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Remediation trials for hydrocarbon-contaminated soils in arid environments: Evaluation of bioslurry and biopiling techniques

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ABSTRACT

Effective bioremediation requires an extensive understanding of the soil parameters and microbial community diversity. Long term contamination of petroleum hydrocarbon soils in arid areas present unique opportunities to study the response of the impacted microbial community to bioremediation efforts. Two bioremediation treatments viz., biopile and bioslurry, were applied to assess the efficacy of different bioremediation methods in long term petroleum hydrocarbon contaminated soils. The bioslurry treatment was markedly more effective at treating the long term petroleum hydrocarbon contaminated soils in a short period of time. First order rates of hydrocarbon degradation in the bioslurry treatment were between 0.066 and 0.073 d^{-1} compared with the biopile treatment where the rates ranged from 0.011 to 0.03 d^{-1}, depending on the level and nature of the hydrocarbon fractions present in the soils. Bioslurry treatment of the long term contaminated soils exhibited a shift in the microbial community composition. *Alpha-proteobacteria* dominated the microbial community present in the hydrocarbon contaminated soils but bioslurry treatment of the contaminated soils led to a clear shift in the microbial community present in the soils, with *Gamma-proteobacteria* dominating the remediation environment along with microbial sequences associated with the *TM7* phylum and a subsequent reduction in hydrocarbon concentration in the soils.

Introduction

Petroleum hydrocarbon contamination of soils is one of the most common pollution issues faced by the mining sector in Australia. Of the 160 000 potentially contaminated sites in Australia, 60% comprise hydrocarbon-contaminated sites (EEA, 2007). Petroleum hydrocarbon contamination originates from a number of different mining activities including inadvertent diesel spills, leaking storage tanks and pipelines, and storage of oily wastewaters in unlined holding ponds. Petroleum hydrocarbons are potentially hazardous to the environment and human health. Consequently, Australian regulators, as with other regulators worldwide, recommend that petroleum hydrocarbon concentrations should not exceed a level that may be deleterious to both the human health and the environment (Duan et al., 2013).

One of the major environmental challenges facing the resource sector is to remediate soils contaminated with petroleum hydrocarbon spills in arid environments. Bioremediation methods, such as land farming and bio-piling, are often utilised to remediate these contaminated soils as these techniques are often viewed as being low cost, less intrusive, and environmentally sustainable compared to other remediation treatments (Juhasz et al., 2000; Farhadian et al., 2008; Megharaj et al., 2011; Lors et al., 2012). However, bioremediation may be considered a sustainable remediation treatment process when conducted successfully. The degree of bioremediation success depends on a number of factors, including the concentration and nature of hydrocarbon fractions present, the site properties (eg. soil texture, pH, organic matter, nutrients, temperature, rainfall), the microbial community present, and the bioremediation treatment selected (Yang et al., 2009; Lors et al., 2010; Hueso et al., 2012). In many arid regions of Australia, these factors will impact on the bioremediation process. For example,

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contaminated arid region soils typically lack adequate soil nutrients required for the microbial degradation of the hydrocarbons, or the soil moisture content may not be adequate to maintain the degradation of the hydrocarbon contaminants throughout the year. In addition, the microbial community present in arid soils may be markedly different in arid environments compared to temperate regions (Lors et al., 2010; Pasternak et al., 2013; Ros et al., 2014). Microorganisms are the key to successful remediation of hydrocarbon contamination as they degrade hydrocarbons by utilising them as a carbon and/or energy sources (Ortega-Calvo et al., 2007; Thavamani et al., 2012). In soils, the microbial community is very diverse and is comprised of bacteria, fungi, actinomycetes, algae, protozoa, and nematodes, but the relative number of bacteria is far greater than the other organisms in the soil. Bacteria have been shown to be the predominant organisms responsible for degradation of hydrocarbons under aerobic conditions (Leahy and Colwell. 1990). Hydrocarbon degrading bacteria are ubiquitous in soils however degradation of hydrocarbons may be limited and/or slow due to external factors that limit microbial activity. In arid environments, this may include factors such as nutrients and moisture. Biostimulation of endemic soil microorganisms by managing environmental conditions conducive for microbial growth has been found to be an ideal method to enhance microbial degradation of hydrocarbon contaminants (Viñas et al., 2005; Gallego et al., 2010; Tyagi et al., 2011; Lladó et al., 2012). However, the effectiveness of biostimulation of soil microorganisms in arid soils contaminated with weathered hydrocarbons has rarely been demonstrated. The objective of this research was to compare the efficacy of two bioremediation approaches on the remediation of aged petroleum hydrocarbon contaminated soils in an arid region of Australia. In addition, advanced molecular techniques were utilised to assess changes in the microbial community structure and to identify microbial organisms in each soil and bioremediation treatment mainly responsible for hydrocarbon degradation.

Materials and methods

Sampling and soil characterisation

Petroleum hydrocarbon contaminated soils were collected from 3 coastal sites in the Pilbara region of Australia. The Pilbara region experiences elevated daytime temperatures with an mean maximum temperature between 22 °C (winter) and 39 °C (summer) and a mean annual rainfall of 310 mm (Bureau of Meteorology, 2015). Approximately 51% of the annual rainfall occurs during the summer period. The soils of the area are typically red shallow loams or red shallow sands which have low soil fertility (George et al., 2009). The Pilbara region has extensive iron ore mining activities centred on the towns of Newman and Tom Price. Iron ore is transported by rail from these areas to Port Hedland, Cape Lambert and Dampier, where it is loaded and shipped to regions worldwide. Maintenance on the rail infrastructure is extensive and this has led to historical contamination of soil and groundwater in localised areas in and around rail maintenance facilities. The nature of the contamination varies but the contamination mainly consists of petroleum hydrocarbon contamination from diesel or fuel oil generated through the disposal of contaminated waste materials (sludges or cleaning materials) or leaking un-bunded fuel tanks. Total petroleum hydrocarbon (TPH) concentrations in contaminated soils and groundwater have been reported to range between <100 to >100 000 mg kg^{-1} dry soil (OTEK 2011) and <0.01 to 370 mg L^{-1} water (HydroSolutions 2001), respectively.

Soil sampling was undertaken at sites which were previously identified by elevated (>5000 mg TPH kg^{-1}) petroleum hydrocarbons. A backhoe was utilised to take soil samples at depths ranging from 25 to 300 cm from the soil surface. Samples were collected on-site from the backhoe bucket at the time of excavation and placed in sealed containers, before being transported to Adelaide for laboratory analysis and assessment of remediation potential. Prior to soil analysis, the soils were air-dried and sieved to remove particles >2-mm. Total petroleum hydrocarbon contamination was determined by accelerated solvent extraction (See section below on extraction and total petroleum hydrocarbon (TPH) quantification in contaminated soils) while soil physio-chemical properties (pH, EC, water holding capacity etc) were determined using standard protocols (Rayment and Lyons, 2010). Total petroleum hydrocarbon concentrations, various equivalent hydrocarbon molecular weight ranges and physico-chemical soil properties are listed in Table 1.

Mineralisation assays

^{14}C hydrocarbon preliminary studies

Biopile and bioslurry microcosm studies of hydrocarbon mineralisation by indigenous microorganisms were undertaken using ^{14}C-labelled decane, hexadecane or octacosane.

For the biopile study, 100 g of contaminated soil was moistened to 60% water holding capacity (WHC) and 1 μCi of the ^{14}C-hydrocarbon (decane, hexadecane or octacosane) in 1% dimethylformamide added with and without amendments. The amendments utilised to enhance indigenous microbial mineralisation included: nutrient addition, additional carbon sources, and a bulking agent to enhance soil physical condition. Control treatments to assess the abiotic degradation of ^{14}C-hydrocarbons were also included in the study. In the bioslurry study, 100 g of soil with nutrients was added to 300 mL of distilled water (soil: solution ratio 1:3). The slurry was mixed vertically on an orbital shaker at 120 rev min^{-1}. The same experimental conditions, ^{14}C-hydrocarbon spiking, sampling and nutrient regime as utilised in the biopile study was utilised in the bioslurry study.

Treatments were incubated at room temperature (22 °C) for 120 days and the evolution of $^{14}CO_2$ monitored throughout the study period by trapping the evolved $^{14}CO_2$ in NaOH solution. Aliquots (2 mL) of the NaOH trap solution were combined with 10 mL of UltimaGold scintillation fluid and the $^{14}CO_2$ evolved counted and quantified using a Pelkin-Elmer beta counter following standard protocols. The $^{14}CO_2$ evolved during the study period for the different treatments in the biopile and bioslurry microcosm studies were compared to assess the efficiency of each remediation method.

Bench scale laboratory studies

Mineralisation studies were conducted over a 56 day period in 3 contaminated soils to compare the bioremediation efficacy of the biopile and bioslurry systems.

In the biopile study, nutrients were added to 300 g of soil ($n = 2$; 4 soils) and thoroughly mixed together in 1 L glass jars before being moistened to 60% water holding capacity with deionised water. The loss of soil moisture was checked on a weekly basis (by weight) and water added as required. At the same time, the microcosms were aerated through manual soil mixing. Nutrients (potassium nitrate and diammonium phosphate) were added at the beginning of the study to achieve a carbon:nitrogen:phosphorus (C:N:P) molar ratio of 100:10:1 based on the hydrocarbon loading as a measure of C. Soil samples (5 g) were collected at 1, 2, 3, 6, and 12 weeks after which the samples were analysed for TPH concentration in duplicate. Sodium azide was added to selected treatment as an abiotic control. The microcosms were incubated at 22 °C.

Table 1
Site characterisation of TPH contaminated soils.

Site	Depth (cm)	pH	EC (μS cm^{-1})	TPH (mg kg^{-1})				PAH (mg kg^{-1})	Metals (mg kg^{-1})							
				C_{10}–C_{14}	C_{15}–C_{28}	C_{29}–C_{36}	TPH		Pb	Cr	As	Cd	Sn	Mo	Ni	Zn
LSS1	0–25	8.67	1156	<50	<100	<100	<100	<3	5	34	9	<1	5	<1	15	22
	25–50	8.91	244	<50	<100	<100	<100	<3	5	35	5	<1	2	<1	10	16
	50–75	8.90	227	<50	<100	<100	<100	<3	4	82	4	<1	<1	<1	7	12
	75–125	8.87	146	860	7720	2340	10 900	4	12	283	5	<1	2		8	72
	125–175	9.18	173	790	5130	1900	7820	2	9	208	6	<1	<1	4	8	37
	175–225	8.93	129	1350	6410	2390	10 200	11	17	268	5	<1	<1	9	7	56
	225–275	9.15	108	2220	6380	2090	10 700	9	4	38	5	<1	<1	3	5	5
OCS1	25–50	9.01	75	<50	260	520	780	<3	158	297	8	<1	6	16	11	165
	120–140	9.34	83	<50	220	340	560	<3	21	33	7	<1	2	<1	6	36
	140–160	9.44	99	940	2650	2180	5770	9	21	44	7	<1	2	3	6	31
	160–180	9.34	127	2360	4050	2750	9160	22	7	24	9	<1	5	3	6	12
	200–225	9.27	118	6820	8730	4670	20 200	53	5	24	6	<1	2	3	5	7
	225–250	9.31	204	5980	7680	4050	17 700	46	12	34	8	<1	2	8	7	22
	280–300	9.24	180	5320	6660	3860	15 800	46	8	31	10	<1	2	4	5	13
OCS2	25–50	7.50	64	<50	<100	<100	<100	<3	8	26	4	<1	<1	2	4	15
	50–75	7.43	112	11 100	46 000	23 800	80 900	118	690	547	11	11	37	152	62	1153
	140–150	8.13	232	8310	31 800	18 800	58 900	956	100	92	8	2	9	18	12	195
	150–175	8.23	75	3310	10 300	7590	21 200	390	1587	6591	11	15	79	164	39	2953
	190–220	7.96	386	5380	10 400	6200	22 000	36	31	72	6	<1	5	17	8	58
	230–240	7.85	270	5590	20 300	14 500	40 400	56	76	139	9	<1	6	69	9	129

In the bioslurry study, 200 g of soil with nutrients was added to 600 mL of distilled water (soil: solution ratio 1:3). The slurry was mixed vertically on an orbital shaker at 120 rev min^{-1}. The same experimental conditions, sampling and nutrient regime as utilised in the biopile study was utilised in the bioslurry study.

Assessment of microbial activity and phylogenetic diversity

Assessment of diesel degrading bacterial populations was performed using a most probable number (MPN) method in 96-well microtiter plates by supplying diesel as carbon source (Wrenn and Venosa, 1996).

The bacterial diversity at each sampling stage was determined using next generation sequencing technique by sequencing the soil microbial DNA from 1 g of homogenised soil with a MO BIO PowerMax Soil DNA Isolation Kit. Amplification of 16S rRNA genes and pyrosequencing of all the extracted soil DNA was done externally through Australian Genomic Research Centre (AGRF), Brisbane, Australia. PCR amplicons were generated using the primers and conditions outlined in the AmpliTaq Gold 360 mastermix protocol (Life Technologies, Australia). Resulting amplicons were measured by fluorometry, normalised, measured by qPCR, normalised a second time, and then pooled in equimolar ratios. This amplicon pool was then run on the GS-FLX platform using XLR70 chemistry (Roche, Australia).

Extraction and total petroleum hydrocarbon (TPH) quantification in contaminated soils

TPH extraction from contaminated soils was performed using an accelerated solvent extraction method (ASE200 Accelerated Solvent Extraction System, Dionex Pty Ltd). Contaminated soil (2 g) was added to extraction cells containing approximately 1 g of solvent washed silica gel sandwiched between two cellulose filter papers. To minimise the solvent solution utilised during the extraction process, quartz sand size material (cleaned by heating to 600 °C) was added to the 1 mL extraction cell and a 50 μL of O-terphenyl surrogate solution added prior to sealing. Contaminated soils were extracted using acetone:hexane solvent mix (1:1 v/v). Solution extracts were filtered through 0.45 μm PTFE filters into 2 mL GC vials. Solutions containing TPH extracts were either injected into the GC-FID after filtering or the solution diluted using hexane:acetone extracting solution.

Analysis of TPH in extracted solution was conducted by an Agilent Technologies Gas Chromatograph fitted with a Flame Ionisation Detector (GC-FID). Chromatography was performed on a fused-silica capillary column BPX-5 from SGE (15 m × 0.32 mm internal diameter) coated with HP-5 (0.10-μm film thickness). Helium was used as the carrier gas at 2.5 mL min^{-1}, and the FID detector temperature kept at 300 °C. Splitless injection with a sample volume of 1 μL was applied. The oven temperature was increased from 50° to 300 °C at a gradient of 25 °C min^{-1} and held at this temperature for 5 min. The total run time was 19.6 min.

The hydrocarbon concentration was quantified according to the following hydrocarbon fractions: C_{10}–C_{14}, C_{15}–C_{28}, C_{29}–C_{36}. QA&QC was confirmed using O-terphenyl as surrogate and recovery (89–92%) was used for quantification while all samples were analysed in triplicate with a standard deviation of <9% between analyses.

Statistical analysis

Statistical analysis was utilised to assess significance difference ($P < 0.05$) between microcosm treatment methodologies using *t*-tests.

Results and discussion

Contaminated site characterisation

Nineteen soil samples were initially collected from 3 sites at 2 contaminated areas in Western Australia which had been previously identified as contaminated with petroleum hydrocarbons, metal and polycyclic aromatic hydrocarbons. The physical and chemical characteristics of these 19 soils are shown in Table 1. The contaminated soils were alkaline, with pH ranging from 7.5 to 9.44 and the electrical conductivity (EC) of the soils ranged from 64 to 1156 μS cm^{-1}. The alkaline nature of the soils along with the elevated soil EC was not unexpected due to the proximity (approximately 500 m) of the sites to the estuarine environment. The texture of the soil samples was dominated by sand or sandy loams (Table 1). Petroleum hydrocarbon concentrations varied markedly with both horizontal and vertical sampling across the

sites (Table 1). Generally, low concentrations of contaminants were identified in the surface soils to a depth of approximately 0.25 m, after which TPH contamination was present to groundwater (~3 m) at concentrations ranging from <100 to 80 900 mg TPH kg^{-1}. The hydrocarbon fractions present in soils were dominated by the C_{16} to C_{36} which is indicative of both the sources of soil TPH and natural on-site weathering processes. Soil fertility, as determined by soil nitrogen and phosphorus concentrations, was low in all soils. Metals, notably chromium, molybdenum, zinc and lead along with polycyclic aromatic hydrocarbons (PAHs) were identified in several localised sampling areas and were associated with very high concentrations of TPH (Table 1). Samples with elevated TPH, metal and PAH concentrations were highly glutinous in nature with very poor handling characteristics. The poor handling characteristics were particularly evident in soil samples collected from the OCS2 site where TPH concentrations below 50 cm ranged from 21 200 to 80 900 mg TPH kg^{-1} (Table 1). Metal and PAH concentrations in these samples were also elevated compared to soil samples collected from the other sites (Table 1).

Preliminary bioremediation studies

Preliminary studies

Contaminated soils from each site were homogenised, resulting in 3 bulk contaminated soils (LSS1 0–275 cm; OCS1 25–300 cm; OCS2 25–240 cm). The soils were relabelled LSS1, OCS1 and OCS2 and contained TPH concentrations of 2250, 5170 and 17 800 mg kg^{-1}, respectively.

To investigate the feasibility of bioremediation techniques in the selected soils, ^{14}C-decane (C_{10}) was incorporated into the LSS1 contaminated soil prior to commencing the microcosm treatment studies. The addition of a range of amendments resulted in various amounts of $^{14}CO_2$ evolved from the microcosms (Fig. 1A). The most effective amendments for the biopile treatments were a combination of activated sludge, biosolids and bulking agent. The addition of these amendments resulted in the mineralization of 50 and 80% of the spiked ^{14}C-decane in the biopile treatment compared to 80 and 95% in the bioslurry treatment. Markedly higher ^{14}C-decane mineralisation was also attained with the addition of fewer amendments in the bioslurry treatments (Fig. 1A). For example, the addition of fertilizer and a bulking agent (T4 treatment; Fig. 1A) resulted in the mineralisation of approximately 60% of the added ^{14}C-decane which is similar to the results obtained for the biopile T7 to T10 treatments. Generally, most soils contain numerous bacteria capable of degrading petroleum hydrocarbons under stimulated conditions, but the low fertility of these soils appear to be limiting microbial mineralisation of the hydrocarbons present (Gallego et al., 2010; Llado et al., 2012; Lors et al., 2012). However, the marked differences in the mineralisation of ^{14}C-decane in the bioslurry and biopile treatments can also be attributed to the enhanced mass transfer rate of spiked ^{14}C-decane and the increased contact between the spiked ^{14}C-decane and the microorganisms in the bioslurry compared to the biopile treatment (Speight and Arjoon, 2012; He et al., 2014). Comparable ^{14}C-decane mineralisation was only observed in the bioslurry and biopile microcosms in treatments that received activated sludge, biosolids and bulking agent amendments (Fig. 1A). In all other biopile microcosms there was little mineralisation of ^{14}C-decane observed. The additions of activated sludge alone, or activated sludge and biosolid together, increased the quantity of ^{14}C-decane mineralised from approximately 10% (Treatment 6) to 50–80% (Treatments 7–10) of the spiked ^{14}C-decane. Activated sludge was added as microbial inoculum source and the biosolids added as a source of low cost nutrients. However, activated sludge also contains an available nutrient source, mainly nitrogen, which enhances short-term microbial activity (Gallego et al., 2001).

TPH contaminated soils collected from the contaminated sites were mainly composed of the C_{16}–C_{36} hydrocarbon fractions (Table 1). To understand the degradation pattern of these fractions, independent microcosm studies were conducted using the LSS1, OCS1 and OCS2 soils spiked with ^{14}C-hexadecane and ^{14}C-octacosane to evaluate mineralisation of these compounds in the biopile or bioslurry treatments (Fig. 1B and C). In these studies only the 4 most effective amendment treatments that enhanced ^{14}C-decane mineralisation were utilised. The results of the ^{14}C mineralisation studies showed that as the carbon-chain increased the percentage of ^{14}C mineralised decreased (Fig. 1B and C). The bioslurry treatment resulted in the mineralisation of between 25 and 70% of spiked ^{14}C-hexadecane (Fig. 1B) while only between 2 and 33% of the spiked ^{14}C-octacosane was mineralised in the soils (Fig. 1C). The reduction in mineralisation rates of hydrocarbon compounds with increasing molecular weight is not unexpected as carbon-chain length affects both the solubility of the compound and therefore potential hydrocarbon bioavailability, as well as the catabolic activity of the microorganisms present in the contaminated soil (Brassington et al., 2007; Muckian et al., 2007; Lors et al., 2010; Towell et al., 2011). The additions of biosolids, activated sludge and a bulking agent were the most effective amendments

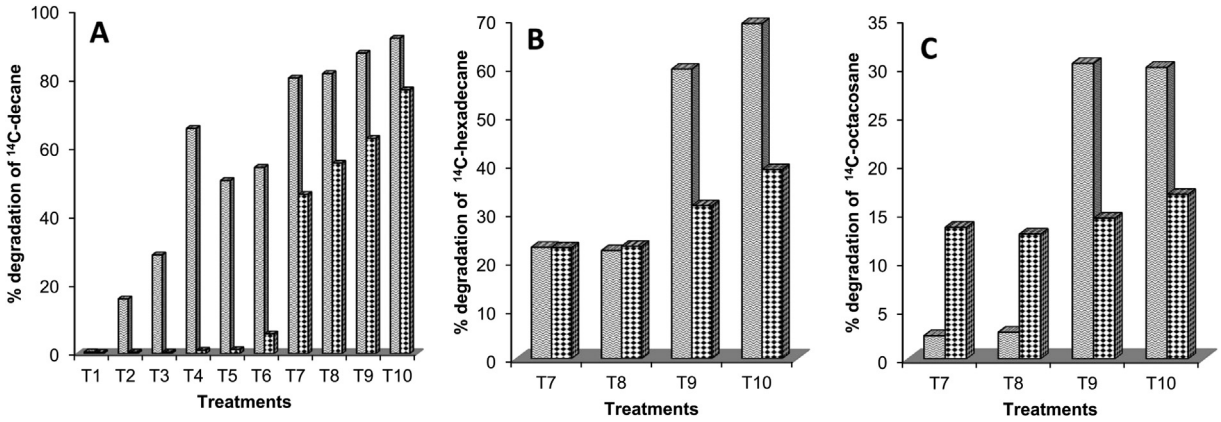

Fig. 1. Preliminary studies of mineralisation of ^{14}C-compounds added to LSS1 soil. ▨ Bioslurry treatment; ▦ Biopile treatment. T1 = biotic control; T2 = abiotic control; T3 = Soil + 5% bulking agent; T4 = Soil + fertiliser + 5% bulking agent; T5 = 5% biosolids + 5% bulking agent; T6 = 10% biosolids + 5% bulking agent; T7 = 5% activated sludge + 5% bulking agent; T8 = 10% activated sludge + 5% bulking agent; T9 = 5% activated sludge + 5% biosolids + 5% bulking agent; T10 = 10% activated sludge + 10% biosolids + 5% bulking agent.

irrespective of the treatment method (biopile vs bioslurry); however, the biopile treatment method was less successful at reducing the ^{14}C hydrocarbon concentration than the bioslurry method overall (Fig. 1A–C).

Utilising the preliminary results of the ^{14}C studies, further microcosm studies using LSS1, OCS1 and OCS2 were conducted to investigate the changes to the microbial community profile during the biopile and bioslurry treatments and the effectiveness of the amendment treatment on the bioremediation of recalcitrant TPH compounds in soils from arid regions.

Microcosm studies for bioremediation

Petroleum hydrocarbon contaminated soil which had been weathered for up to two decades were utilised in this study. The TPH concentrations in these soils ranged from 2200 to 17 800 mg TPH kg^{-1}. No benzene, toluene, ethylbenzene, xylene (BTEX), or polycyclic aromatic hydrocarbons were detected in these soils. The soils were predominantly composed of the C_{15}–C_{28} hydrocarbon fraction, although one soil also contained elevated concentration of C_{29}–C_{36} (OCS2). The microcosm studies were conducted utilising the optimised amendment conditions identified in the preliminary studies; Contaminated soil + 5% activated sludge + 5% biosolids + 5% bulking agent (<2 mm washed sand).

Microcosm studies showed there was an initial rapid degradation of TPH during the first 10 days of the studies followed by a slower rate of degradation (Figs. 2–4). In all soils studied, the TPH degradation rate was considerably higher in the bioslurry treatment compared to the biopile treatment (Figs. 2–4; Table 2) with the bioslurry treatment generally degrading twice as much TPH than the biopile treatment over the same time frame. For the C_{15}–C_{28} hydrocarbon fraction, the bioslurry treatment degraded between 80 and 100% of the TPH fraction in the soils while the biopile treatment only degraded between 40 and 60% of the TPH fraction. Similar degradation behaviour was observed for the C_{29}–C_{36} hydrocarbon fraction although the percentage of TPH degradation was markedly lower than the C_{15}–C_{28} fraction. Examples of the TPH degradation chromatographs for the biopile and bioslurry treatments for the LSS1 soil are shown in Fig. 5A and B, respectively. The chromatographic profiles in Fig. 5A and B clearly show that the original source of the contamination is from diesel fuel supporting the use of ^{14}C-labelled aliphatic compounds in the preliminary studies. The lower degradation rate of the high molecular weight TPH compounds is due to the lower hydrocarbon bioavailability of the C_{29}–C_{36} fraction compared to the C_{15}–C_{28} fraction (Van Zyl and Lorenzen, 1999). The degradation pattern observed in this study is similar to the results obtained by other researchers investigating TPH degradation in biopile treatments (Nocentini et al., 2000; Sarkar et al., 2005) indicating that there is a commonality in the behaviour of TPH in soils which can be mainly attributed to the solubility of TPH compounds present. Low molecular weight hydrocarbons (ie. $<C_{20}$) have a much higher water solubility than high molecular weight hydrocarbons (ie. $>C_{20}$) and therefore the bioavailability of the higher molecular weight hydrocarbons is considerably lower. High molecular weight hydrocarbons are predicted to reside in the soil solid and the non-aqueous phase liquid (NAPL) compartments in the biopile (Pollard et al., 2008; Coulon et al., 2010). Therefore hydrocarbon bioavailability is controlled by the desorption kinetics from the NAPL and soil solid phases into the soil aqueous phase. In the bioslurry treatment, desorption of higher molecular weight hydrocarbons is still limited by desorption kinetics from the soil solid and the NAPL compartments, but the mass hydrocarbon transfer rate is markedly faster due to the larger volume of water utilised in the bioslurry treatment. Comparison of TPH mineralization rates (Table 2) between the two treatment methods, show significantly faster TPH mineralization in the bioslurry treatment compared to the biopile treatment. Overall, TPH mineralisation rates followed first order kinetic model and ranged from 0.066 to 0.073 in the bioslurry treatment compared to 0.011–0.037 in the biopile treatment. The degradation rate constants were markedly lower than reported by Namkoong et al. (2002) but similar to those reported by Nocentini et al. (2000). Nocentini et al. (2000) reported hydrocarbon degradation rate constants of between 0.018 and 0.035 in soils spiked with either kerosene, diesel or lubricating mineral oil. The slightly lower first order degradation rates observed in this study are therefore not unexpected as the TPH has weathered over time (aged), resulting in soils dominated by the higher molecular weight hydrocarbon fraction (Table 1).

Dynamics of microbial population during bioremediation

The viable microbial population of the soils that were able to grow on diesel impregnated agar plates were assessed prior to remediation, and at the end of the treatment study period. As the assessment was selective for hydrocarbon degraders, the initial (pre-remediation) microbial count values were considerably lower than previously reported for the total microbial population (10^7 CFU g^{-1}) reported in dry soils (Ting et al., 1999). However, across the 3 soils, the total numbers of diesel degraders ranged from 9×10^4 to 55×10^4 CFU g^{-1}. The biopile microcosm treatment resulted in a 50- to 100-fold increase in the diesel degrading microbial population across all 3 treatments compared to a 22 000- to 57 000-fold increase in the diesel degrading microbial population in the bioslurry treatments.

To investigate the dynamics in the microbial communities in each soil, 454 pyrosequencing was conducted using the soils prior to, and after, bioslurry bioremediation. The microbial sequencing was only conducted using the bioslurry treated soils as this was the most effective treatment for reducing the TPH concentration in

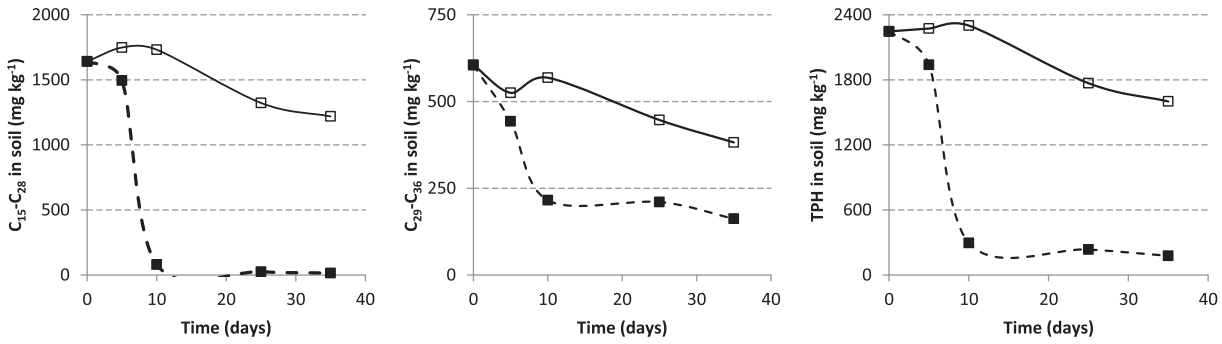

Fig. 2. TPH degradation in biopile and bioslurry microcosms in LSS1 soil. □ = biopile treatment ■ = bioslurry treatment.

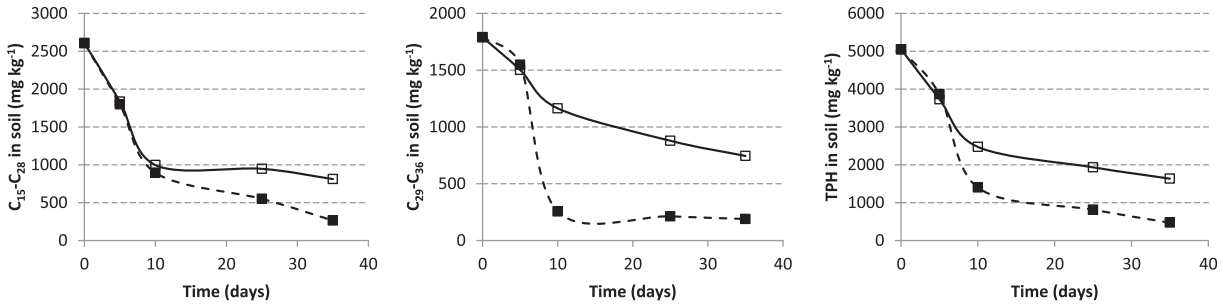

Fig. 3. TPH degradation in biopile and bioslurry microcosms in OCS1 soil. □ = biopile treatment ■ = bioslurry treatment.

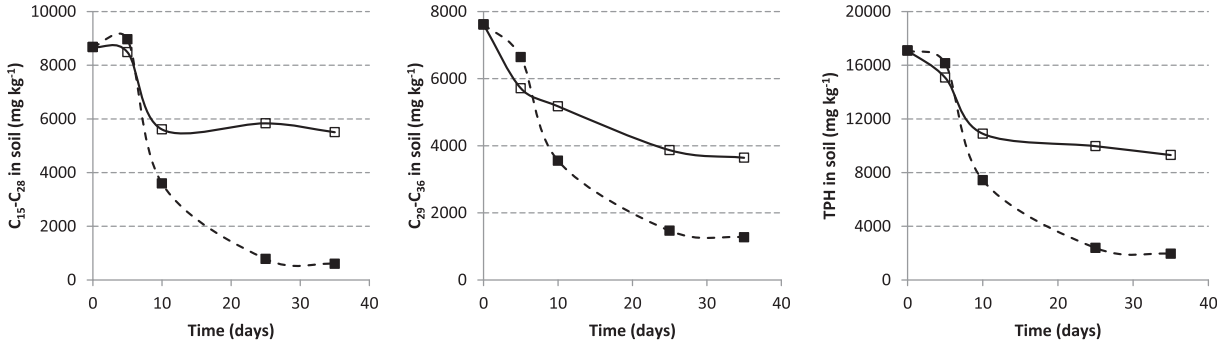

Fig. 4. TPH degradation in biopile and bioslurry microcosms in OCS2 soil. □ = biopile treatment ■ = bioslurry treatment.

these soils (Figs. 2–4). At the phylogenetic level, the contaminated soils (non-remediated) contains a wide range of taxa (Fig. 6), including sequences from the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteriodetes*, which have previously identified as hydrocarbon degrading organisms (Militon et al., 2010; Benedek et al., 2013; Aleer et al., 2014). However, the bacterial community in these non-remediated contaminated soils were dominated by *Proteobacteria* group (Fig. 6). The distribution of the *Proteobacteria* varies depending on the level of site contamination, with *Proteobacteria* comprising nearly 90% of the bacterial community present in LSS1, 65% in OCS1 and only 40% of the bacteria in OCS2. The change in bacterial distribution is associated with the increasing TPH concentration in each of these soils as there is little difference in the physiochemical properties of the soils (Table 1). At low TPH contaminant concentrations (LSS1 2200 mg TPH kg^{-1}) sequencing indicates that the *Proteobacteria* phyla comprise nearly 90% of the bacterial community present while *Bacteroidetes* and *Acidobacteria* are the dominant bacteria present in the remaining 10%. In contrast, in the OCS2 contaminated soil (17 800 mg TPH kg^{-1}), *Proteobacteria* comprised only 45% of the bacterial community identified, while *Bacteroidetes* comprised 40% of the microbial community. Bacteria from *Firmicutes* and *Acidobacteria* phyla were the other organisms identified while approximately 13% of the bacterial community could not be identified. Although it is not clearly known how the bacterial community structure in the polluted soils varies from the uncontaminated soil, the presence of contaminants such as petroleum hydrocarbons in soils has been reported to induce a shift in the community structure (Röling et al., 2004; Muckian et al., 2007; Lors et al., 2010; Ros et al., 2014). Post bioslurry remediation treatment assessment of the relative abundance of bacteria again identified a diverse range of phyla present in the soils (Fig. 6). Bacterial phyla sequenced in the bioslurry treated soils included, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteriodetes*, *Acidobacteria*, *TM7* and *Gemmatimonadetes* indicating marked bacterial community diversity in both the pre-treated and treated soil. The inverse Simpson index showed that the bacterial community diversity was high in LSS1 and OCS1 in both the pre-treated and treated soils (Table 3). However, in the OCS2 soil the bacterial community diversity was initially high in the pre-treated soil but decreased during the bioremediation treatment in the treated soil (Table 3).

In the bioslurry treated soil *Proteobacteria* remained the dominant phylum present in all the soils; 69% in LSS1, 61% OCS1 and 74% OCS2. The increase in the abundance of *Proteobacteria* in the treated OCS2 soil was accompanied by a decrease in the abundance of *Bacteroidetes* (40–9%) and an increase in the abundance of *TM7* bacteria (Fig. 6). In the other treated soils (LSS1 and OCS1) the relative abundance of *Bacteroidetes* remained similar as found in the original contaminated soils. For *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Gemmatimonadetes* phyla there was some variation in phyla abundance but these phyla generally made

Table 2
First order TPH degradation rates for biopile and bioslurry treatments ($k = d^{-1}$).

Soil	Bioslurry						Biopile					
	TPH		C_{15}–C_{28}		C_{29}–C_{36}		TPH		C_{15}–C_{28}		C_{29}–C_{36}	
	k	R^2	k	R^2	k	R^2	k	R^2	k	R^2	k	R^2
LSS1	0.073	0.75	0.141	0.85	0.034	0.77	0.030	0.89	0.011	0.86	0.024	0.96
OCS1	0.066	0.93	0.061	0.96	0.065	0.71	0.011	0.91	0.030	0.75	0.012	0.92
OCS2	0.069	0.95	0.086	0.95	0.055	0.95	0.016	0.81	0.013	0.63	0.019	0.91

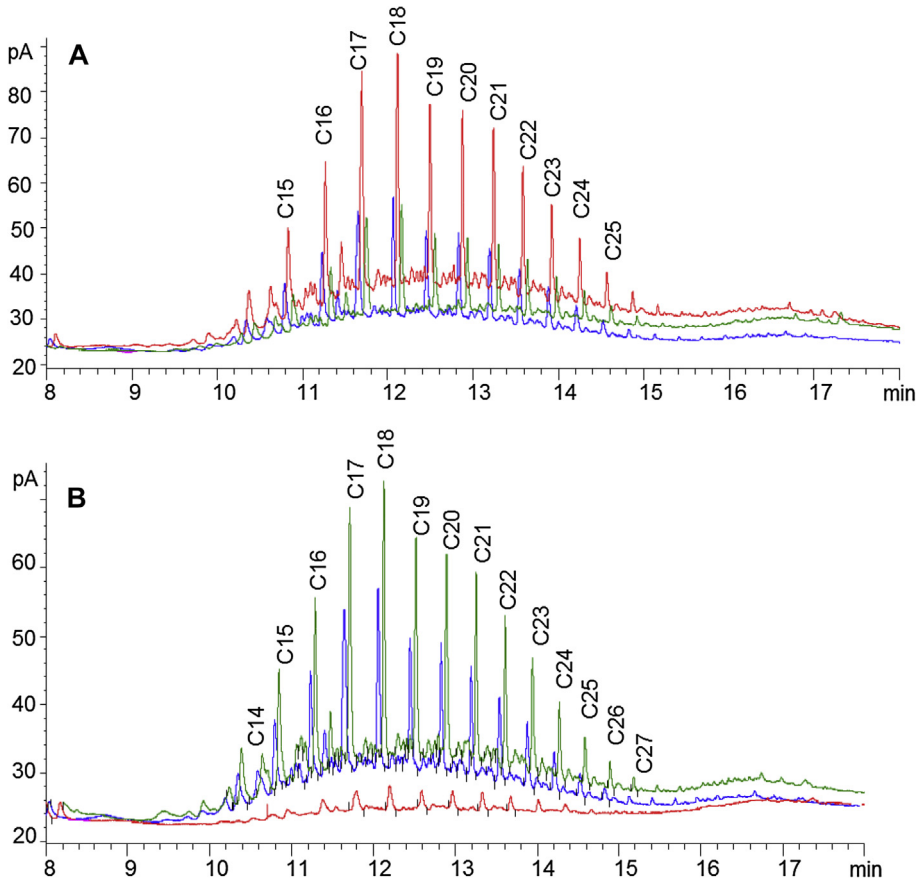

Fig. 5. A. TPH fingerprint during the degradation in biopile microcosms in LSS1 soil (Blue peaks = Day 0 TPH concentration; Red peaks = Day 10 TPH concentration; Green peaks = Day 35 TPH concentration). B. TPH fingerprint during the degradation in bioslurry microcosms in LSS1 soil (Blue peaks = Day 0 TPH concentration; Green peaks = Day 10 TPH concentration; Pink peaks = Day 35 TPH concentration). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

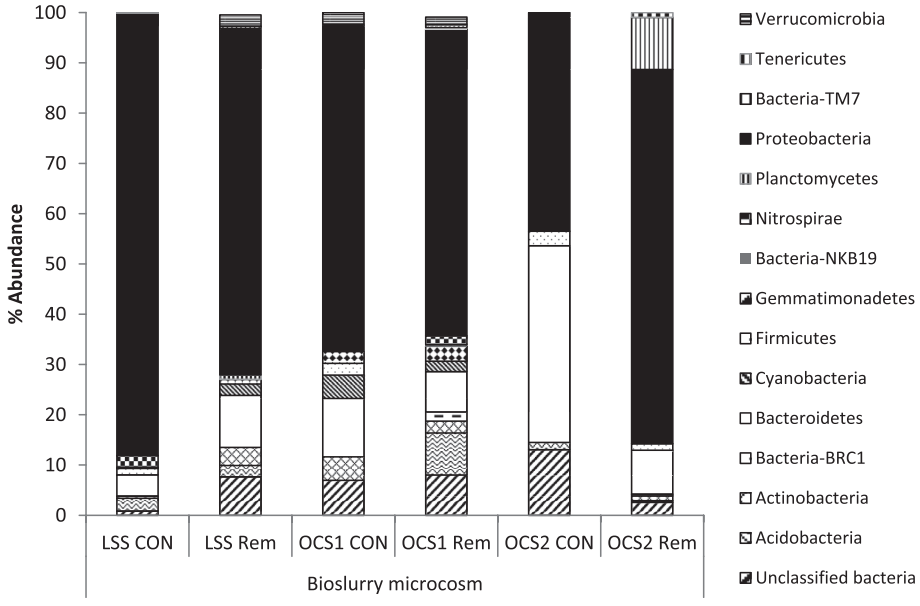

Fig. 6. Changes in bacterial phylogenetic groups during bioremediation.

Table 3
Inverse Simpson index assessment of bacterial community diversity.

	LSS1 CON	LSS1 rem	OCS1 CON	OCS1 rem	OCS2 CON	OCS2 rem
Inverse Simpson diversity index	10.75	12.98	25.64	28.57	15.87	2.83

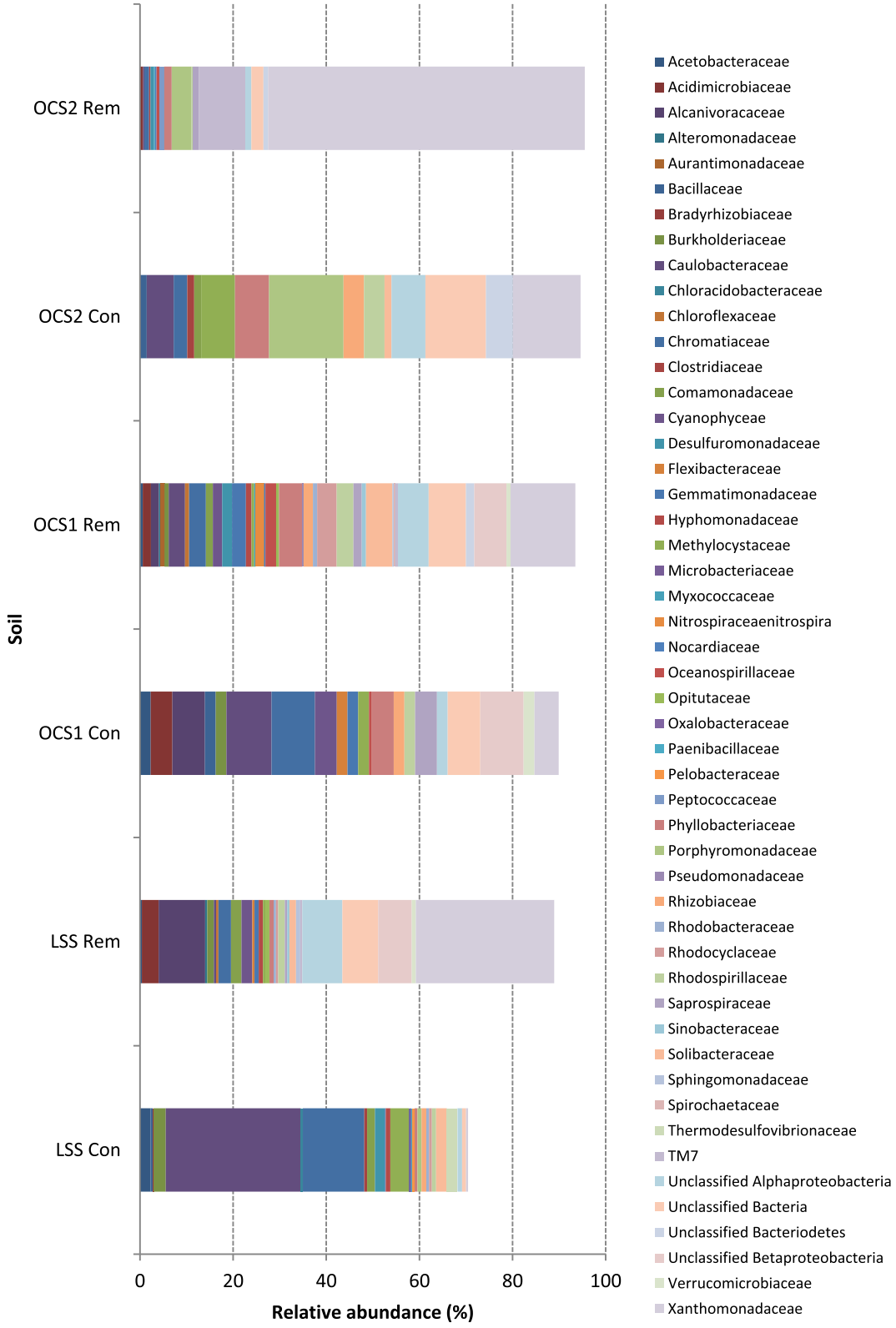

Fig. 7. Changes in relative abundance of different bacterial families in TPH contaminated soils.

only minor contributions to the bacterial diversity (Fig. 6). The exception was *TM7* in the OCS2 soil whose sequence was not identified in the OCS2 contaminated soil prior to treatment. Bioslurry treatment increased the relative abundance of this microorganism to approximately 10% of the bacteria in the treated soil.

The dominance of *Proteobacteria* in hydrocarbon contaminated soils which have been treated or have undergone a bioremediation treatment has been noted by other researchers (Militon et al., 2010; Benedek et al., 2013; Aleer et al., 2014). The *Proteobacteria* phylum encompasses an enormous number of bacteria with morphological, physiological and metabolic diversity (Kersters et al., 2006). *Alpha-*, *Beta-*, *Delta-* and *Gamma-Proteobacteria* bacteria were identified in the pretreated soils. *Alphaproteobacteria* and *Gammaproteobacteria* were the most active *Proteobacteria* present in LSS1 CON, OCS1 CON and OCS2 CON contaminated soils (Fig. 7), contributing 75, 51 and 49% of the total bacteria identified in these soils. The contribution of *Alpha-* or *Gamma-Proteobacteria* varied in the contaminated soils but a markedly higher percentage of *Alphaproteobacteria* compared to *Gammaproteobacteria* were present in LSS1 CON (54% vs 21%) and OCS2 CON (31% vs 18%) soils. In contrast, similar percentages of *Alpha-* and *Gamma-Proteobacteria* were present in OCS1 CON (26% vs 25%). The *Caulabacteraceae* (29, 10 and 6%), *Methylocystaceae* (4, 2 and 7%), *Phyllobacteriaceae* (<1, 5 and 7%), and *Acetobacteraceae* (2, 2 and <1%) represented the majority of the *Alphaproteobacteria* phylum present in LSS1 CON, OCS1 CON and OCS2 CON contaminated soils. In contrast, analysis of the treated soils observed that *Gammaproteobacteria* dominated the Proteobacteria phylum with *Xanthomonadaceae* as the dominant *Gammaproteobacteria* organisms present in the treated soil. *Xanthomonadaceae* contributed 44, 23 and 70% of the *Proteobacteria* present in LSS1 Rem, OCS1 Rem and OCS2 Rem, respectively. Previous studies have often reported a shift in microorganisms from *Alpha-* to the *Gamma-Proteobacteria* when soils are contaminated with hydrocarbons (Kalbelitz et al., 2009; Militon et al., 2010; Simarro et al., 2013; Ros et al., 2014) however, in long-term contaminated soils, such as utilised in this study, this shift in the distribution of *Proteobacteria* classes may have initially occurred but may have reverted back to an *Alpha-*dominated *Proteobacteria* community as the contaminated soils aged and the bioavailability of the hydrocarbons declined. Altering the soil environment during the bioslurry bioremediation process, through enhancing hydrocarbon solubility shifted the *Proteobacteria* group to be dominated by *Gammaproteobacteria* class. The predominance of *Xanthomonadaceae* in all the 3 treated contaminated soils is similar to the findings of Militon et al. (2010) who reported that *Actinobacter* were the dominant active bacterial community after the bioremediation treatment of aged hydrocarbon contaminated soils. *Actinobacteria* were identified in all soils in this study (predominantly as *Acidimicrobiaceae*) but played little active role in the bioslurry bioremediation treatments. Presumably, this may be in part be attributable to the bioremediation column reactor treatment methodology utilised by Militon et al. (2010) compared to the bioremediation bioslurry treatment methodology utilised in this study.

Conclusions

Long term petroleum hydrocarbon contaminated soils in Australia are mostly situated in the remote, arid regions where technological constraints on the implementation of traditional remediation methods are high. Pilot scale comparisons of two widely used bioremediation techniques in the aged contaminated soils are pivotal for the success of field level remediation. The bioslurry method of bioremediation rapidly reduced the TPH concentration due to the accelerated microbial mineralization. Of particular interest was the adaptive behaviour of the microbial community in aged contaminated soils. Molecular profiling before remediation of these microbial diversity rich soils showed that *Alphaproteobacteria* has dominated during the TPH mineralization process, however bioslurry bioremediation of the aged contaminated soils increased the dominance of *Proteobacteria* but shifted the balance of the active *Proteobacteria* from *Alpha-* to *Gamma-*domination. In particular the *Xanthomonadaceae* family played a substantial role in the degradation of aged petroleum hydrocarbons in these soils. The bioslurry principle provides optimum condition for the accelerated microbial growth through increased contact time, continuous aeration and homogenised nutrient conditions.

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