaminated soil could be promoted through supplementing additional carbon sources (Tween 80, sawdust, compost, pea straw), fungal $^{14}$C-hexadecane mineralisation was negligible when sodium azide was added to soil microcosms to inhibit bacterial activity. In contrast, when fungal activity was inhibited through nystatin additions, $^{14}$C-hexadecane mineralisation ranged from 6.5 ± 0.2 to 35.8 ± 3.8% after 98 days depending on the supplied amendment. Bacteria inhibition with sodium azide resulted in a reduction in bacterial diversity (33–37%) compared to microcosms supplemented with nystatin or microcosms without inhibitory supplements. However, a*lkB* bacterial groups were undetected in sodium azide supplemented microcosms, highlighting the important role of this bacterial group in $^{14}$C-hexadecane mineralisation.



## 1. Introduction

Hydrocarbon contamination of soil is well studied in terms of its toxic effects on soil ecological processes, macro and microorganisms and associated human health risks due to the presence of recalcitrant fractions such as long-chain aliphatic and aromatic hydrocarbons (Keith and Telliard, 1979; Peng et al., 2009; Sheppard et al., 2011). Although soil microbial groups are capable of degrading a variety of hydrocarbons (Boonchan et al., 2000; Genovese et al., 2008; Wu et al., 2008) this process is often slow, with bioremediation timeframes of months to years. This is due to a number of factors including environmental nutrient limitations, associated hydrocarbon toxicity and the presence of inhibitory substances such as co-contaminants (e.g. lead) which may inhibit the activities of autochthonous hydrocarbon degraders (Al-Saleh and Obuekwe, 2005).

Bioremediation is a well established cost effective and comparatively environmentally friendly method of treating/detoxifying hydrocarbon contaminated soils (Sanscartier et al., 2009). Bioremediation involves exploiting the natural capacity of microorganisms to degrade hydrocarbons by either stimulating this capacity in soils with nutrients and surfactants or by supplying microorganisms capable of degrading the contaminant when this capacity is limited (Bento et al., 2005). Prior to any successful bioremediation, it is often necessary to conduct treatability tests in order to determine the suitability of bioremediation strategies and to estimate the efficacy of the bioremediation process (Diplock et al., 2009). This may involve conducting small scale laboratory studies during which different parameters such as soil respiration, mineralisation rates, changes in hydrocarbon degrading populations and total petroleum hydrocarbon reduction are monitored and correlated with hydrocarbon removal (Diplock et al., 2009; Towell et al., 2011). The data generated from these experiments, combined with appropriate kinetic modelling, can then be used as a predictor for large scale field based studies (Beolchini et al., 2010; Diplock et al., 2009).

The detection of specific microbial groups or capacity (directly or indirectly) to degrade specific hydrocarbons is a measure of the bioremediation potential of a given matrix. Bacterial groups are thought to be the major mediators in hydrocarbon biodegradation (Andreoni et

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al., 2000; Song et al., 1986; Walker and Colwell, 1976; Whyte et al., 1997). Other reports have also indicated that bacteria play important roles in hydrocarbon mineralisation (Aislabie et al., 2008; Hilyard et al., 2008) despite the fact that fungi may also mineralize hydrocarbons (Volke-Sepulveda et al., 2006). Defining the roles of different soil microbial groups in a polluted environment is a challenge as the presence of an organism is not necessarily an indication of the expression of degradative capacity. Few investigations of microbial roles in hydrocarbon degradation have been performed by specifically inhibiting microbial groups in order to clearly define their roles in the biodegradation process.

To address this question, in this study we investigated the roles of different microbial groups in $^{14}$C-hexadecane mineralisation experiments by inhibiting either bacterial or fungal groups in laboratory based soil microcosms. In addition, the impact of these inhibitions on microbial community dynamics was assessed using culture independent molecular microbiology tools.

## 2. Materials and methods

### 2.1. Sampling and soil characterisation

Hydrocarbon contaminated soil was sampled from a former oil refinery site in Australia. Historically contaminated soil (~30 kg) was collected from stockpiled material on-site with a bulk soil sample being collected from the top 20 cm of the stockpile. The bulk soil (<2 mm) had an initial hydrocarbon concentration of 15 g kg$^{-1}$ ($C_{10}$–$C_{40}$): the concentration of various equivalent hydrocarbon molecular weight ranges and other soil properties is listed in Table 1. Determination of soil type was carried out using the methodology described by McDonald et al. (1990) while soil moisture content, water holding capacity, pH and organic matter content were determined using standard methods (Rayment and Higginson, 1992).

### 2.2. Total petroleum hydrocarbon analysis

An accelerated solvent extraction method (ASE200 Accelerated Solvent Extraction System, Dionex Pty Ltd, Lane Cove, NSW, Australia) was used to extract hydrocarbons from contaminated soils. Prior to use, 1 g of solvent washed silica gel (Davisil, Sigma-Aldrich Pty Ltd, Sydney, Australia) sandwiched between 2 cellulose filter circles was added to 11 mL ASE extraction cells. Inclusion of silica in the extraction step was found to assist in sample clean-up prior to GC analysis of hydrocarbon extracts (data not shown). Freeze-dried soil (2–10 g) was ground with diatomaceous earth (Dionex), weighed into extraction cells (on top of the silica layer) and surrogate (phenanthrene 100 mg mL$^{-1}$) added prior to sealing. Soils were extracted using standard conditions (150 °C, 10.34 MPa, static time 5 min) and a solvent mixture consisting of hexane:acetone (1:1 v/v). Soil extracts were concentrated to dryness under a steady flow of nitrogen gas, resuspended in 2 mL of hexane:acetone (1:1 v/v), filtered through 0.45 μm Teflon syringe filters into 2 mL GC vials (Agilent Technologies Australia, Forest Hills, VIC, Australia) prior to analysis.

Gas chromatograms of hydrocarbon extracts were generated using an Agilent Technologies 7890A gas chromatograph with flame ionisation detector. Samples were separated using a 15 m × 0.32 mm × 0.1 μm Zebron ZB-5HT (5% phenyl, 95% dimethylpolysiloxane) Inferno column with a 5 m × 0.25 mm inert guard column (Phenomenex Australia, Lane Cove, NSW, Australia). Operating conditions were as follows: The oven temperature was programmed at 40 °C for 3 min followed by a linear increase to 375 °C at 25 °C min$^{-1}$, held at 375 °C for 5 min. Injector and detector temperatures were maintained at 300 and 380 °C, respectively. Hydrocarbon concentration was quantified according to defined hydrocarbon fractional ranges ($C_{10–14}$, $C_{15–28}$, $C_{29–36}$, $C_{37–40}$) using Window defining standards (Accustandard Inc., New Haven, CT, USA). Hydrocarbon concentrations were reported per g freeze-dried soil. TPH concentration was quantified according to Dandie et al. (2010). Surrogate recovery during TPH quantification ranged from 94 to 103% while results of duplicate analysis of the same sample showed a standard deviation of less than 8%.

### 2.3. Mineralisation assays

Mineralisation of $^{14}$C-hexadecane by indigenous soil microorganisms in TPH-contaminated soil was determined in triplicate biometer flasks (Bellco Glass). Contaminated soil (50 g moistened to 60% water holding capacity) was supplemented with 1.0 μCi of $^{14}$C-hexadecane with and without the addition of soil supplements (nutrients, surfactants, additional carbon sources) (Table 2). Control flasks, to assess abiotic hexadecane mineralisation, consisted of contaminated soil to which 2% $HgCl_2$ was added. When inhibition of bacterial or fungal growth was required, sodium azide or nystatin was added at 10 and 2 g kg$^{-1}$ respectively. Soils were incubated at room temperature for up to 98 days and the evolution of $^{14}CO_2$ (trapped in 1 M NaOH) monitored routinely over the incubation period. Aliquots (1 ml) from $^{14}CO_2$ traps were combined with scintillation cocktail (ReadySafe, Beckman-Coulter, USA) and the samples were counted and quantified by liquid scintillation counting (Beckman LS3801) using standard counting protocols and automatic quenching correction (Macleod and Semple, 2002).

### 2.4. DNA extraction and PCR

Genomic DNA was extracted from replicate soil samples from non-labelled experiments using a DNA isolation kit (MoBio PowerSoil, Carlsbad, CA, USA) according to the manufacturer's protocol at selected

**Table 1**
Physico-chemical characteristics of soil used in this study.

| Property | Value |
|---|---|
| Soil type | Sandy loam |
| Sand, silt, clay (%) | 70, 14, 16 |
| Bulk density (g cm$^3$) | 1.50 |
| Moisture content | 17.0 ± 0.2 |
| pH (1:5, water) | 7.2 ± 0.1 |
| pH (1:5, $CaCl_2$) | 6.8 ± 0.2 |
| Organic matter (%, LOI) | 14.7 ± 0.7 |
| Nitrate (mg kg$^{-1}$) | <2.0 |
| Phosphate (mg kg$^{-1}$) | <2.0 |
| Sulphate (mg kg$^{-1}$) | 480 |
| TPH (mg kg$^{-1}$) | |
| $C_{10}$-$C_{14}$ | 103 |
| $C_{15}$–$C_{29}$ | 10 477 |
| $C_{29}$–$C_{36}$ | 4444 |
| $C_{37}$–$C_{40}$ | 533 |

**Table 2**
Treatments assessed during $^{14}$C-hexadecane mineralisation assays.

| Treatment | Amendments | | | | |
|---|---|---|---|---|---|
| | Nutrients | Surfactants | Sawdust | Compost | Pea Straw |
| NA | – | – | – | – | – |
| ENA[a] | C:N:P 100:5:0.5 | – | – | – | – |
| ENA–T80[b] | C:N:P 100:5:0.5 | 1% Tween80 | – | – | – |
| ENA–SD[c] | C:N:P 100:5:0.5 | – | 5% w/w | – | – |
| ENA-C[d] | C:N:P 100:5:0.5 | – | – | 25% w/w | – |
| ENA-PS[e] | C:N:P 100:5:0.5 | – | – | – | 5% w/w |

[a] Enhanced natural attenuation treatments were prepared by supplementing TPH-contaminated soil with nitrogen ($[NH_4]_2SO_4$) and phosphorus ($K_2HPO_4$, $KH_2PO_4$) to achieve a C:N:P molar ratio of 100:5:0.5.
[b] Tween 80 was added to ENA microcosms to achieve a 1% Tween 80 concentration in soil solution.
[c] Sawdust was added to ENA microcosms at a 5% w/w loading.
[d] Compost was added to ENA microcosms at a 25% w/w loading.
[e] Pea straw was added to ENA microcosms at a 5% w/w loading.

time points (day 0, 3, 5, 7, 14, 21, 28, 56 and 98 days) to evaluate the effect of the different inhibitors on microbial community dynamics. PCR amplification of 16S rDNA genes was performed using primer pair 341FGC and 518R (Muyzer et al., 1993) as described by (Sheppard et al., 2011). Alkane degrading communities (alkB genes in the lineage of *Pseuodmonas oleovorans GPo1*) were evaluated using TS2S, Deg1RE and deg1RE GC primers as described by (Smits et al., 1999) using a semi nested approach described by Makadia et al. (2011). DNA was extracted from pure fungal cultures using the phenol-chloroform-bead beating method and PCR carried out with ITS 1 and ITS 4 (Anderson and Parkin, 2007) using the following thermocycling conditions; 1 cycle at 95 °C for 5 min, 30 cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C and a final extension at 72 °C for 10 min.

### 2.5. DGGE and sequence analysis

DGGE was carried out on selected PCR amplicons on a Universal Mutation Detection System D-code apparatus (Biorad, CA, USA) with a 9% polyacrylamide gel using a 40–60% denaturing gradient at 60 °C for 20 h. DGGE gels were silver stained (Girvan et al., 2003), scanned and saved as Tiff files with an Epson V700 scanner. Digitised gel images were then analysed with TotalLab analysis package (Nonlinear Dynamics, USA). Unweighted Pair Group with Mathematical Averages (UPGMA) dendrograms were then generated with Totallab and bacterial community diversity determined with Shannon Weaver Diversity Index ($H'$) using the formula $-\sum p_i \, LN \, p_i$ (Girvan et al., 2003). Where necessary data were transformed and statistical significance was determined in replicate samples comparison by either $T$ test or analysis of variance (ANOVA) and Tukey tests (Sigma Stat 2.03, Systat, London). Bands of interests were excised and incubated in nuclease free water overnight at 70 °C and re-amplified with 341F-GC and 518R and their sequence identities determined as described in (Aleer et al., 2010).

## 3. Results and discussion

### 3.1. Hexadecane mineralisation under natural and enhanced natural attenuation conditions

The extent of $^{14}$C-hexadecane mineralisation by the indigenous soil microflora under natural attenuation and enhanced natural attenuation conditions is shown in Fig. 1. In unamended soils (natural attenuation), $^{14}$C-hexadecane mineralisation was negligible (~1.2% of the radiolabelled carbon was evolved as $^{14}CO_2$ after 98 days) indicating that microorganisms within the stockpiled soil were not capable of sustaining hydrocarbon mineralisation presumably due to nutrient deficiencies. In contrast, $^{14}$C-hexadecane mineralisation could be enhanced through the addition of nutrients (enhanced natural attenuation) with 8.5 ± 3.7% of $^{14}$C-hexadecane being mineralised after 98 days. Although the extent of $^{14}$C-hexadecane mineralisation was improved through the addition of nitrogen and phosphorus, presumably the limited $^{14}$C-hexadecane mineralisation was due to hydrocarbon bioavailability constraints. Contaminant and nutrient bioavailability are important factors that may limit hydrocarbon biodegradation in soils. Nitrogen and phosphorus are often limiting in hydrocarbon polluted environments and the addition of these nutrients have been shown to substantially enhance hydrocarbon mineralisation or degradation (Borresen and Rike, 2007; Roling et al., 2002).

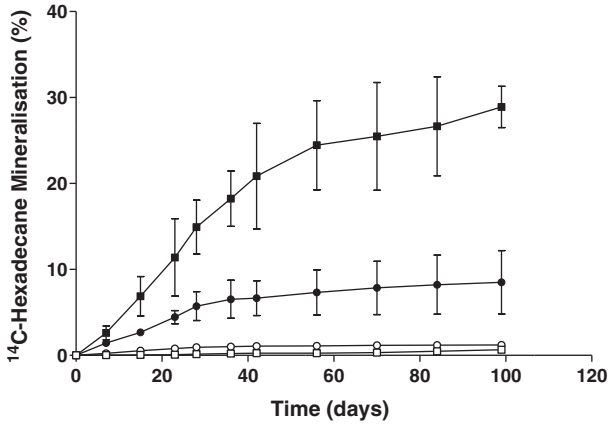


**Fig. 1.** $^{14}$C-hexadecane mineralisation in weathered TPH-contaminated soil incubated under natural attenuation conditions (○), in the presence of nutrients (enhanced natural attenuation; ●) and under enhanced natural attenuation conditions supplemented with 1% Tween 80 (■). $^{14}$C-hexadecane mineralisation is also shown for mercuric chloride-killed controls (□).

Hydrocarbon bioavailability will influence the rate and extent of ENA processes. If hydrocarbon desorption from the sorbed to the aqueous phase is slow, the rate of degradation will be limited by physico-chemical processes. The rate of desorption may, however, be enhanced through the application of surfactants which increase the solubility of hydrocarbons through micelle formation (Li and Chen, 2009). As a result, further studies were conducted in ENA soils to which Tween 80 (non-ionic surfactant) was amended to determine the impact of surfactant addition on the rate and extent of $^{14}$C-hexadecane mineralisation. In the presence of Tween 80, $^{14}$C-hexadecane mineralisation was enhanced compared to ENA alone, resulting in 28.9 ± 2.4% of the radiolabelled carbon being evolved as $^{14}CO_2$ after 98 days. Many reports have indicated that the addition of synthetic and non synthetic surfactants can improve hydrocarbon mineralisation and TPH removal from oil and sludge contaminated environments (Davezza et al., 2011; Lai et al., 2009; Tahhan and Abu-Ateih, 2009; Zhang et al., 2010). Hydrocarbon degrading microorganisms are known to produce biosurfactants which aid dispersion of hydrocarbons into small droplets (<0.22 μm) that are more accessible to microorganisms (Cameotra and Singh, 2009; Partovinia et al., 2010). Although the application of surfactants can inhibit degradation in some cases (Franzetti et al., 2010), the exogenously supplied surfactants in this study presumably increased the availability of $^{14}$C-hexadecane from soil complexes, improving microbial access resulting in an increase in the extent of $^{14}$C-hexadecane mineralisation.

In addition to enhancing $^{14}$C-hexadecane mineralisation in stockpile soil, the addition of Tween 80 promoted the growth of a fungal mat with the mat being identified as *Phanerochaete chrysosporium* (data not shown). White rot fungi, as observed in Tween 80 augmented soils, have been shown to have the ability to degrade a variety of environmental pollutants (Faraco et al., 2009; Gao et al., 2010) as a result of the production of non-specific extracellular enzymes used for lignin degradation. However, the role of fungi (versus bacteria) in the mineralisation of $^{14}$C-hexadecane in stockpiled soils was unclear. As a result, subsequent experiments were designed to determine fungal and bacterial roles on $^{14}$C-hexadecane mineralisation.

### 3.2. Bacterial and fungal roles in hexadecane mineralisation

The presence of a fungal mat in surfactant amended microcosms raised the possibility that soil fungi were important in $^{14}$C-hexadecane mineralisation as soil fungi such as *Aspergillus* spp. and *Phanerochaete chrysosporium* have been implicated in hydrocarbon (hexadecane) degradation (Kanaly and Hur, 2006; Volke-Sepulveda et al., 2006). Earlier reports by Walker and Colwell (1976) and Song et al. (1986) suggested that soil bacteria rather than fungi were responsible for significant hexadecane mineralisation but these studies were conducted on soil without any amendment to promote fungal growth. Therefore, in this study the extent of $^{14}$C-hexadecane mineralisation was determined in soil amended with sodium azide (to inhibit bacterial growth) or nystatin (to inhibit fungal growth) to determine the role of bacteria and fungi

in hydrocarbon biodegradation. In addition, $^{14}$C-hexadecane mineralisation was determined in soils without the addition of inhibitory substances to determine the impact of bacterial-fungal co-cultures on $^{14}$C-hexadecane mineralisation. In order to stimulate fungal growth, soil was amended with a variety of carbon sources, namely Tween 80, sawdust, compost and pea straw.

The addition of Tween 80, sawdust, compost and pea straw to stockpile soil (in the absence of nystatin) resulted in the enhanced growth of indigenous fungal species based on visual observations. In the presence of nystatin (an inhibitor of fungal activity), fungal growth was not observed. When sodium azide was added to contaminated soil to inhibit bacterial activity, $^{14}$C-hexadecane mineralisation was negligible (Fig. 2) indicating that fungal species, although able to grow on the amended carbon sources, were unable to mineralise hexadecane. Conversely, when fungal growth was inhibited due to the addition of nystatin, bacterial $^{14}$C-hexadecane mineralisation ranged from $6.5 \pm 0.2$ to $35.8 \pm 3.8\%$ (after 98 days) depending on the supplied amendment (Fig. 2). Excluding Tween 80 addition, there was no significant difference ($P<0.05$) in $^{14}$C-hexadecane mineralisation between soil microcosms amended with different carbon sources (sawdust, compost, pea straw). The decreased bacterial $^{14}$C-hexadecane mineralisation in the sawdust, compost, pea straw amended microcosms may be due to the preferential or co-utilisation of the additional carbon supplied in the amendments over the target substrate (hexadecane). In the absence of sodium azide and nystatin (i.e. no inhibition of bacterial or fungal species), $^{14}$C-hexadecane mineralisation as a result of bacterial-fungal co-culture activity was similar to that observed for bacteria alone (Fig. 2) indicating that $^{14}$C-hexadecane mineralisation as a result of fungal activity was negligible. Consequently, the observed fungal mat was not responsible of the enhanced $^{14}$C-hexadecane mineralisation following Tween 80 addition with the development of the mat due to fungal growth on the supplied carbon.

### 3.3. Bacterial community response to inhibition

The soil microbial community exists in a dynamic environment in which there are different interactions between biotic and abiotic components of the environment. Any alteration in a component may have positive or negative effects on other components. As a result, the bacterial community in ENA Tween 80 microcosms was assessed for its response to inhibitory treatments. Other treatments (e.g. sawdust, compost, pea straw amendments) were not assessed as there was no significant difference in the extent of $^{14}$C-hexadecane mineralisation between the ENA treatment alone and ENA supplemented with the aforementioned carbon sources. 16S rRNA gene community profiles showed that the addition of sodium azide to contaminated soils resulted in partial inhibition of the bacterial community (Fig. 3). It is difficult to achieve total inhibition or destruction of soil microorganisms without sterilization at high temperature or application of mercuric control and even when using those methods it is impossible to selectively eliminate a particular group (as desired in this study). However, the level of inhibition achieved in this study with sodium azide was sufficient to cause changes in bacterial activities (Fig. 1) and diversity (Fig. 4).

The composite dendrogram in Fig. 3 illustrates that inhibiting specific microbial groups, either bacterial or fungal, caused distinct shifts in the bacterial community. The effect of inhibition was largely negligible within the first week (cluster 1) with significant shifts in the microbial community being observed after this period. Bacterial inhibition (BI) led to a significant reduction in bacterial abundance and diversity ($P<0.05$) (Fig. 4) leading to soil microcosms with sodium azide forming a distinct cluster 2 (weeks 1–14) which was 45% similar to cluster 3 composed of fungal (FI) and non inhibited microcosms (NI) (Fig. 3). The high similarity coefficients between FI and NI samples showed that inhibiting the fungal community, through nystatin addition, did not cause a significant change in the bacterial community. Analysis of dominant soil bacterial groups in the bacterial community profiles with Tween 80 showed that the bacterial communities in these microcosms were dominated by sequences similar to the genera *Alcanivorax* and *Pseudomonas* (gammaproteobacteria) although some sequences similar to Actinobacteria were also detected (data not shown).

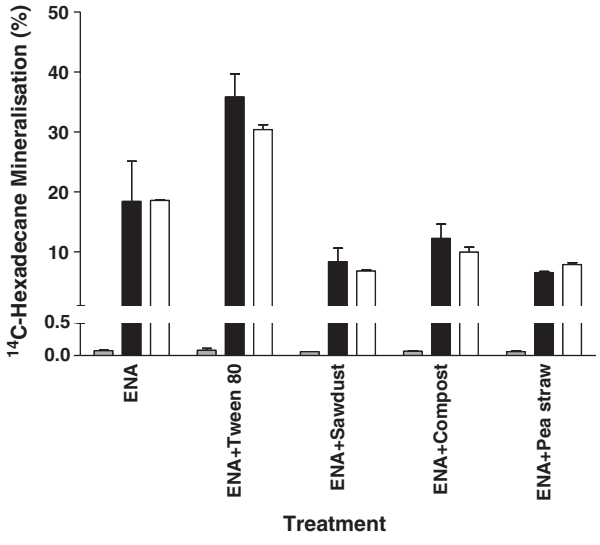


**Fig. 2.** $^{14}$C-hexadecane mineralisation in weathered TPH-contaminated soil incubated under enhanced natural attenuation conditions supplemented with various carbon amendments. Sodium azide or nystatin was added to selected soil microcosms to determine the impact of bacterial or fungal inhibition on hydrocarbon mineralisation. The extent of $^{14}$C-hexadecane mineralisation for each soil treatment following 98 days incubation is shown for bacterial (■), fungal (■) and no inhibition (□).

### 3.4. Assessment of alkane hydroxylase

Molecular methods targeting key genes involved in hydrocarbon degradation can be useful in assessing the hydrocarbon degrading potential of different environments and investigating microbial responses to hydrocarbon pollution. Alkane hydroxylase (*alkB*) systems are involved in the initial steps of alkane (most common hydrocarbon component) degradation with the gene (*alkB*) being widely found in different microbial groups (Hamamura et al., 2008; van Beilen et al., 2002). Targeting this gene would give useful information about the impact of inhibitory substances on hydrocarbon degrading potential of microbial communities. The PCR-assay for *alkB* bacterial groups showed that they were not detectable in BI microcosms with Fig. 5 showing that these groups were detected in FI and NI microcosms. The absence of these groups in BI microcosms was presumably related to the application of sodium azide even although all bacterial groups were not inhibited by its inclusion. While there are reports of the sensitivity (inhibition) of microbial groups to hydrocarbon pollution (Leahy and Colwell, 1990), the specific impacts on the expression (sensitivity) of specific functional genes, such as *alkB,* have not been reported. However, *alkB* genes are detectable in hydrocarbon contaminated environments with increasing copy numbers of *alkB* genes being correlated with hydrocarbon contamination (Salminen et al., 2008; Wasmund et al., 2009). The frequency of genes (and microbial genotype) detected in contaminated soils is a reflection of the contaminant composition with the expression of *alkB genes* changing as the contaminant composition changed with time (Sotsky et al., 1994). The GPo1 alkB genes (pp alkB) detected in this study are commonly found in contaminated soils unlike other *alkB* variants (Rh *alkB1*, Rh *alkB2*, Ac *alkM*) which are found in both contaminated and pristine soils (Aislabie et al., 2006; Whyte et al., 2002). Therefore, it was possible that members of the *alkB* bacterial community that were detected in soil when the microcosms were initiated were important for hexadecane

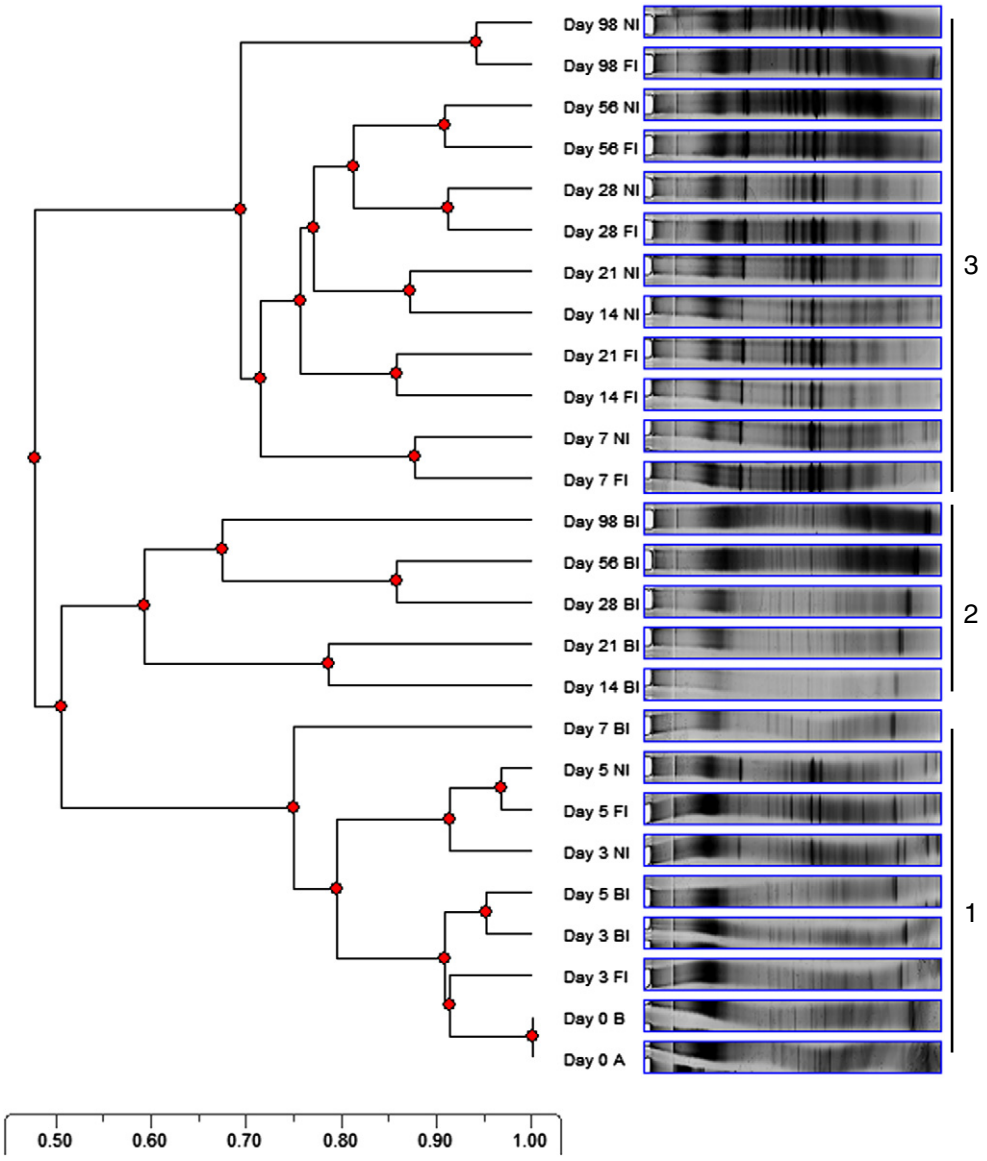


**Fig. 3.** Composite dendrogram of bacterial (BI), fungal (FI) inhibited and control (NI) microcosms incubated for 14 weeks. Scale refers to similarity percentages while numbers 1, 2 and 3 represent different bacterial community clusters. Each lane except day 0 is a mixture of replicate samples.

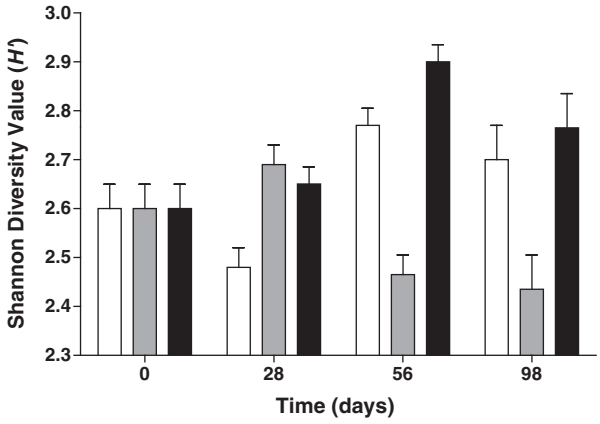


**Fig. 4.** The effects of inhibiting specific microbial groups on soil bacterial community diversity in soil microcosm incubated for 98 days. Bars represent Shannon diversity values for microcosms containing bacterial inhibition (■), fungal inhibition (■) and no inhibition (□).

mineralisation (Marchant et al., 2006) (Fig. 1) and their inhibition contributed to the lack of $^{14}$C-hexadecane mineralisation observed in BI microcosms.

## 4. Conclusions

This study showed that treatment of soils using enhanced natural attenuation alone may not be sufficient to significantly reduce hydrocarbon concentrations as a result of contaminant bioavailability limitations. In such cases, surfactant addition may facilitate enhanced removal of hydrocarbons as in this study where the application of Tween 80 increased $^{14}$C-hexadecane mineralisation from 9% to 36% during a 98-day incubation period. Soil bacteria were also found to be crucial for $^{14}$C-hexadecane mineralisation and *alkB* bacterial groups were important in the mineralisation process. In contrast, although fungal growth could be stimulated through the addition of various carbon amendments, their role in $^{14}$C-hexadecane mineralisation was negligible.

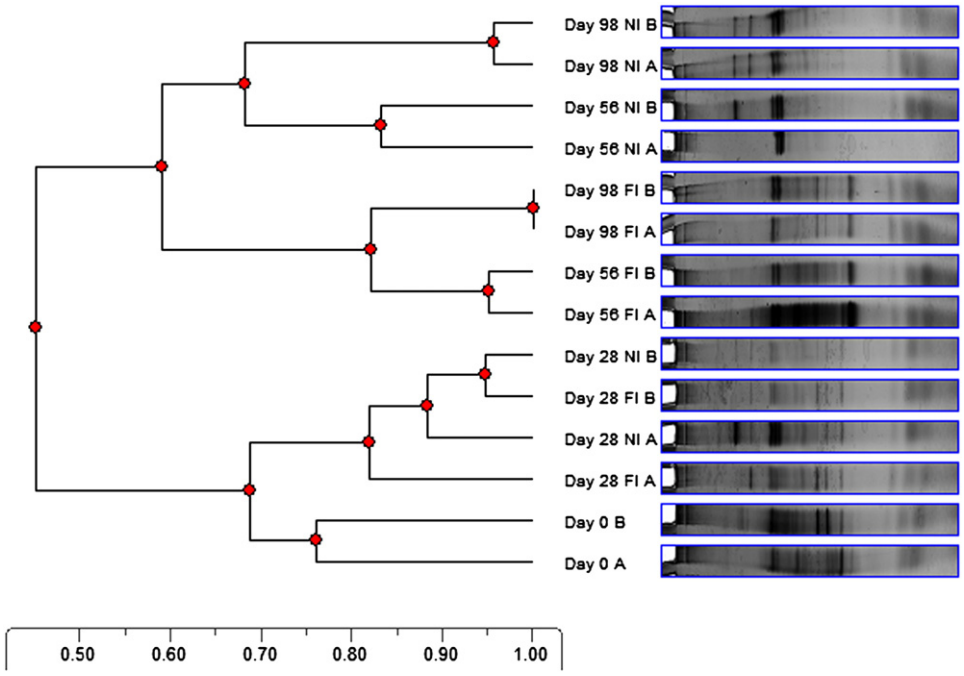


**Fig. 5.** UPGMA dendrogram of 16S rDNA based DGGE profiles of *alkB* bacterial community in inhibited and control microcosms following 98 days incubation. Scale refers to similarity percentages.

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