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Potential impact of soil microbial heterogeneity on the persistence of hydrocarbons in contaminated subsurface soils

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ABSTRACT

In situ bioremediation is potentially a cost effective treatment strategy for subsurface soils contaminated with petroleum hydrocarbons, however, limited information is available regarding the impact of soil spatial heterogeneity on bioremediation efficacy. In this study, we assessed issues associated with hydrocarbon biodegradation and soil spatial heterogeneity (samples designated as FTF 1, 5 and 8) from a site in which *in situ* bioremediation was proposed for hydrocarbon removal. Test pit activities showed similarities in FTF soil profiles with elevated hydrocarbon concentrations detected in all soils at 2 m below ground surface. However, PCR-DGGE-based cluster analysis showed that the bacterial community in FTF 5 (at 2 m) was substantially different (53% dissimilar) and 2–3 fold more diverse than communities in FTF 1 and 8 (with 80% similarity). When hydrocarbon degrading potential was assessed, differences were observed in the extent of ^{14}C-benzene mineralisation under aerobic conditions with FTF 5 exhibiting the highest hydrocarbon removal potential compared to FTF 1 and 8. Further analysis indicated that the FTF 5 microbial community was substantially different from other FTF samples and dominated by putative hydrocarbon degraders belonging to *Pseudomonads*, *Xanthomonads* and *Enterobacteria*. However, hydrocarbon removal in FTF 5 under anaerobic conditions with nitrate and sulphate electron acceptors was limited suggesting that aerobic conditions were crucial for hydrocarbon removal. This study highlights the importance of assessing available microbial capacity prior to bioremediation and shows that the site's spatial heterogeneity can adversely affect the success of *in situ* bioremediation unless area-specific optimizations are performed.

1. Introduction

Soil contamination with hydrocarbons can occur through accidental leakage or spillage of crude oil and refined petroleum products during transportation or industrial activities (Bento et al., 2005; Eggen and Majcherczyk, 1998). There are numerous physical, chemical and biological approaches for the remediation of hydrocarbon contaminated soil (Riser-Roberts, 1998) with bioremediation being a favourable treatment strategy due to its lower economic and environmental costs (Kauppi et al., 2011; Margesin and Schinner, 2001). Bioremediation is primarily driven by microorganisms and microbial roles in hydrocarbon removal from different environments such as soil and marine environments have been demonstrated (Gallego et al., 2007; Horel and Schiewer, 2009; Margesin and Schinner, 2001; Towell et al., 2011). Bioremediation of hydrocarbon impacted soils may be performed using either *ex situ* or *in situ* methods (Boopathy, 2000; Vidali, 2001) although *ex situ* strategies are more commonly used.

In situ bioremediation is sometimes preferred to *ex situ* strategies in cases where physical removal of the contaminated material is not possible due to location, high economic and environmental costs and legislative restrictions. Under such conditions, strategies such as air sparging, bioventing and biostimulation may be applied to degrade target contaminants (Guazzaroni et al., 2012; Johnson et al., 1993; Machackova et al., 2012; Vidali, 2001). However, the efficiency of *in situ* bioremediation may be constrained by factors including low temperature, availability of nutrients and the target

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contaminant in addition to sufficient microbial hydrocarbon degrading potential (Gallego et al., 2011; Huesemann, 1997; Ortega-Calvo et al., 2007; Romantschuk et al., 2000). The availability of oxygen and other suitable electron acceptors may also affect microorganisms involved in hydrocarbon degradation influencing *in situ* removal rates (Boopathy, 2000; Boopathy et al., 2012; Farhadian et al., 2008; Romantschuk et al., 2000).

Prior to the application of any bioremediation strategy, assessment of available microbial potential can be performed on samples obtained from the contaminated site. This may involve the use of microbial biosensors, indicator microbial groups, enumeration of heterotrophic and hydrocarbonoclastic populations (Diplock et al., 2009; Lors et al., 2012, 2010). Molecular tools used for detecting and quantifying hydrocarbon catabolic genes such as *alkB* and *bss* genes via PCR, quantitative PCR or metagenomic based assays (Beller et al., 2002; Diplock et al., 2009; Marcos et al., 2009; Yergeau et al., 2012) can also be used to assess microbial contaminant removal potential. An increase in the hydrocarbonoclastic microbial population or the abundance of catabolic genes is assumed to be beneficial to the bioremediation process. The use of information on microbial potential in combination with data on hydrocarbon bioavailability may allow for the estimation of bioremediation performance (Diplock et al., 2009). However, most evaluations on microbial potential are carried out under controlled conditions in laboratory based assays using bulked or homogenized samples which may not adequately reflect field conditions especially when *in situ* bioremediation is being considered.

In addition, the diversity of the microbial drivers of hydrocarbon degradation is influenced by many environmental factors. These include geographic locations of soils, soil type, plant growth and type, nature and movement of pollutants, competition, aeration, temperature and nutrients (Garbeva et al., 2004; Maila et al., 2006; Robertson et al., 2011) which can vary from one contaminated site to another. These factors affect soil microbial distribution leading to a spatially heterogeneous community which may influence microbial activity and bioremediation efficiency. This is especially important for *in situ* strategies which involve the treatment of contaminated environments “in place” with minimal disturbance (Boopathy, 2000; Vidali, 2001).

In this study, hydrocarbon degrading potential and microbial community dynamics were assessed at a hydrocarbon impacted site to which an *in situ* bioremediation strategy was proposed as a treatment strategy. Soil samples were collected from varying depths along a transect across the site and the influence of environmental variables on hydrocarbon mineralisation and microbial community composition was determined. While some variability across the site transect was expected due to soil heterogeneity issues, it was hypothesised that microbial responses to environmental variables would be conserved across the site and that a unified strategy could be applied for *in situ* bioremediation.

2. Materials and methods

2.1. Soil sampling and characterisation

Hydrocarbon contaminated soils were collected from a former oil refinery site in Australia. Soils were collected during test pit activities aimed at delineating hydrocarbon impacts near the refined fuel storage facility. Samples were obtained from three test pits designated as FTF 1, 5 and 8 using a backhoe. Test pits were located on a transect down gradient from the storage facility and were approximately 15 m apart. Soils samples (20 kg) were collected in 20 l plastic storage buckets (Silverlock, Adelaide, Australia) throughout the depth profile from 0 m to ~2.0 m and stored at 4 °C prior to both chemical and biological analysis. Samples were homogenised according to soil sampling guidelines (IWRG, 2009) prior to the determination of soil type, soil moisture content, water holding capacity, bulk density, pH and organic matter content were determined using standard methods.

2.2. Hydrocarbons quantification

Sample extraction and analysis was performed at an external analytical laboratory (ALS Laboratory Group) in Australia which is a nationally accredited environmental facility (NATA). Hydrocarbon concentrations were qualified using the internal standard method with a lower limit of reporting of 10, 50, 100 and 100 mg kg^{-1} for C_6–C_9, C_{10}–C_{14}, C_{15}–C_{28} and C_{29}–C_{36} hydrocarbon fractional ranges respectively. For mono-aromatic hydrocarbons, the lower limit of reporting for benzene, toluene, ethylbenzene, meta- and para-xylene and ortho-xylene were 0.2, 0.5, 0.5, 0.5, 0.5 mg kg^{-1} respectively. Recovery of 1,2-dichloroethane-D4, toluene-D8 and 4-bromofluorobenzene during hydrocarbon quantification ranged from 89.8 to 111.0% while the results of replicate analysis of the same sample showed a standard deviation ranging from 1.5 to 13.4%.

2.3. Mineralisation assays

Mineralisation of ^{14}C-benzene by indigenous soil microorganisms in hydrocarbon-contaminated soil was determined in replicate biometer flasks (Bellco Glass) in order to assess the biodegradation potential in FTF 1, 5 and 8 samples. Soil samples collected from 2 m below ground surface were utilised for mineralisation assays as maximum hydrocarbon concentrations were detected at this depth. These samples also represented the ‘worst case’ bioremediation scenario. Replicate soil samples (50 g) supplemented with 1.0 μ Ci ^{14}C-benzene were moistened to 10%, 60% and 100% soil water holding capacity (WHC) to assess the effects of different moisture regimes on ^{14}C-benzene mineralization. The effect of nutrient amendments on ^{14}C-benzene mineralization at the different moisture regimes was also assessed by supplementing nitrogen ($[NH_4]_2SO_4$) and phosphorus (K_2HPO_4, KH_2PO_4) to achieve a C:N:P molar ratio of 100:2.5:0.25, 100:10:1, and 100:20:2. ^{14}C-Benzene mineralisation was also assessed under anaerobic conditions at 100% WHC following the addition of nutrients in distilled water (i.e. without electron acceptor amendment) or supplementation of alternative electron acceptors (nitrate; 100 mg l^{-1}, sulphate; 960 mg l^{-1}) (Coates et al., 1997). Anaerobic conditions were achieved and maintained by replacing the biometer headspace with an atmosphere of nitrogen:hydrogen (95:5%) and incubation in a chamber with the same atmosphere. Control flasks to assess abiotic ^{14}C-benzene mineralisation (for all experimental variables) included the addition of 2% $HgCl_2$ to inhibit biotic processes. Soils were incubated at room temperature 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 protocols and automatic quenching correction (Macleod and Semple, 2002).

In order to investigate microbial community changes associated with hydrocarbon degradation in these samples, soil microcosms were also prepared as described above without the addition of labelled ^{14}C-benzene and monitored for 7 weeks. Soil sampling for microbial community analysis was performed at day 0, weeks 2, 4 and 7 and stored at −20 °C for further analysis.

2.4. DNA extraction, PCR, DGGE and sequencing

Genomic DNA was extracted from replicate samples obtained from the depth sampling of FTF soils and selected soil samples

obtained from the non-labelled experiments under aerobic and anaerobic conditions. This extraction was carried out using PowerSoil™ DNA Kit (MoBio, Carlsbad, CA, US) according to the manufacturers' protocol. Eubacterial PCR (16S rRNA genes) was performed with primer pair 341F GC and 518R (Muyzer et al., 1993). PCR amplification was performed as described by Girvan et al. (2003). The PCR products were analysed with Universal Mutation Detection System (Bio Rad Inc., CA, USA) using 9% polyacrylamide gels (the ratio of acrylamide to bisacrylamide was 37:1). Microbial communities were profiled by DGGE analysis of 16S rRNA genes using a 40–60% urea and formamide denaturing gradient. The gels were run for up to 19 h at 60 °C and 60 V before being silver stained (Girvan et al., 2003), scanned and saved as 'tiff' files. Selected dominant bands on the DGGE profiles of selected FTF samples from the non-labelled experiments were aseptically excised and incubated in 100 μl of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) overnight at 70 °C for DNA elution. The eluted DNA was then cleaned by repeated PCR, band excision and DGGE (using a narrower denaturing gradient) in order to obtain pure bands for sequencing. The purified bands were sequenced and the sequence data analysed as described by Aleer et al. (2011).

2.5. Microbial community and statistical analysis

Digitized images (.tiff files) from DGGE analysis were analysed using Phoretix 1D advanced analysis package (Phoretix Ltd, UK). The microbial community diversity of the FTF depth samples was determined with Shannon Weaver diversity (H') using the formula: $H' = -\sum p_i \text{ LN } p_i$ (Girvan et al., 2003) where p_i; is the proportion of the community that is made of species i (intensity of the band i/ total intensity of all bands in the lane) and ln p_i is the natural log of p_i. The microbial community profiles in samples from aerobic non-labelled mineralization assays were also analysed. The relatedness of the microbial (bacterial) communities between FTF 1, 5 and 8 prior to the start of the experiments was expressed as similarity clusters using the unweighted pair group method with mathematical averages (UPGMA). The range-weighted richness index (Rr) analysis of these samples was also carried out using the formula $\text{Rr} = (N^2 \times D_g)$ (Marzorati et al., 2008). Based on the result obtained from these analyses, further evaluation of microbial community structure was carried out on FTF 1 and 5 samples. UPGMA dendrograms were used to compare the similarities of the bacterial communities in FTF 1 and 5 samples and their community diversity determined as described before. Pareto Lorenz (PL) distribution curves were used to estimate evenness in selected microbial communities (Marzorati et al., 2008). The bacterial community in anaerobic non-labelled mineralization assay was also analysed with UPGMA dendrogram and Shannon Weaver diversity index. Statistical significance was determined between different samples by either T Test or Analysis of Variance (ANOVA) in SPSS version 20 with significance accepted at $P < 0.05$.

3. Results and discussion

3.1. Physical, chemical and microbiological characterisation of soils

Fig. 1 shows the vertical profile of hydrocarbons in test pits 1, 5 and 8. In all soil profiles, hydrocarbon concentrations increased with increasing soil depth resulting in maximum concentrations at ~2 m below ground surface. Maximum BTEX concentrations ranged from 390 (FTF 5) to 620 mg kg^{-1} (FTF 1) while the concentration of low molecular weight n-alkanes (C_6–C_9 hydrocarbon fractional range) ranged from 1190 (FTF5) to 1460 mg kg^{-1} (FTF 8). The highest hydrocarbon concentration observed in FTF 1, 5 and 8 samples was from the C_{10}–C_{14} hydrocarbon fractional range with concentrations of 2790, 1370 and 2040 mg kg^{-1} respectively at 2 m below ground surface. Some variability in FTF 1, 5 and 8 soil profiles were observed during sample collection (Fig. 1). All three profiles consisted of a surface clay–sandy layer (to 0.3–0.4 m) of fine to medium grained sand with low plasticity. Sandwiched between a clay–sandy layer at the bottom of the test pits (starting at 1.9

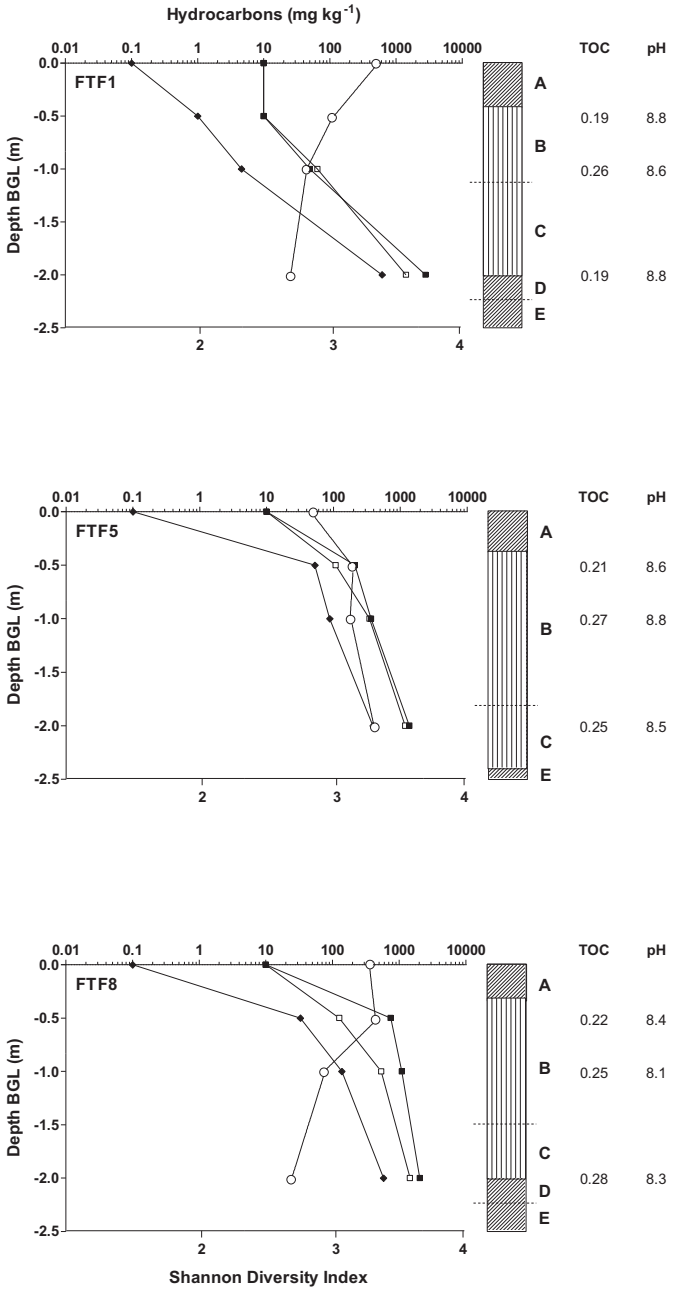

Fig. 1. Vertical profile of BTEX (◆), C_6–C_9 hydrocarbons (□), C_{10}–C_{14} hydrocarbons (■), total organic carbon (TOC; %), soil pH and bacterial diversity (; Shannon Diversity Index) in samples collected from FTF 1, 5 and 8. Mean values are presented; the maximum standard deviation in sample analysis was 8.0%. Changes in soil colour, texture and structure (based on ASTM standard D2487-93) through the soil profile are also shown. Letter in soil profiles correspond to: A: Clay sandy: brown, low plasticity, fine grained sand, sub-angular, >30% sand, organic odour. B: Silt sandy: light grey fine grained sand, sub-angular, >30% sand, coarser sand with depth. C: Silt clayey: dark grey, low plasticity, fine grained sand, sub-angular. D: Clay silt: dark grey, medium plasticity, fine-medium grained sand. E: Clay sandy: orange, high plasticity, fine grained sand, sub-rounded, >5% sand.

[FTF 1] to 2.4 m [FTF 5]) was a silt layer which changed in composition from silt–sandy to silt–clayey with increasing depth (Fig. 1). The bottom clay–sandy layer contained more clay materials with high plasticity and less sand at ~2.0 m than the upper soil layers which had low plasticity clay and more coarse sand. This bottom layer was therefore likely impermeable to the contaminant compared to the upper layers, resulting in the accumulation of the hydrocarbon contaminant at ~2.0 m soil depth.

Analysis of bacterial diversity of samples from 0 m to ~2.0 m was performed to assess the relationship between contaminant concentration, soil depth and microbial diversity (Fig. 1). Results from these investigations showed that in both FTF 1 and 8, bacterial diversity decreased with increasing depths. The Shannon Weaver Diversity indices (H') of 3.31 and 3.30 for FTF 1 and 8 respectively obtained from the surface samples (0 m) was significantly reduced (2.68 and 2.65 respectively) at ~2.0 m below ground surface ($P < 0.05$). The decrease in bacterial diversity was inversely related to hydrocarbon concentrations in these soils. Decreasing bacterial diversity with increasing soil depth in FTF 1 and 8 may have occurred due to changes in physico-chemical properties. Bacterial diversity has been shown to be strongly depth dependent, varying with changes in organic C, pH, soil texture and oxygen content (Hansel et al., 2008). Bacterial biomass, concentration of 16S rRNA genes and number of DGGE bands have been reported to be lower in subsurface soils compared to surface samples in some studies (Agnelli et al., 2004; Zhou et al., 2004). The decrease in bacterial diversity in subsurface samples compared to surface samples observed in FTF 1 and 8 is therefore not unusual (Fierer et al., 2003; Griffiths et al., 2003; LaMontagne et al., 2003). Differences in the soil texture (Fig. 1) and the oxygen content of surface and subsurface samples would have played some roles in the observed trend (Hansel et al., 2008). In addition, the decrease in bacterial diversity may also have been exacerbated by the presence of hydrocarbons that exert a toxicological impact (Fahy et al., 2005; Labud et al., 2007; Regno et al., 1998). Depending on soil type and use, hydrocarbon contamination can lead to the selection of bacterial groups capable of tolerating or utilizing the contaminant leading to an increase in the population of these specific hydrocarbonoclastic groups while eliminating other microbial groups. This may sometimes result in a decrease in microbial diversity.

While hydrocarbon concentrations also increased with increasing soil depth in FTF 5, bacterial diversity increased from 2.82 (surface samples) to 3.14 (~2.0 m subsurface samples) unlike the result observed for FTF 1 and 8 (Fig. 1). As the soil profile of FTF 5 and its physico-chemical characteristics were similar to those of FTF 1 and 8 (Fig. 1), the reason for the increase in diversity is unclear. However, spatial heterogeneity of the microbial communities in the soil samples and differences in soil porosity could have played some roles in the diversity trend observed in FTF 5.

3.2. *In situ* bioremediation potential – ^{14}C benzene mineralization

Based on the elevated hydrocarbon concentrations at ~2.0 m below ground surface, samples from this depth were selected for ^{14}C-benzene mineralisation experiments as they represented a 'worst case scenario' for *in situ* bioremediation. Initially, the degradative capabilities of the indigenous microorganisms were assessed under aerobic conditions ('best case scenario' for electron acceptors) with varying moisture and nutrient regimes. This allowed for the assessment of the impact of nutrient amendments on hydrocarbon degradative performance. Soil moisture content may vary greatly between surface and subsurface samples with the amount of water in soil pores determining the level of oxygen in soil systems. As a result, the impact of different moisture regimes on hydrocarbon degradative performance was assessed under conditions of limited (10% WHC), optimum (60% WHC) and excessive (100% WHC) soil water contents.

In all mercuric chloride-killed controls irrespective of moisture regime, ^{14}C-benzene mineralisation was minimal, evolving a maximum of 2.1% of the ^{14}C label as $^{14}CO_2$ after 42 days (Table 1). In the absence of added nutrients (natural attenuation), ^{14}C-benzene mineralization at 10% soil WHC was low between 0.2 ± 0.1% and 3.1 ± 2.9%. Increasing the soil moisture content to 60 and 100% resulted in a small but significant increase in ^{14}C-benzene mineralisation of 4.2 ± 1.6% and 4.1 ± 1.5% for FTF 1 and 9.4 ± 0.9% and 6.6 ± 1.9% for FTF 5 respectively. In contrast, ^{14}C-benzene mineralisation in FTF 8 increased to 17.2 ± 1.4% and 23.0 ± 8.1% indicating that essential nutrients were available in the soil to sustain biodegradation. Although ^{14}C-benzene mineralisation could be enhanced through moisture addition, there was no significant difference ($P < 0.05$) in $^{14}CO_2$ evolution between FTF 8 soil at 60% and 100% water holding capacity.

When nutrients were added to soils, the extent of ^{14}C-benzene mineralisation increased significantly (Table 1). In studies where $(NH_4)_2SO_4$, KH_2PO_4 and K_2HPO_4 were augmented to achieve a C:N:P molar ratio of 100:10:1, the extent of ^{14}C-benzene mineralisation increased up to 7-fold compared to natural attenuation treatments when soil moistures were ≥60%. As seen for studies without nutrient additions, the extent of ^{14}C-benzene mineralisation in ENA treatments varied between soils with ^{14}C-benzene mineralisation following the order FTF5 > FTF8 > FTF1 (Table 1).

Varying the C:N:P molar ratio in FTF soils had a significant impact on ^{14}C-benzene mineralisation by the indigenous soil microorganisms. As illustrated in Table 1, the extent of ^{14}C-benzene mineralisation in FTF 5 increased with increasing nutrient inputs. At nutrient application rates of 100:2.5:0.25, 100:10:1 and 100:20:2 (C:N:P) and a soil moisture of 60% WHC, ^{14}C-benzene mineralisation increased 1.7, 5.4 and 6.5-fold compared to natural attenuation (Table 1). Similar trends were observed for FTF 1 and FTF 5 soils at 100% WHC. The impact of nutrient amendments on ^{14}C-benzene mineralisation was not significant in FTF1 (60% WHC) and FTF8 (60 and 100% WHC) soils.

Varying the soil moisture also had an impact on the extent of ^{14}C-benzene mineralisation. The ability of the indigenous soil microorganisms to mineralise ^{14}C-benzene was limited under dry

Table 1
^{14}C-Benzene mineralisation under aerobic conditions by indigenous soil microorganisms in FTF1, 5 and 8 soils collected from 2 m below ground surface.

Treatment	^{14}C-Benzene mineralisation (%)[a]		
	10% WHC	60% WHC	100% WHC
FTF1			
$HgCl_2$ killed	0.19 ± 0.07	0.16 ± 0.13	0.14 ± 0.04
NA (no nutrients)	0.25 ± 0.20	4.17 ± 1.06	4.16 ± 1.47
ENA (100:2.5:0.25 C:N:P)	0.27 ± 0.07	10.87 ± 2.86	9.2 ± 2.80
ENA (100:10:1 C:N:P)	0.34 ± 0.05	14.91 ± 0.99	7.33 ± 4.20
ENA (100:20:1 C:N:P)	0.68 ± 0.64	14.86 ± 0.84	37.09 ± 10.32
FTF5			
$HgCl_2$ killed	0.12 ± 0.07	0.29 ± 0.06	0.34 ± 0.02
NA (no nutrients)	0.22 ± 0.14	9.41 ± 0.94	6.62 ± 1.94
ENA (100:2.5:0.25 C:N:P)	0.31 ± 0.11	16.27 ± 0.17	33.71 ± 10.57
ENA (100:10:1 C:N:P)	3.86 ± 3.55	50.94 ± 5.33	47.74 ± 1.31
ENA (100:20:1 C:N:P)	1.27 ± 0.35	60.81 ± 0.51	55.32 ± 5.33
FTF8			
$HgCl_2$ killed	2.11 ± 0.24	0.47 ± 0.01	1.27 ± 0.08
NA (no nutrients)	3.11 ± 2.88	17.21 ± 1.43	23.01 ± 8.14
ENA (100:2.5:0.25 C:N:P)	2.69 ± 1.47	23.96 ± 0.38	22.90 ± 14.66
ENA (100:10:1 C:N:P)	1.35 ± 0.13	24.67 ± 4.04	31.12 ± 5.30
ENA (100:20:1 C:N:P)	2.92 ± 0.65	23.66 ± 3.52	35.62 ± 2.17

[a] ^{14}C-benzene mineralisation determined after 42 days.

(10% WHC) soil conditions (Table 1). The extent of ^{14}C-benzene mineralisation in FTF soils with 10% water holding capacity ranged from 0.2 to 3.9% depending on the soil and nutrient status. Increasing the soil moisture to 60% water holding capacity increased ^{14}C-benzene mineralisation by up to 60-fold (Table 1). While ^{14}C-benzene mineralisation was moderately enhanced under saturated conditions compared to 60% WHC, the increase in ^{14}C-benzene mineralisation was not significant ($P > 0.05$).

The results from ^{14}C mineralization assays showed that nutrient amendments under moisture regimes of 60 and 100% were more beneficial to ^{14}C-benzene mineralisation in FTF 5. The beneficial effect of nutrient addition to labelled compound mineralisation has been shown in other studies (Adetutu et al., 2012; Towell et al., 2011). This indicates that the microbial potential for contaminant removal was significantly higher in FTF 5 than in FTF 1 and 8 samples (at 60% WHC). Differences in soil microbial community structure may have caused the observed difference in the extent of hydrocarbon removal in FTF soils. Microbial community structure and function may be influenced by differences in soil and plant type, soil use and geographic location (Maila et al., 2006; Robertson et al., 2011) although contaminant exposure could also play a role. Soil type dependent response of hydrocarbon degrading genes in soil bacteria (Ding et al., 2010) can translate to different hydrocarbon degradation rates in different soil types. However, the site used for this study was largely devoid of plant cover with the transect samples being collected from the same geographic location and were of the same soil type. In the absence of any substantial difference in the physico-chemical properties of FTF 1, 5 and 8 samples, we therefore performed further investigation on the FTF microbial communities in order to assess their homogeneity across the sampling site.

3.3. Soil microbial community analysis

Initial microbial community depth profile analysis showed that bacterial diversity in FTF 1 and 8 decreased with increasing depth while for FTF 5, bacterial community diversity increased in subsurface samples (Fig. 1). This result suggested that that the bacterial community structure and diversity in FTF 5 was different to those of FTF 1 and 8. Further investigation was performed using samples obtained at ~2.0 m below ground surface (highest concentration of hydrocarbon contaminants) to assess differences in microbial community composition. Analysis of the bacterial community DGGE profiles using UPGMA dendrogram showed that FTF 1 and 8 bacterial communities were 80% similar (Fig. 2). In contrast, FTF 5 bacterial community was highly dissimilar to both FTF 1 and 8 (47% similar). Further analysis of microbial community profiles was carried out using range weighted richness analysis (Rr). The Rr value of FTF 5 was 47.0 (high range weighted richness) which was at least twice that of FTF 1 and 8 (Rr values of 22.7 and 15.4 respectively) (medium range weighted richness) (Marzorati et al., 2008) indicating that the bacterial community in FTF 5 was more diverse than bacterial communities in FTF 1 and 8 (data not shown). These results suggest that the microbial community at the FTF site was not homogenous, although FTF 1 and 8 communities appeared to be more similar to each other than to FTF 5. Given the high similarity between FTF 1 and 8 microbial community profiles and ^{14}C-benzene mineralisation data, further microbial community comparisons were performed using FTF 1 and 5. FTF 1 was selected as a representative of soil with low ^{14}C benzene mineralization potential and compared with FTF 5 (samples with high ^{14}C benzene mineralization) at 60% WHC.

UPGMA dendrogram of the bacterial communities in both FTF 1 and 5 soil samples obtained from non-labelled microcosms conducted over a 7 week incubation period showed two distinct clusters based on FTF designation rather than treatment (Fig. 3a). The addition of nutrients to FTF microcosms appeared to have exerted mild effects on the bacterial community profiles (Fig. 3b). The bacterial diversity and evenness was also largely unaffected by nutrient addition as the Pareto Lorenz distribution curves (data not shown) ranged between 47 and 58% for FTF 1 and 38–60% for FTF 5 over the 7 weeks incubation period. Other studies (Makadia et al., 2011; Sheppard et al., 2011) on contaminated soils have also shown that nutrient addition did not cause a significant effect on bacterial diversity although a reduction in band number and shifts in bacterial community can be associated with biostimulation (Evans et al., 2004). Reductions in soil hydrocarbon contaminant during bioremediation may not always be accompanied by substantial changes in microbial community diversity (Makadia et al., 2011).

3.4. Sequence analysis

Detailed analysis of the comparative bacterial community profile showed a greater number of dominant bands which were unique to FTF 5 (B, C, D, E, G and I) than in FTF 1 (F) with bands tagged A and H common to both profiles (Fig. 3b). Sequence analysis of these bands showed that they were most similar to Gammaproteobacteria group with only two bands belonging to the Firmicutes (Table 2). The dominance of sequences putatively belonging to the Gammaproteobacteria is not unusual. Members of this group have been identified and shown to be involved in hydrocarbon degradation in contaminated environments (Hamamura et al., 2013; Labbe et al., 2007; Uhlik et al., 2012). Therefore, their detection in both FTF samples could have been related to historical hydrocarbon contacts or natural occurrence in soil.

The putative identities of bands unique to FTF 5 were *Pseudomonas*, *Bacillus*, *Xanthomonas*, *Enterobacter* and *Marinobacter*/

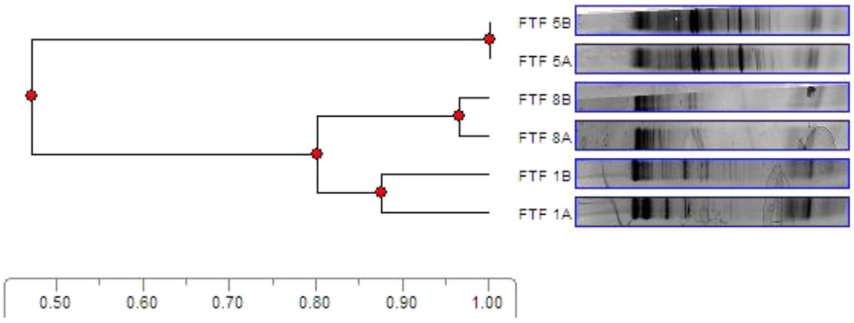

Fig. 2. UPGMA dendrograms comparing bacterial communities in samples of FTF 1, 5 and 8 collected from 2 m below ground surface. Letters A and B refer to duplicate analysis while the scale refers to percentage similarity.

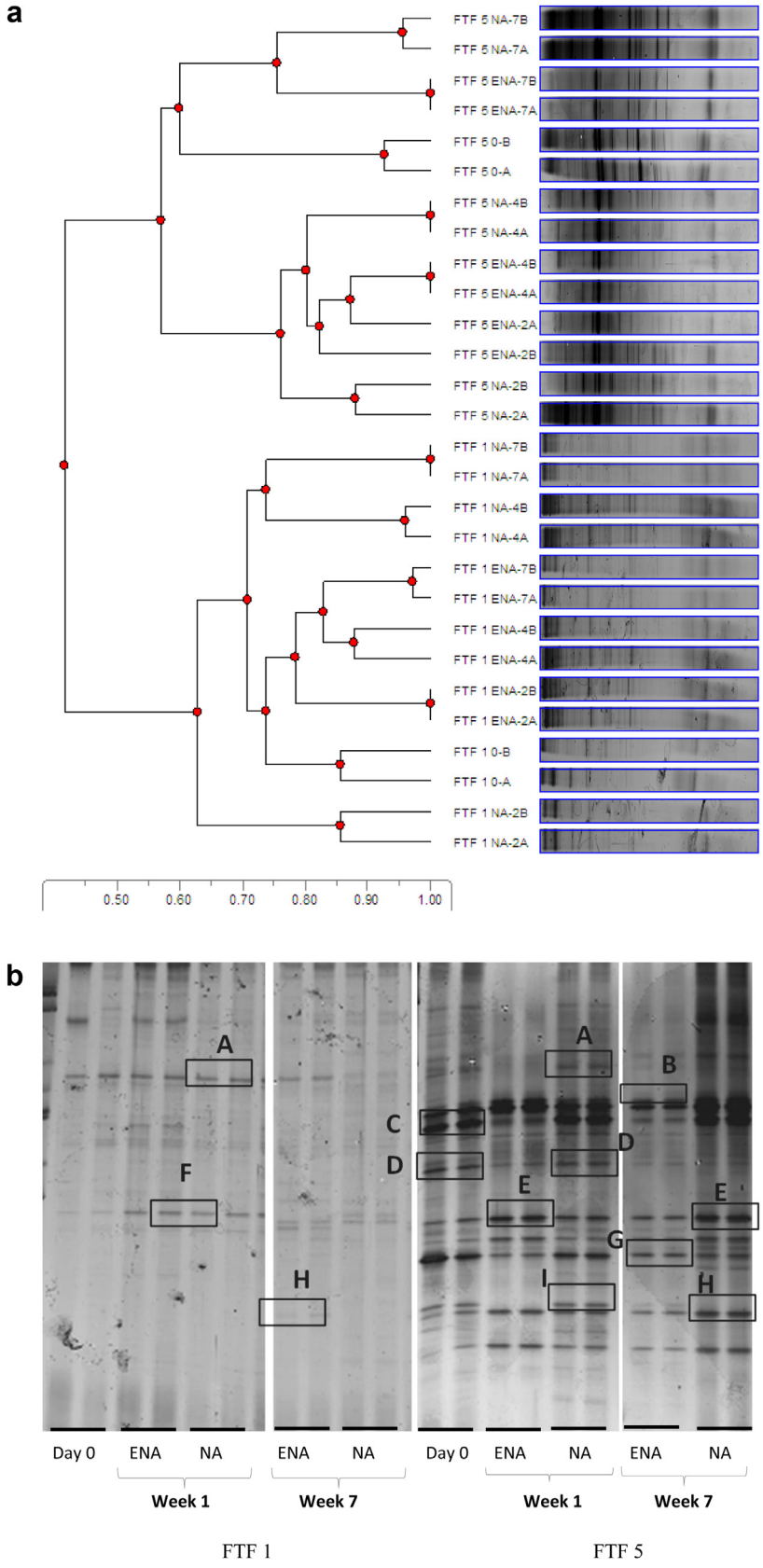

Fig. 3. (a) Bacterial community profiles derived from 16S rRNA analysis of soil samples from FTF 1 and, 5 incubated under natural attenuation (NA) and enhanced natural attenuation (ENA) aerobic conditions for 7 weeks. The scale refers to percentage similarity, numbers refer to sampling time points (0, 2, 4, 7 weeks) while letters refer to duplicate samples. (b) Cross section of FTF 1 and FTF 5 bacterial community profile showing the locations of bands excised and sequenced. Letters refer to bands excised and sequenced (see Table 2 for sequence identities).

Table 2
Sequence analysis of bands excised from FTF 1 and FTF 5 DGGE gels (Fig. 3b).

Band label	Taxon/division	Nearest match	Accession number	Sequence similarity (%)
A	Gammaproteobacteria	*Pseudomonas* sp	HE798533.1	94
	Gammaproteobacteria	*Pseudomonas putida*	FJ596989.1	94
B	Firmicutes	*Staphylococcus* sp	JQ183036.1	96
C	Gammaproteobacteria	*Pseudomonas* sp	AF229864.1	99
D	Firmicutes	*Bacillus* sp	JQ782996.1	98
E	Gammaproteobacteria	*Xanthomonas campestris*	JN164700.1	97
F	Gammaproteobacteria	Uncultured *Pseudomonas*	EU919222.1	90
G	Gammaproteobacteria	*Enterobacter* sp	JQ917796.1	99
H	Gammaproteobacteria	*Pantoea agglomerans*	JF683668.1	99
I	Gammaproteobacteria	*Marinobacter* sp	GU059908.1	94
	Gammaproteobacteria	*Alteromonas* sp	FM992720.1	94

Alteromonas sp (Table 2). Members of these groups have been associated with hydrocarbon degradation in the natural environment and laboratory based studies (Abed, 2010; Kao et al., 2010; Liu et al., 2010; Pemmaraju et al., 2012; Shibata and Robert, 2009; Yousaf et al., 2010). Although the specific roles of these groups in hydrocarbon degradation in FTF 5 were not investigated, it was hypothesised that their dominance in microcosms where substantial hydrocarbon degradation occurred was indicative of their role in hydrocarbon removal.

3.5. Anaerobic ^{14}C-benzene mineralization

A key parameter which influences the efficacy of bioremediation of petroleum hydrocarbons is oxygen availability. Degradation of monoaromatic hydrocarbons may proceed in the presence of a variety of electron acceptors, however, degradation utilising oxygen is the most efficient (Hutchins, 1991). ^{14}C-Benzene experiments with FTF soils demonstrated the capacity of the indigenous microorganisms to mineralise the hydrocarbon under aerobic conditions following nutrient stimulation. However, due to the silt–clay soils present at the site, oxygen may be limiting in the subsurface or it may be difficult to supply oxygen (via biosparging) where elevated concentrations of hydrocarbons are present. As a result, mineralisation experiments were also performed to assess the efficacy of hydrocarbon degradation under anaerobic conditions in the presence of alternative electron acceptors.

ENA biometers were prepared with soil from FTF 5 (2 m below ground surface) as ^{14}C benzene mineralization results were most promising under aerobic conditions (60 and 100% WHC) compared to those obtained for FTF 1 and FTF 8. When incubated under anaerobic conditions, the rate and extent of ^{14}C-benzene mineralisation was reduced compared to aerobic conditions (Fig. 4). After 70 days incubation, up to 14.5 ± 0.8% of ^{14}C-benzene was mineralised compared to 50.9 ± 5.3% (after 42 days) when aerobic conditions were supplied. Small differences in ^{14}C-benzene mineralisation were observed between the anaerobic treatments with ^{14}C-benzene mineralisation ranging from 8.6 ± 0.7% (nitrate reducing conditions) to 10.3 ± 1.0% (sulphate reducing conditions) and 13.2 ± 2.0% (without the addition of electron acceptors – distilled water). Consequently, the absence of substantial ^{14}C benzene mineralization in this study was likely due to the soils not having the microbial capacity for this process rather than the absence of electron acceptors as anaerobic microbial degradation of benzene has been reported in literature (Foght, 2008). However, when incubation conditions were changed from anaerobic to aerobic following 70 days of incubation, an increase in ^{14}C-benzene mineralisation was observed (Fig. 4). After further 4 weeks incubation under aerobic conditions, between 12.6 ± 0.3% and 39.6 ± 6.7% of ^{14}C-benzene was mineralised depending on the nutrient amendments supplied.

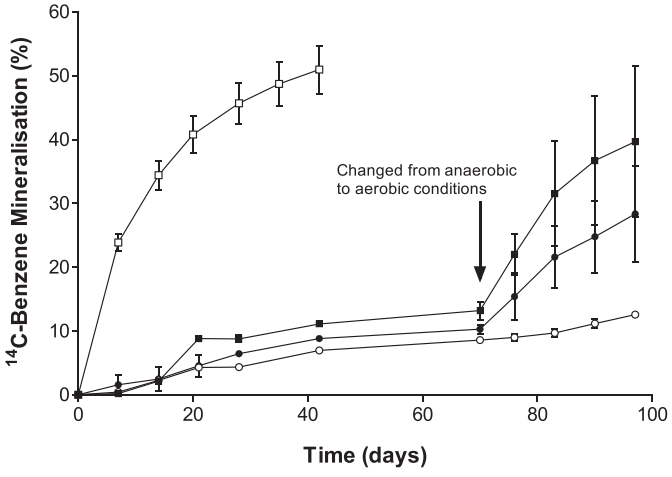

Fig. 4. ^{14}C-benzene mineralisation in FTF 5 incubated under ENA (C:N:P 100:10:1) anaerobic (10 weeks) and aerobic conditions (4 weeks) in the presence of nitrate (), sulphate (●) and natural background (■) electron acceptors. The arrow indicates when incubation conditions were changed from anaerobic to aerobic.

Hydrocarbon degradation is mediated by microorganisms with oxygen (aerobic conditions) and nitrates and sulphates (anaerobic conditions) serving as electron acceptors (Boopathy et al., 2012; Farhadian et al., 2008; Hutchins, 1991). Significant removal of contaminating hydrocarbons have been reported when nitrates and sulphates have been used as electron acceptors although sulphate reducing conditions may be more efficient for decontamination than nitrates in some instances (Boopathy et al., 2012). However, the extent of removal under optimum concentrations of electron acceptor is dependent on available microbial capacity. Since the addition of electron acceptors was not beneficial for hydrocarbon removal in this study, further investigations were carried out the microbial community response to the supply of electron acceptors.

3.6. Anaerobic soil microbial profiles

The effect of soil amendments on bacterial community dynamics under anaerobic conditions was assessed for FTF 5 (i.e. nitrate and sulphate reducing conditions versus no electron acceptor amendment). Cluster analysis indicated a high level of similarity in the bacterial communities between day 0 and week 7 with most of the samples (irrespective of treatment) forming a distinct cluster 80–95% similar (Fig. 5a). This cluster was substantially different to the cluster formed by sulfidogenic samples (week 7) and ENA incubations without electron acceptor amendments at weeks 7 and 10. The addition of nitrate or sulphate also caused a substantial reduction in bacterial community diversity especially at week 10

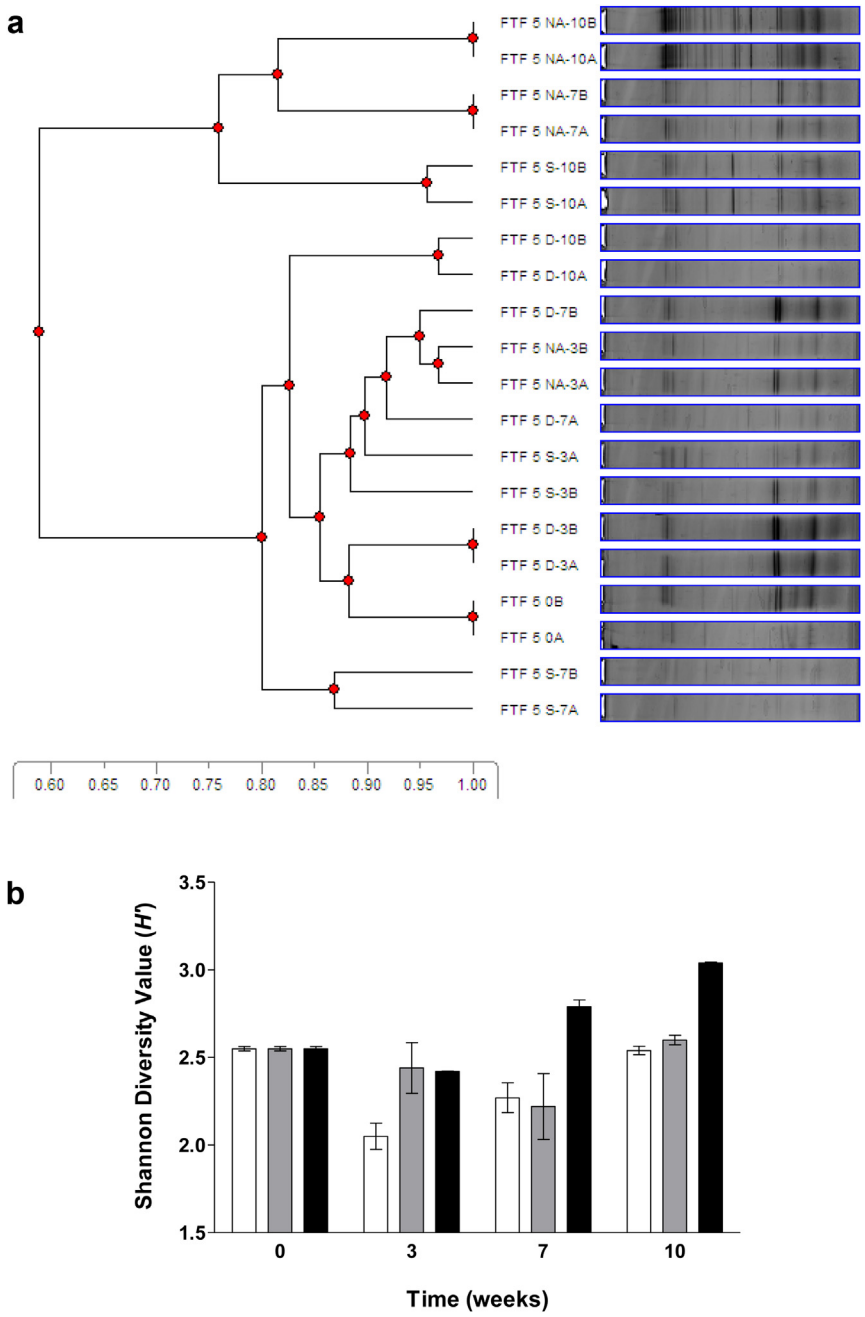

Fig. 5. (a) UPGMA dendrogram (a) of bacterial community diversity (b) in FTF 5 incubated under ENA (C:N:P 100:10:1) anaerobic conditions with nitrate (N, □), sulphate (S, ■) or no additional electron acceptors (NA, ■).

when compared to unamended samples (H' values of 2.5–2.6 compared to 3.0 respectively). The clustering and observed reduction in bacterial diversity suggests that the addition of nitrate or sulphate to ENA treatments leads to the selection of fewer bacterial species compared to ENA treatments without electron acceptor amendments.

3.7. Impacts for in situ bioremediation

This study has shown that the potential to degrade hydrocarbon contaminants exists in FTF soils but was more efficient under aerobic conditions than anaerobic conditions. Although hydrocarbon (benzene) removal has been reported under anoxic conditions (Edwards and Grbić-Galić, 1992; Masumoto et al., 2012; Xiong et al., 2012), the thermodynamic stability of benzene molecules is thought to make it less susceptible to enzymatic attack under anoxic conditions by most microorganisms (Foght, 2008; Langenhoff et al., 1989). The persistence of the hydrocarbon contaminant at ~2 m soil depth was therefore likely due to reduced oxygen tension at this depth. Therefore a strategy that introduces oxygen coupled with biostimulation will be crucial for hydrocarbon removal from FTF soils. However, the application of this strategy has to be mitigated by the issue of spatial heterogeneity observed at the FTF site. Substantial differences in FTF 1, 5 and 8 bacterial diversity and banding patterns were correlated with significant differences in hydrocarbon removal potential especially

at 60% WHC (significantly higher in FTF 5 compared to other FTF samples). The reason for the different microbial community in FTF 5 is unclear given its location between FTF 1 and 8 with these two designations having largely similar microbial communities. However, it is possible that natural spatial heterogeneity and the site's previous contacts with hydrocarbons were responsible for these differences. The latter point is important as the contamination event on the site could have been heterogeneous leading to the development of hotspots at some site locations, different distribution of hydrocarbon fractions and weathering of these fractions, all of which can affect microbial community response and diversity. Most *in situ* bioremediation projects are usually performed without the assessment of a site's spatial heterogeneity and its impact on hydrocarbon removal rates. The results obtained from this study show that applying a common or conserved approach to *in situ* bioremediation of sites such as the FTF may not be appropriate. The high variability in hydrocarbon removal indicates that application of air sparging or other *in situ* bioremediation strategies may produce variable results. As a consequence, this may result in longer than anticipated time frame and/or additional process costs.

4. Conclusion

This study showed that differences in microbial community structure and diversity may occur in subsurface samples from a hydrocarbon contaminated site. These differences may result in significant variations in microbial hydrocarbon degrading potential in different areas of this site under aerobic and anaerobic conditions. Such variables need to be taken in consideration when *in situ* bioremediation strategies are planned for contaminated sites in order to ensure successful and site-wide hydrocarbon removal. This study also demonstrated the value of using molecular tools for assessing available microbial potential prior to the initiation of a bioremediation strategy. The use of these tools will allow for the assessment of the suitability of proposed strategies and allow for its optimization for process and cost effective contaminant removal.

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