Biodegradation (2012) 23:813–822
DOI 10.1007/s10532-012-9563-8

ORIGINAL PAPER

Comparison of indigenous and exogenous microbial populations during slurry phase biodegradation of long-term hydrocarbon-contaminated soil

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Received: 9 April 2012 / Accepted: 29 May 2012 / Published online: 10 June 2012

Abstract In this study, a number of slurry-phase strategies were trialled over a 42 day period in order to determine the efficacy of bioremediation for long-term hydrocarbon-contaminated soil (145 g kg^{-1} C_{10}–C_{40}). The addition of activated sludge and nutrients to slurries (bioaugmentation) resulted in enhanced hydrocarbon removal (51.6 ± 8.5 %) compared to treatments receiving only nutrients (enhanced natural attenuation [ENA]; 41.3 ± 6.4 %) or no amendments (natural attenuation; no significant hydrocarbon removal, $P < 0.01$). This data suggests that the microbial community in the activated sludge inoculum contributed to the enhanced removal of hydrocarbons in ENA slurries. Microbial diversity in slurries was monitored using DGGE with dominant bands excised and sequenced for identification. Applying the different bioremediation strategies resulted in the formation of four distinct community clusters associated with the activated sludge (inoculum), bioaugmentation strategy at day 0, bioaugmentation strategy at weeks 2–6 and slurries with autoclaved sludge and nutrient additions (bioaugmentation negative control). While hydrocarbon-degrading bacteria genera (e.g. *Aquabacterium* and *Haliscomenobacter*) were associated with the hydrocarbon-contaminated soil, bioaugmentation of soil slurries with activated sludge resulted in the introduction of bacteria associated with hydrocarbon degradation (*Burkholderiales* order and *Klebsiella* genera) which presumably contributed to the enhanced efficacy for this slurry strategy.

Keywords Activated sludge · Bioaugmentation · Hydrocarbons · Slurry phase bioremediation

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Introduction

The refining of oil as well as petrochemical manufacturing has left a legacy of contamination in the form of hydrocarbon-contaminated soils and groundwater. The use of bioremediation, which relies on the metabolic potential of microorganisms for the degradation of contaminants in the environment, represents

an attractive option because of its clean and green image and low cost. A popular bioremediation strategy is natural attenuation (NA) and because it is non-intrusive, it has been successful used in the treatment of several contaminated sites (Aburto et al. 2009; Fahy et al. 2005). Other benefits of NA may include reduced use of energy and lower remediation costs during site treatment. However, NA timeframes may be lengthy depending on site characteristics and environmental conditions. Therefore, in order to increase the rate of degradation, other bioremediation strategies may be used including enhanced natural attenuation (ENA) and bioaugmentation. The former attempts to enhance the activities of indigenous pollutant degraders by the addition of inorganic nutrients, electron acceptors, water or electron donors (Atlas and Philp 2005), while the latter involves the inoculation of exogenous strain(s) in order to degrade specific pollutants thereby increasing the rate of degradation. Bioaugmentation has been used for soil remediation in agriculture and wastewater treatment for several years (Vogel 1996). Moreover, successful bioaugmentation strategies in laboratory-based studies have been reported with several treatments including aerobic or anaerobic conditions, soil, groundwater and the degradation of a wide range of substrates (Watanabe et al. 1996; Weiner and Lovley 1998). Activated sludge is a popular inoculum used for bioaugmentation to enhance hydrocarbon biodegradation. Inocula of this nature have been successfully applied for the remediation of contaminated matrices including oilfield producing water and contaminated soils (Juteau et al. 2003; Tellez et al. 2002; Zhao et al. 2006). A recent review discusses the effectiveness and limitations of bioaugmentation strategies in laboratory trials and field applications (Tyagi et al. 2011).

In this study, slurry phase biodegradation of long-term hydrocarbon contaminated soil was investigated. Slurry phase treatments were chosen as previous studies demonstrated the lack of hydrocarbon removal in conventional 'solid phase' biopiles even though the microbial community possessed the catabolic ability (*alkB*) to degrade hydrocarbons (unpublished results). It was hypothesised that biodegradation was limited by hydrocarbon bioavailability under constraints of the solid phase biopile; an operational parameter overcome through the use of slurries. It was further hypothesised that the extent of slurry phase hydrocarbon biodegradation could be enhanced through bioaugmentation with complex microbial communities such as those present in activated sludge. In order to test these hypotheses, a combination of chemical and molecular techniques were utilised to analyse slurry phase treatments to determine the impact of biodegradation strategy on hydrocarbon removal and microbial community dynamics.

Materials and methods

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 145 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 performed 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).

Preparation of slurries

Slurry phase hydrocarbon biodegradation experiments were conducted in four separate 2 L bioreactors for each treatment strategy. Soil slurries were prepared by combining 240 g of hydrocarbon contaminated soil and 1.2 L of liquid (deionised water or activated sludge sourced from Bolivar Wastewater Treatment Plant (South Australia) to achieve a 20 % (w/v) slurry. When nutrients were supplied to stimulate microbial growth, $(NH_4)_2$ SO_4 (2.8 g), KH_2PO_4 (0.4 g) and K_2HPO_4 (0.4 g) were added during the slurry preparation phase to achieve a C (determined from hydrocarbon concentration):N:P molar ratio of approximately 100:10:1. Bioreactor treatments included:

- Hydrocarbon-contaminated soil plus water (NA).
- Hydrocarbon-contaminated soil plus water and nutrients (ENA).
- Hydrocarbon-contaminated soil plus activated sludge and nutrients (bioaugmentation).

Table 1 Hydrocarbon concentration following slurry phase treatment of hydrocarbon contaminated soil

Slurry	Treatment	Time (weeks)	Hydrocarbon (mg kg^{-1})				
			C_{10}–C_{14}	C_{15}–C_{28}	C_{29}–C_{36}	C_{37}–C_{40}	C_{10}–C_{40}
NA	No amendments	0	4005 ± 980	72910 ± 11825	43440 ± 7400	24600 ± 4630	144955 ± 24650
NA	No amendments	6	1445 ± 290	73535 ± 8110	49950 ± 7350	24885 ± 5820	149815 ± 16585
NP	Nitrogen and phosphorus (N–P) addition	6	870 ± 370	42260 ± 5060	28860 ± 2875	15935 ± 1395	87925 ± 9635
AS	Activated sludge plus N–P addition	6	550 ± 210	34750 ± 6240	23780 ± 3980	13450 ± 2340	72530 ± 12765
KAS	Killed activated sludge plus N–P addition	6	760 ± 100	42580 ± 6465	28420 ± 5350	15720 ± 4215	87480 ± 16050

Data represents the mean and standard deviation of quadruplicate slurry samples

- Hydrocarbon-contaminated soil plus autoclaved activated sludge and nutrients (bioaugmentation negative control).

Autoclaved sludge was prepared by sterilising activated sludge via autoclave (121 °C, 15 psi) on 3 occasions over a 6-day period. Slurries were incubated at room temperature (22 ± 2 °C) for 42 days (in the dark) with continuous aeration supplied via diffused compressed air (1 L min^{-1}). Samples (50 mL) were removed weekly over the incubation period to analyse changes in microbial populations and hydrocarbon concentrations.

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 as described previously (Adetutu et al. 2012a). 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. 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 %.

DNA extraction and PCR

Microbial community DNA was extracted directly from the slurries collected on day 0 and weeks 2, 3, 4, and 6 using the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach CA) and PCR amplification of 16S rDNA genes was performed using primer pair 341FGC and 518R (Muyzer 1993) as described by (Sheppard et al. 2011). The cycling conditions were as

follows: 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.

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 (Non-linear 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—$\sum$ pi LN pi (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 interest 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. 2011).

Statistical analyses

Principal component analysis (PCA) was carried out using SPSS version 19 on the matrix data obtained from Phoretix 1D analysis (TL 120) as described previously (Adetutu et al. 2012b).

Results and discussion

Slurry phase hydrocarbon biodegradation

In order to assess the degradative potential of bioremediation strategies for the treatment of hydrocarbon contaminated soil, three different slurry treatments were prepared: (i) hydrocarbon-contaminated soil plus water, (non-amended slurries, i.e. natural attenuation), (ii) hydrocarbon-contaminated soil plus water and nutrients (nutrient slurries, i.e. ENA), (iii) hydrocarbon-contaminated soil plus activated sludge and nutrients (augmented slurries, i.e. bioaugmentation). In addition, a fourth slurry treatment was prepared (hydrocarbon-contaminated soil plus autoclaved activated sludge and nutrients) to act as a control for bioaugmentation investigations.

Table 1 and Fig. 1 show the concentration of C_{10}–C_{14}, C_{15}–C_{28}, C_{29}–C_{36}, C_{37}–C_{40}, and C_{10}–C_{40} hydrocarbon fractional ranges at the start of slurry phase treatment ($T = 0$) and at the end of the 6 week treatment period. In slurries without nutrient or inoculum amendments, some variability in hydrocarbon concentration was observed, due to soil heterogeneity, however, no significant difference in C_{15}–C_{28}, C_{29}–C_{36}, C_{37}–C_{40}, and C_{10}–C_{40} hydrocarbon fractional range concentrations ($P > 0.1$) was observed over the 6 week treatment period (Table 1). Nitrogen and phosphorus concentrations were low in this soil and slurry system (<1 mg kg^{-1}) which presumably restricted hydrocarbon degradation due to nutrient limitations (Tyagi et al. 2011). In contrast, in nutrient amended slurries where nitrogen and phosphorus were added to stimulate bacterial growth, a significant decrease ($P < 0.001$) in all hydrocarbon fractional ranges was observed (Table 1; Fig. 1). Following 6 weeks treatment, the concentration of C_{10}–C_{40} hydrocarbons was reduced by 41.3 ± 6.4 % although 87.9 ± 9.6 g C_{10}–C_{40} hydrocarbons kg^{-1} was still present in soil recovered from the slurry reactor. Presumably, longer treatment times may result in further removal of hydrocarbons, however,

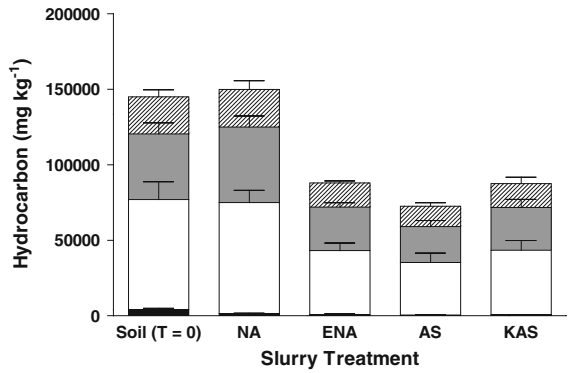

Fig. 1 Hydrocarbon concentration (*filled square* C_{10}–C_{14}; *open square* C_{15}–C_{28}; ▩ C_{29}–C_{36}; ▨ C_{37}–C_{40}) following 6 weeks of slurry phase treatment of hydrocarbon contaminated soil. Treatment strategies included no amendments (NA), nitrogen and phosphorus (N–P) addition (ENA), activated sludge plus N–P addition (AS) and killed activated sludge plus N–P addition (KAS). The hydrocarbon concentration in the soil prior to treatment is also shown (soil $T = 0$). Data represents the mean and standard deviation of replicate ($n = 4$) samples

the rate of hydrocarbon removal may be slower than initial rates due to the utilisation of the labile hydrocarbon pool and the decreased flux of C_{10}–C_{40} hydrocarbons from the sorbed to aqueous phase (Dandie et al. 2010).

In slurries amended with nutrients and activated sludge, hydrocarbon removal for all fractional ranges was enhanced following 6 weeks of treatment compared to other treatments (Table 1; Fig. 1). The concentration of C_{10}–C_{40} hydrocarbons was reduced to 75.5 ± 12.8 g C_{10}–C_{40} hydrocarbons kg^{-1} (i.e., 51.6 ± 8.5 % removal) compared to 87.5 ± 16.0 g C_{10}–C_{40} hydrocarbons kg^{-1} (i.e. 41.6 ± 10.7 % removal) for slurries amended with nutrients and autoclaved sludge. Although hydrocarbon biodegradation was enhanced through the addition of activated sludge, the reduction in total hydrocarbon concentration was not statistically significant compared to slurry treatments where nutrients were added alone ($P = 0.1025$) or where nutrients and autoclaved sludge ($P = 0.1951$) was added. However, the decrease in C_{29}–C_{36} hydrocarbon concentration was significant ($P < 0.1$) compared to treatments without 'active' sludge amendments. The statistical significance of hydrocarbon biodegradation results between slurry treatments with and without activated sludge amendments was in part due to the large standard deviations associated with the determination of hydrocarbon concentration resulting from heterogeneity issues associated with complex samples of this nature.

Microbial diversity in slurry bioreactors

Microbial diversity in hydrocarbon slurries was monitored at the start of the experiment (day 0) and on weeks 2, 3, 4 and 6 and compared with that of the activated sludge (Fig. 2). The dendrogram created from the DGGE gel showed four distinct community clusters associated with (1) the activated sludge (inoculum); (2) bioaugmented slurries with N–P additions at day 0; (3) bioaugmented slurries with N–P additions at weeks 2, 3, 4 and 6 and (4) slurries with autoclaved sludge and N–P additions (bioaugmentation negative control). A clearer representation of the four clusters can be seen through PCA (Fig. 3). Microbial diversity in the bioaugmented (activated sludge plus N–P addition) and killed control slurries (autoclaved sludge plus N–P addition) from weeks 2 to 6 remained stable resulting in the formation of two different clusters. This suggests that once the hydrocarbon-degrading community becomes established following an initial acclimation period (2 weeks), it remains stable throughout the degradation period. The augmented and killed control slurries corresponding to day 0 also form a different cluster which is explained by the fact that the activated sludge had just been added to the soils creating a new microbial community. As expected, the activated sludge profile also forms a distinct cluster since it was obtained from a different source (Australian wastewater treatment plant) to the contaminated soil.

Dominant microorganisms in slurry phase bioreactors

In order to identify the microbial component that may be responsible for hydrocarbons degradation (Table 1), prominent bands in the DGGE gel were excised from the activated sludge and the bioaugmented slurries (Fig. 4). This approach allowed for the identification of dominant organisms within slurry phase microbial communities and their potential ability to degrade hydrocarbons through database searches. Some of the excised bands (bands 1, 2, 3 and 4; Fig. 4) were only present in the bioaugmented slurries but not in the activated sludge alone suggesting that these bacteria were dominant organisms in the original hydrocarbon-contaminated soil. Six bands from day 0 of the bioaugmented slurries were retrieved. Bands 1 and 2 matched with a *Chitinophagaceae* bacterium (98 and 97 % similar respectively) found in soil in an *alkb* gene diversity study (Schulz et al. 2012) suggesting the presence of hydrocarbon degrading potential. The genus *Terrimonas* of the *Chitinophagaceae* family has been suggested to play an important role in the degradation of anthracene in municipal solid waste composting soil (Zhang et al. 2011). Band 3 was similar (100 %) to an uncultured *Haliscomenobacter* sp. Clone (EMBL Accession number: AEE50658). The strain *Haliscomenobacter hydrossis* DSM 1100 contains an aromatic hydrocarbon degradation membrane protein (Daligault et al. 2011) and has been implicated in hydrocarbon degradation (Table 2).

An uncultured *Aquabacterium* sp. clone was the closest match to band 4 (100 %). The recently proposed species *Aquabacterium njensis* strain HMGZ-01 T was isolated from hydrocarbon-contaminated soil in New Jersey and a preliminary screen of known alkane

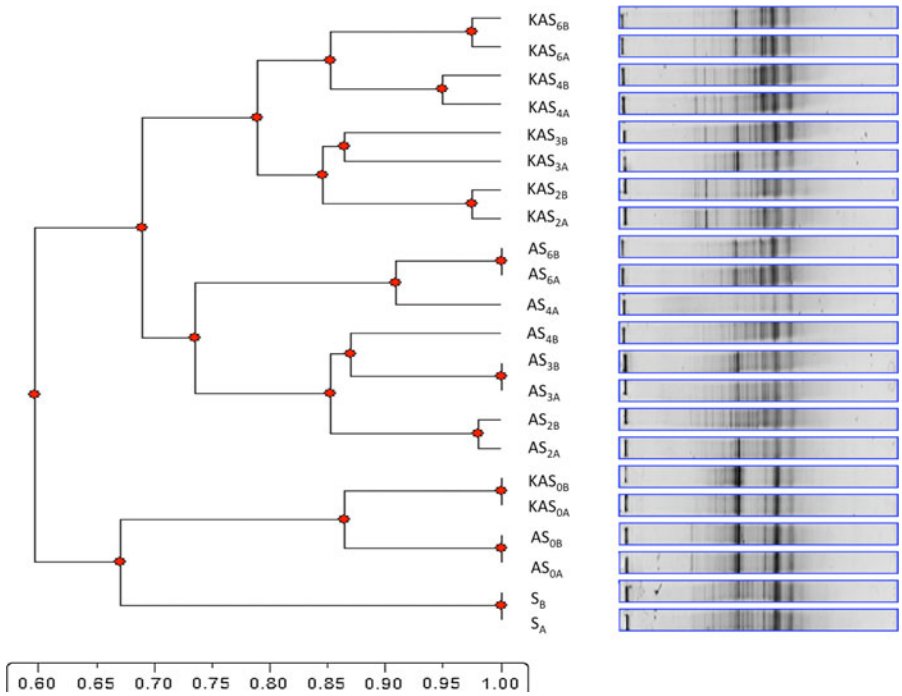

Fig. 2 UPGMA dendrogram of 16S rDNA based DGGE profiles of bacterial communities in activated sludge (S) and slurries containing hydrocarbon-contaminated soil and bioaugmented activated sludge plus N–P addition (AS) and killed activated sludge plus N–P addition (KAS). Scale refers to percentage similarity. *Subscript numbers* refer to sampling time points (0, 2, 3, 4 and 6 weeks) while *subscript letters* (A and B) refer to duplicate samples

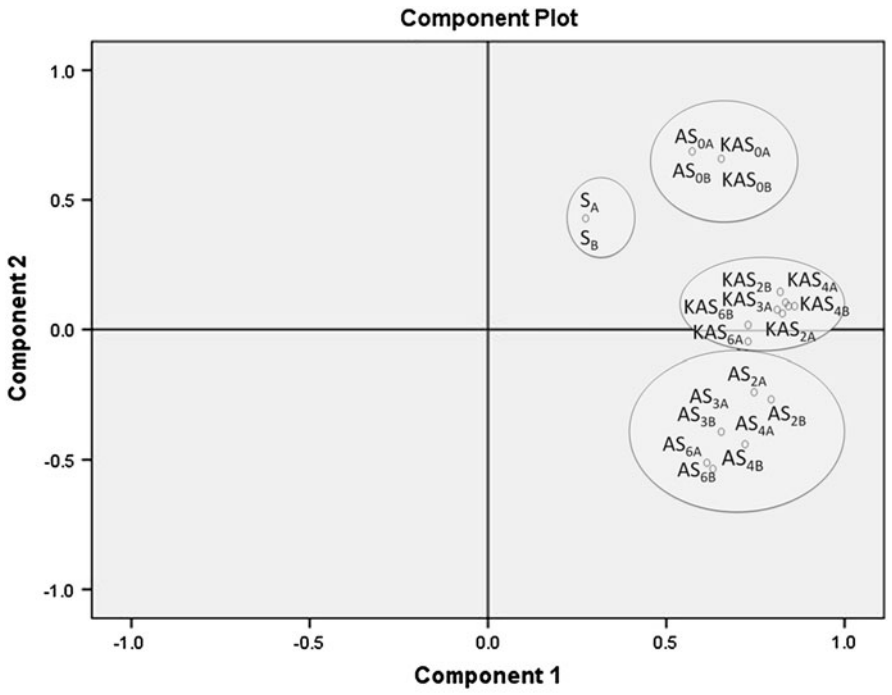

Fig. 3 Principal component analysis of the DGGE showing the difference in microbial communities of samples from activated sludge (S) and slurries containing hydrocarbon-contaminated soil and bioaugmented activated sludge plus N–P addition (AS) and killed activated sludge plus N–P addition (KAS). *Subscript numbers* refer to sampling time points (0, 2, 3, 4 and 6 weeks) while *subscript letters* (A and B) refer to duplicate samples

oxidation enzymes identified two alkane monooxygenase (*alkB*) genes, confirming its ability to degrade hydrocarbons (Masuda 2009). Moreover, the methylotroph *Methylibium petroleiphilum*, a methyl *tert*-butyl ether, benzene, toluene, xylenes and phenol degrader (Deeb et al. 2001) is closely related to the *Aquabacterium* genus within the *Sphaerotilus-Leptothrix* group (Nakatsu et al. 2006). The detection of a large number of methylotrophs in hydrocarbon-contaminated environments such as in plumes from the recent *Deepwater*

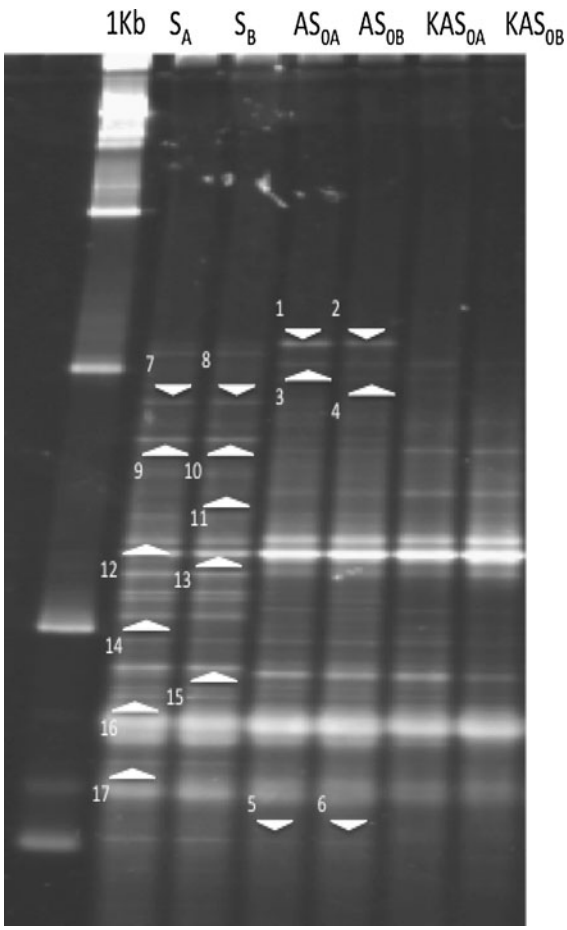

Fig. 4 DGGE analysis of 16S rRNA gene fragment of total bacterial population from the activated sludge and bioaugmented slurries at day 0. Excised and sequenced bands are marked on the gel. *Lanes* represent microbial communities from activated sludge (S) and slurries containing hydrocarbon-contaminated soil and bioaugmented activated sludge plus N–P addition (AS) and killed activated sludge plus N–P addition (KAS). The *subscript number* refer to sampling time point (time = 0 weeks) while *subscript letters* (A and B) refer to duplicate samples

Horizon oil spill (Redmond and Valentine 2011) suggest an important role in the degradation of hydrocarbons for these species. Bands 5 and 6 were closely related (95 and 94 % respectively) to an uncultured bacterium from west Pacific deep sea sediments which have not been linked to hydrocarbons degradation (Zeng et al. 2010).

It is well known that the metabolic capacity of complex microbial communities found in activated sludge is diverse due to the enrichment on a variety of organic and inorganic constituents found in waste water treatment facilities. As a result, determining the identity of dominant organisms may provide qualitative information regarding their catabolic potential. Identifying prominent bacterial species in the activated sludge was achieved by excision and sequencing of 10 bands (7-17, Fig. 4) from the DGGE gel. Two bands (7 and 8) were similar (99 and 98 % respectively) to an uncultured *Chloroflexi* bacterium clone. Several *Chloroflexi* species have been found in hydrocarbon-contaminated environments. An uncultured *Chloroflexi* bacterium, previously reported from a heavy metal-contaminated site, was the most dominant member of a microbial community isolated from a petroleum-contaminated aquitard (Van Stempvoort et al. 2009). In more recent studies, members of the *Chloroflexi* have been detected in a BTEX-contaminated aquifer (Berlendis et al. 2010) and in clone libraries from ^{13}C-labelled and unlabelled fractions of DNA after ^{13}C-toluene consumption from a tar-oil-contaminated aquifer sediment (Winderl et al. 2010).

Other prominent bands within the activated sludge (9 and 10) were closely related (96 and 98 % respectively) to an uncultured bacterium isolate with a trimethylamine (TMA)-degrading protein-like gene. Although the closest match to band 11 was an uncultured bacterium not linked to hydrocarbon degradation (94 %), the sequences obtained from bands 12 and 13 were closely related to a *Burkholderiales* bacterium (97 %) and an uncultured *Klebsiella* clone (100 %) respectively. Several members of *Burkholderiales* and *Klebsiella* have been linked to the degradation of hydrocarbons (Chamkha et al. 2011; Chunyang et al. 2010; Pérez-Pantoja et al. 2011; Rodrigues et al. 2009). Members of the *Burkholderiales* have been found in two consortia with the ability to degrade aliphatic and aromatic hydrocarbon binary mixtures (Bacosa et al. 2010, 2011). Moreover, recent studies have determined the presence and organization of genes encoding oxygenases involved in aromatics degradation in several *Burkholderiales* genomes (Pérez-Pantoja et al. 2011) while bacteria related to the *Burkholderiaceae* were also found to be associated with anaerobic benzene degradation (van der Zaan et al. 2012). Furthermore, there is a large number of *Klebsiella* strains capable of hydrocarbon degradation (Rodrigues et al. 2009). Recently, the species *Klebsiella aquatica* was also found to degrade polyaromatic hydrocarbons (Chunyang et al. 2010) while *Klebsiella oxytoca* can degrade crude oil (Chamkha et al. 2011).

Table 2 Closest relatives of band sequences excised from the DGGE in Fig. 4

Sample	Band	Closest relative	Accession number	% Similarity
AS_0[a]	1	*Chitinophagaceae* bacterium MS281c	JN616363	98
AS_0	2	*Chitinophagaceae* bacterium MS281c	JN616363	97
AS_0	3	Uncultured *Haliscomenobacter* sp. clone WR41	HM208523	100
AS_0	4	Uncultured *Aquabacterium* sp. clone RUGL1-394	GQ420898	100
AS_0	5	Uncultured bacteria partial 16S rRNA gene, isolate DGGE band WP02-3-8	AM292550	95
AS_0	6	Uncultured bacteria partial 16S rRNA gene, isolate DGGE band WP02-3-8	AM292550	94
S[b]	7	Uncultured *Chloroflexi* bacterium clone MSB-5A8	DQ811890	99
S	8	Uncultured *Chloroflexi* bacterium clone MSB-5A8	DQ811890	98
S	9	Uncultured bacterium isolate DGGE gel band D3 TMA-degrading protein-like gene	EF521194	96
S	10	Uncultured bacterium isolate DGGE gel band D3 TMA-degrading protein-like gene	EF521194	98
S	11	Uncultured bacteria partial 16S rRNA gene, isolate DGGE band WP02-3-5	AM292547	94
S	12	Uncultured *Burkholderiales* bacterium clone PSB011.C21_P16	GU300280	97
S	13	Uncultured *Klebsiella* sp. clone G2-4	GQ471877	100
S	14	Uncultured bacterium clone B3_37	HM228719	99
S	15	Uncultured bacterium isolate DGGE gel band 14	FJ499355	92
S	16	Uncultured organism clone SBYH_3324	JN456305	99
S	17	Uncultured *Proteus* sp. isolate DGGE gel band UC05-3	JF833134	90

[a] Activated sludge plus N–P addition at time = 0 days

[b] Activated sludge inoculum

These two bands were also the most prominent ones in the bioaugmented slurries throughout the 6-week treatment period suggesting that they are important members of the hydrocarbon degrading community. Bands 14, 15 and 16 matched (99, 92, 99 % respectively) with uncultured bacteria found in an aquifer, a reactor and a hypersaline microbial mat respectively but have not been associated with hydrocarbons degradation.

An uncultured *Proteus* sp. isolate was the closest match to band 17 (90 %). Strain LM 18-6 identified as *Proteus mirabilis*, was isolated from a gasoline-contaminated soil and was able to degrade methyl-*tert* butyl ether (Muñoz-Castellanos et al. 2006). Moreover, another study reported that a *Proteus mirabilis* strain was resistant to heavy metals, and it was also shown to utilize aromatic hydrocarbons as a sole source of carbon and energy (Filali et al. 2000). Identifying microorganisms closely related to *Klebsiella*, *Chloroflexi* and the *Burkholderiales* order in the activated sludge suggests the capability of this inoculum to enhance slurry phase biodegradation due to the potential role these organisms play in hydrocarbon degradation following bioaugmentation.

Conclusion

In this study, the indigenous microbial population in a long-term hydrocarbon contaminated soil was able to degrade hydrocarbons in the C_{10}–C_{40} fraction range once nutrients were made available. The indigenous population was highly diverse and contained hydrocarbon-degrading bacteria such as members of the *Aquabacterium* and *Haliscomenobacter* genera. Moreover, bioaugmentation of soil slurries with activated sludge resulted in the enhanced capacity to biodegrade hydrocarbons. The addition of activated sludge containing microorganisms in the *Klebsiella* genera and *Burkholderiales* order, enhanced hydrocarbon degradation although longer degradation timeframes may be required to demonstrate the potential of this approach.

Acknowledgments This research was funded by the Cooperative Research Centre for Contamination Assessment and Remediation of the Environment (Grant no. 6-5-01). The authors would like to acknowledge the support of the Centre for Environmental Risk Assessment and Remediation, University of South Australia and the School of Biological Sciences, Flinders University.

References

Aburto A, Fahy A, Coulon F, Lethbridge G, Timmis KN, Ball AS, McGenity TJ (2009) Mixed aerobic and anaerobic microbial communities in benzene-contaminated groundwater. J Appl Microbiol 106(1):317–328

Adetutu EM, Ball AS, Weber J, Aleer S, Dandie CE, Juhasz AL (2012a) Impact of bacterial and fungal processes on 14C-hexadecane mineralisation in weathered hydrocarbon contaminated soil. Sci Total Environ 414:585–591. doi:10.1016/j.scitotenv.2011.11.044

Adetutu EM, Thorpe K, Shahsavari E, Bourne S, Cao X, Fard RMN, Kirby G, Ball AS (2012b) Bacterial community survey of sediments. Int J Speleol 41(20):13–23

Aleer S, Adetutu E, Makadia T, Patil S, Ball A (2011) Harnessing the hydrocarbon-degrading potential of contaminated soils for the bioremediation of waste engine oil. Water Air Soil Pollut 218(1):121–130. doi:10.1007/s11270-010-0628-1

Atlas MRP, Philp J (2005) Bioremediation applied microbial solutions for real-world environmental cleanup. ASM Press, Washington

Bacosa H, Suto K, Inoue C (2010) Preferential degradation of aromatic hydrocarbons in kerosene by a microbial consortium. Int Biodeterior Biodegradation 64(8):702–710. doi:10.1016/j.ibiod.2010.03.008

Bacosa H, Suto K, Inoue C (2011) Preferential utilization of petroleum oil hydrocarbon components by microbial consortia reflects degradation pattern in aliphatic–aromatic hydrocarbon binary mixtures. World J Microbiol Biotechnol 27(5):1109–1117. doi:10.1007/s11274-010-0557-6

Berlendis S, Lascourreges J-F, Schraauwers B, Sivadon P, Magot M (2010) Anaerobic biodegradation of BTEX by original bacterial communities from an underground gas storage aquifer. Environ Sci Technol 44(9):3621–3628. doi:10.1021/es100123b

Chamkha M, Trabelsi Y, Mnif S, Sayadi S (2011) Isolation and characterization of Klebsiella oxytoca strain degrading crude oil from a Tunisian off-shore oil field. J Basic Microbiol 51(6):580–589. doi:10.1002/jobm.201100073

Chunyang Z, Xinglei L, Huiquan M, Guanling S, Guihua S, Zhongfeng D, Mingfeng L (2010) *Klebsiella aquatica* sp. nov., a novel PAH degrading bacterium from wastewater. In: E-product E-service and E-entertainment (ICEEE), 2010 international conference on, 7–9 Nov 2010, pp 1–4

Daligault H, Lapidus A, Zeytun A, Nolan M, Lucas S, Glavina Del Rio T, Tice H, Cheng J-F, Tapia R, Han C, Goodwin L, Pitluck S, Liolios K, Liolios K, Pagani I, Ivanova N, Huntemann M, Mavromatis K, Mikhailova N, Pati A, Chen A, Palaniappan K, Land M, Hauser L, Brambilla E, Rohde M, Verberg S, Göker M, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk H-P, Woyke T (2011) Complete genome sequence of *Haliscomenobacter hydrossis* type strain (O T) Stand. Genomic Sci 4(3):352–360. doi:10.4056/sigs.1964579

Dandie CE, Weber J, Aleer S, Adetutu EM, Ball AS, Juhasz AL (2010) Assessment of five bioaccessibility assays for predicting the efficacy of petroleum hydrocarbon biodegradation in aged contaminated soils. Chemosphere 81(9):1061–1068. doi:10.1016/j.chemosphere.2010.09.059

Deeb RA, Hu HY, Hanson JR, Scow KM, Alvarez-Cohen L (2001) Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. Environ Sci Technol 35(2):312–317

Fahy A, Lethbridge G, Earle R, Ball AS, Timmis KN, McGenity TJ (2005) Effects of long-term benzene pollution on bacterial diversity and community structure in groundwater. Environ Microbiol 7(8):1192–1199

Filali BK, Taoufik J, Zeroual Y, Dzairi FZ, Talbi M, Blaghen M (2000) Waste water bacterial isolates resistant to heavy metals and antibiotics. Curr Microbiol 41(3):151–156. doi:10.1007/s0028400

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003) Soil type is the primary determinant of the composition of the total and active bacterial community in arable soils. Appl Environ Microbiol 69:1800–1809

Juteau P, Bisaillon J-G, Lépine F, Ratheau V, Beaudet R, Villemur R (2003) Improving the biotreatment of hydrocarbons-contaminated soils by addition of activated sludge taken from the wastewater treatment facilities of an oil refinery. Biodegradation 14(1):31–40. doi:10.1023/a:1023555616462

Masuda H (2009) Identification and characterization of monooxygenase enzymes involved in 1,4-dioxane degradation in *Pseudonocardia* sp. strain ENV478, *Mycobacterium* sp. strain ENV421, and *Nocardia* sp. strain ENV425, Dissertation. The State University of New Jersey

McDonald RC, Isbell RF, Speight JC, Walker J, Hopkins MS (1990) Australian soil and land survey field handbook, 2nd edn. Inkata Press, Melbourne, Australia

Muñoz-Castellanos LN, Torres-Muñoz JV, Keer-Rendón A, Manzanares-Papayanopoulos LI, Nevárez-Moorillón GV (2006) Aerobic biodegradation of methyl tert-butyl ether (MTBE) by pure bacterial cultures isolated from contaminated soil. World J Microbiol Biotechnol 22(8):851–855. doi:10.1007/s11274-005-9114-0

Muyzer G (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nakatsu CH, Hristova K, Hanada S, Meng X-Y, Hanson JR, Scow KM, Kamagata Y (2006) *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl tert-butyl ether-degrading methylotroph of the *Betaproteobacteria*. Int J Syst Evol Microbiol 56(5):983–989. doi:10.1099/ijs.0.63524-0

Pérez-Pantoja D, Donoso R, Agulló L, Córdova M, Seeger M, Pieper DH, González B (2011) Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Microbiol 14(5):1091–1117. doi:10.1111/j.1462-2920.2011.02613.x

Rayment GE, Higginson FR (1992) Australian laboratory handbook of soil and water chemical methods. Inkata Press, Melbourne

Redmond MC, Valentine DL (2011) Natural gas and temperature structured a microbial community response to the deepwater horizon oil spill. Proc Natl Acad Sci USA. doi:10.1073/pnas.1108756108

Rodrigues DF, Sakata SK, Comasseto JV, Bícego MC, Pellizari VH (2009) Diversity of hydrocarbon-degrading *Klebsiella* strains isolated from hydrocarbon-contaminated estuaries. J Appl Microbiol 106(4):1304–1314. doi:10.1111/j.1365-2672.2008.04097.x

Schulz S, Giebler J, Chatzinotas A, Wick LY, Fetzer I, Welzl G, Harms H, Schloter M (2012) Plant litter and soil type drive abundance, activity and community structure of alkB harbouring microbes in different soil compartments. ISME J. doi:http://www.nature.com/ismej/journal/vaop/ncurrent/suppinfo/ismej201217s1.html

Sheppard PJ, Adetutu EM, Makadia TH, Ball AS (2011) Microbial community and ecotoxicity analysis of bioremediated, weathered hydrocarbon-contaminated soil. Aust J Crop Sci 49 (3):261–269. doi:http://dx.doi.org/10.1071/SR10159

Tellez GT, Nirmalakhandan N, Gardea-Torresdey JL (2002) Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oilfield produced water. Adv Environ Res 6(4):455–470. doi:10.1016/s1093-0191(01)00073-9

Tyagi M, da Fonseca M, de Carvalho C (2011) Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22(2):231–241. doi:10.1007/s10532-010-9394-4

van der Zaan BM, Talarico Saia F, Stams AJM, Plugge CM, de Vos WM, Smidt H, Langenhoff AAM, Gerritse J (2012) Anaerobic benzene degradation under denitrifying conditions: Peptococcaceae as dominant benzene degraders and evidence for a syntropic process. Environ Microbiol. doi:10.1111/j.1462-2920.2012.02697.x

Van Stempvoort DR, Millar K, Lawrence JR (2009) Accumulation of short-chain fatty acids in an aquitard linked to anaerobic biodegradation of petroleum hydrocarbons. Appl Geochem 24(1):77–85. doi:10.1016/j.apgeochem.2008.11.004

Vogel TM (1996) Bioaugmentation as a soil bioremediation approach. Curr Opin Biotechnol 7(3):311–316

Watanabe K, Hino S, Takahashi N (1996) Effects of exogenous phenol-degrading bacteria on performance and ecosystem of activated sludge. J Ferment Bioeng 82(3):291–298

Weiner JM, Lovley DR (1998) Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. Appl Environ Microbiol 64(2):775–778

Winderl C, Penning H, Netzer F, Meckenstock RU, Lueders T (2010) DNA-SIP identifies sulfate-reducing *Clostridia* as important toluene degraders in tar-oil-contaminated aquifer sediment. ISME J 4(10):1314–1325

Zeng X, Xiao X, Wang F (2010) Response of bacteria in the deep-sea sediments and the Antarctic soils to carbohydrates: effects on ectoenzyme activity and bacterial community. J Environ Sci 22(11):1779–1785. doi:10.1016/s1001-0742(09)60319-3

Zhang S-Y, Wang Q-F, Wan R, Xie S-G (2011) Changes in bacterial community of anthracene bioremediation in municipal solid waste composting soil. J Zhejiang Univ Sci B 12(9):760–768. doi:10.1631/jzus.B1000440

Zhao X, Wang Y, Ye Z, Borthwick AGL, Ni J (2006) Oil field wastewater treatment in biological aerated filter by immobilized microorganisms. Process Biochem 41(7):1475–1483. doi:10.1016/j.procbio.2006.02.006