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ORIGINAL PAPER

Exploiting the intrinsic hydrocarbon-degrading microbial capacities in oil tank bottom sludge and waste soil for sludge bioremediation

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Abstract In this study, biological methods (biostimulation and bioaugmentation) were used to treat oil tank bottom sludge contaminated soils to total petroleum hydrocarbon (TPH) levels suitable for landfill disposal. The sludge's hydrocarbon-degrading microbial capacities were initially compared to those from other contaminated environments using culture-based methods. Results indicated that a fungus, *Scedosporium* dominated the sludge microbial community. Its application in a nutrient formulation resulted in greater reduction in oil tank bottom sludge viscosity (44 %) and residual soil hydrocarbon compared to hydrocarbonoclastic microorganisms from other sources (26.7 % reduction in viscosity). Subsequent field-based experiments showed greater TPH reduction (54 %) in fungal-nutrient-treated sludge–waste soils than in naturally attenuated controls (22 %) over 49 days. 16S ribosomal ribonucleic acid and internal transcribed spacer region-based polymerase chain reactions and denaturing gradient gel electrophoresis analyses showed minimal effects on the microbial communities during this time. TPH reduction to landfill disposal levels occurred at a slower rate after this, falling below the 10,000 mg kg^{-1} legislated TPH disposal threshold earlier in amended samples (91.2 %; 9,500 mg kg^{-1}) compared to the control (82 %; 17,000 mg kg^{-1}) in 182 days. The results show that the intrinsic hydrocarbon-degrading microbial capacities in sludge are better suited for sludge degradation than those from other sources. The substantial TPH reduction observed in control samples demonstrates the beneficial effects of natural attenuation with waste soils for oil tank sludge treatment. Microbial capacities in sludge and treated waste soils can therefore be successfully employed for treating oil tank bottom sludge.

Keywords Sludge · Total petroleum hydrocarbon · 16S ribosomal ribonucleic acid · Internal transcribed spacer regions · Denaturing gradient gel electrophoresis

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Introduction

Crude oil, consisting of hundreds of different hydrocarbon fractions ranging from straight chain volatile alkanes to heavier fractions such as polycyclic aromatic hydrocarbons (PAHs), is often stored in holding tanks prior to being pumped to various locations for downstream processing. Regular use of these tanks can lead to the accumulation of heavy hydrocarbon fractions (called oil tank bottom sludge), which cannot be removed by conventional pumps. The periodic cleaning of these hydrocarbon deposits in storage tanks is time consuming, labor intensive and is a major cost in crude oil production (Banat et al. 1991). Their removal and disposal is also hazardous and creates additional waste management issues. This is because some of

the fractions in the deposits such as the PAHs are recalcitrant, carcinogenic and potentially toxic to the natural environment (Banat et al. 1991; Ferrari et al. 1996; Bojes and Pope 2007).

Different physical, chemical and biological methods (incineration, solidification hydrocarbon re-extraction and bioremediation) can be used to treat oil sludge from crude oil storage tanks. However, most physical and chemical methods are generally expensive (Al-Futaisi et al. 2007; Gallego et al. 2007). In contrast, biological treatments (which involve the use of microorganisms) are attractive because they are cheaper and more environmentally friendly. Microorganisms (bacteria and fungi) are crucial in hydrocarbon detoxification with their roles and mechanisms involved in degrading different hydrocarbon fractions being well documented (Atlas 1981; Leahy and Colwell 1990; Gallego et al. 2007; Rojo 2009).

Bio-treatment of oil sludge or a sludge–soil complex can involve the addition of aqueous soil slurries loaded with microorganisms (Ferrari et al. 1996). It can also involve the biostimulation of indigenous sludge degrading microorganisms with nutrients and aeration or inoculation with known hydrocarbon-degrading organisms (Deka et al. 2005; Makadia et al. 2011). Surfactants can be added to oil tank bottom sludge to enhance microbial contaminant removal while land farming has also been used for sludge degradation (Al-Futaisi et al. 2007; Zhang et al. 2010). The success of any of these methods is dependent on the extent to which available microbial capacity can be exploited for hydrocarbon contaminant removal. Although there are limited reports of microbial treatment of oil tank bottom sludge, substantial microbial removal of alkane, cycloalkanes and aromatic compounds in oil tank sludge has been reported (Gallego et al. 2007).

The choice of the soil to be mixed with oil tank bottom sludge is important. Prior contact with hydrocarbon can boost a soil's hydrocarbon-degrading capacity, which can be exploited for detoxification purposes. The use of this type of soil is restricted by legislation due to the inherent health risks associated with the contaminants in polluted soils. However when detoxified, such soils should be suitable candidates for treating oil wastes. In Australia, the levels of total petroleum hydrocarbon (TPH) and other residual hydrocarbon fractions such as benzo (a) pyrene and aromatic fractions and metals permissible in treated waste soils prior to landfill disposal are defined by the National Environmental Protection Council (NEPC) (NEPC 1999; Sheppard et al. 2011). Waste soils which have satisfied the legislated safety threshold (such as having TPH levels of $\leq$10,000 mg kg^{-1}) can possess substantial microbial hydrocarbon-degrading potential which can be successfully harnessed for treating new hydrocarbon contaminants (Makadia et al. 2011; Sheppard et al. 2011).

The use of waste soils for treating oil tank bottom has other additional benefits; reusing them for oil tank bottom sludge treatment will provide another economical alternative (to land farming) for the management of oil tank bottom wastes. The use of waste soils will also reduce the amount of material being placed in landfills. Reuse of these waste soils also fits into a new model of waste management W2R EPP (waste 2 resources, environmental protection policy) developed by the South Australian Environmental Protection Authority. This model emphasizes waste minimization but also encourages the reuse, recycling, recovery, treatment of wastes with landfill disposal as a last resort (http://www.epa.sa.gov.au/environmental_info/waste).

Apart from the improved hydrocarbon degradation potential in waste soils, crude oil with its rich supply of hydrocarbonoclastic microorganisms (Yemashova et al. 2007) could be a source of microorganisms for oil tank bottom sludge treatment. Bacteria are more widely used in bioaugmentation and biostimulation studies for hydrocarbon degradation (Cameotra and Singh 2008; Machin-Ramirez et al. 2008; Gojgic-Cvijovic et al. 2011) than fungi, despite the importance of fungi in degrading complex hydrocarbons (Wu et al. 2008). Therefore, the aim of this study was to investigate the suitability of microbial isolates (especially fungi) from oil tank bottom sludge for the biological treatment of waste oil tank bottom sludge in a microbe nutrient formulation. We have used laboratory- and field-based studies to investigate the efficacy of this microbe nutrient formulation for the treatment of oil tank bottom sludge–waste soil mixture and compared it to naturally attenuated samples. Changes in the samples' microbial community were assessed with PCR-DGGE techniques. This research was carried out in Australia and was part of a larger study carried out between 2008 and 2011.

Materials and methods

Isolation of microorganisms

The oil tank bottom sludge used for this study was obtained from an oil storage tank (10,000 m^3) in Australia. This sludge which had accumulated at the bottom of this storage tank for over 5 years was removed by the addition of cutter fluid (diesel). The diesel–sludge mixture was subjected to chemical analysis for TPH determination. Isolation of microorganisms in the removed oil tank sludge was carried out by an enrichment method using replicate samples of homogenized sludge as described by Kadali et al. (2012). Bushnell–Haas (BH) medium (Eriksson et al. 2000) was supplemented with agar, sterilized and mixed with sterile oil tank bottom sludge (0.2 %) using a pour plate technique

(Kadali et al. 2012). The BH-sludge plates were inoculated by streaking with oil tank bottom sludge and incubated for up to 3 weeks at 25 °C. The microorganisms detected on these plates were subcultured and purified for further studies.

Viscosity measurements and microcosms

Laboratory-based microcosms were set up to assess the abilities of the microbial isolate obtained from the sludge to reduce the viscosity of the oil tank bottom sludge. This was then compared to the oil tank bottom sludge viscosity reducing abilities of hydrocarbonoclastic bacterial isolates from other sources (Bird et al. 2012). This was carried out by inoculating the oil sludge BH medium (ratio 1:1, w/v) with 300 μL of standardized culture of microbial isolates. The inoculated medium was incubated at 37 °C for 7 days on a shaker at 150 rpm. Controls were set up without microbial inoculation. After 7 days, viscosity measurements of oil samples were performed with a HAAKE, Viscotester fitted with SV cup and SV DIN rotor (Thermo Electron Corp, USA) following the manufacturer's protocol. The data from the Viscotester were analyzed using Rheo Win 3 job manager software.

Laboratory-based soil microcosms were also set up in 1 L flasks using previously treated waste soils contaminated with oil tank bottom sludge. This soil was obtained from a waste depot in Australia and had been subject to bioremediation to reduce the TPH to <10,000 mg kg^{-1}. This treated waste soil was originally intended for landfill disposal at the depot. The replicate microcosms consisted of (1) 200 g of tank bottom sludge-contaminated soil and test isolate (0.1 g dry cell weight) in BH medium (8 %, w/w) (2) 200 g of tank bottom sludge-contaminated soil and BH medium, (3) 200 g of tank bottom sludge-contaminated soil and consortium of hydrocarbonoclastic bacteria (0.5 g L^{-1}) in BH medium and (4) 200 g of tank bottom sludge-contaminated soil only. Inoculum generation for the fungal isolate was performed according to Makadia et al. (2011). The microcosms were incubated for up to 9 weeks at 40 % soil water holding capacity (WHC) with samples being obtained weekly for TPH analysis.

Field-based studies

Field-based studies were set up as shown in Table 1 based on the results of laboratory investigations. The treatment pile consisted of bioremediated (or treated waste) soil (500 kg) contaminated with oil tank bottom sludge (100 kg) and the test isolate nutrient formulation (as earlier described). The control pile was set up with the bioremediated or treated waste soil contaminated with oil tank bottom sludge. Soil piles were set up at 40 % WHC, covered with shade cloth and maintained for up to 182 days. The two piles were mixed regularly (1–2 weeks) and water added as necessary (usually every 2–3 weeks) in order to maintain the soil water moisture. Soil sampling was carried out by collecting multiple samples from the top, middle and base of the piles with composite samples being generated by mixing these different fractions. Sampling was carried out largely on a weekly basis for up to 182 days with samples being stored at −20 °C prior to any analysis.

Table 1 Experimental design of field-based investigations

Component	Control pile	Treatment pile
Soil	+	+
Oil + cutter fluid	+	+
Fungi BH medium	−	+
Water	+	+

+, means component added and −, means component not added

Total petroleum hydrocarbon analysis

TPH analyses were carried out on selected samples obtained from laboratory-based microcosms, field-based studies and procedural blanks. TPH contents of replicate samples were determined in samples using the modified standard protocol of International Organization for Standardization (ISO2004), ISO/DIS 16703 GC. The soil TPH content was estimated as described by Sheppard et al. (2011). Standard calibration curves were made from hydrocarbon mixture (RTW solution) dilutions. The equations from these calibration curves were used in conjunction with the area under each chromatogram for estimating TPH concentrations. A Gas Chromatography with a Varian 8200 Auto sampler and Flame Ionizing Detector was used (Sheppard et al. 2011).

DNA extraction, polymerase chain reaction and denaturing gradient gel electrophoresis

DNA extraction from soil was carried out using the PowerSoil™ DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad, CA, USA) according to the manufacturer's instructions. 16S rRNA amplification via polymerase chain reaction (PCR) was carried out with universal eubacterial primers 341F GC and 518R (Muyzer et al. 1993). DNA was extracted from pure microbial cultures (fungi) as described by Adetutu et al. (2011). Internal transcribed spacer (ITS) regions were amplified using ITS1 and ITS4 primers (Anderson and Parkin 2007). ITS region amplification of soil DNA extracts was also carried out via a

nested reaction with ITS 1–4 and ITS 1FGC-2 primer sets as described by Anderson and Parkin (2007). The thermocycling conditions used for fungal PCR were as follows: 1 cycle at 95 °C for 5 min, 35 cycles of 45 s at 95 °C, 45 s at 58 °C and 45 s at 72 °C and final extension at 72 °C for 10 min. Based on the TPH results, microbial community analyses were carried out on samples from the time frame which showed the greatest TPH reduction (days 0–49). Amplicons were analyzed with denaturing gradient gel electrophoresis (DGGE) using a DCode Apparatus (Bio-Rad, USA) using 9 % polyacrylamide gels. Denaturing gradient range of 45–60 % was used for bacterial analysis, while a 40–50 % gradient was used for fungal analysis. The DGGE gels were silver stained, scanned and analyzed using Phoretix 1D (Nonlinear Dynamics, USA) (Sheppard et al. 2011).

Sequencing, microbial community and statistical analyses

The ITS 1–4 amplicons obtained from PCR amplification of extracted DNA from fungal isolates were cleaned up with the Wizard(R) SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) prior to sequencing. Sequencing was carried out as described by Aleer et al. (2011), and the sequence data trimmed and aligned with Sequencher 4.1.4 software (Gene Codes Corp., Ann Arbor, MI, USA) before being submitted to GenBank for the determination of their putative identities. Similarity relationships between microbial groups on the community profiles were expressed in similarity clusters using the unweighted pair group method with mathematical averages (UPGMA). Shannon index (H') was also calculated from DGGE community profiles using the formula $H' = -\sum p_i$ LN p_i (Adetutu et al. 2011). Pareto–Lorenz (PL) curves were used to estimate evenness within the microbial community with bands being ranked from high to low based on their intensities. The cumulative normalized bands (numbers) were plotted on x-axis, while the normalized cumulative intensities of bands were plotted on the y-axis in order to draw a PL curve with the intercept set at 20 % of population (0.2 x-axis). The values obtained at the intercept are usually related to either the 25 %, or the 45 % or the 80 % of the PL curve. The 25 % PL curve is representative of a community with high evenness and poorly defined internal structure. The 45 % PL curve reflects a community with mid-evenness and functionality and well-defined internal structure which allows it to deal with changing environmental conditions. The 80 % PL curve is reflective of a fragile community with low evenness (Marzorati et al. 2008). The effects of treatment on soil microbial diversity and TPH degradation were assessed via statistical analysis with either t test or analysis of variance (ANOVA) using SPSS version 19. The effects were deemed to be significant compared to the control at $P \leq 0.05$.

Results and discussion

Microbial isolates and microcosms

The sludge mixed with cutter fluid used for this study contained approximately 61 % aliphatic and 39 % aromatic compounds (58.4 % of C_{15}–C_{28} fraction, 36 % of C_{10}–C_{14} fraction, 3.5 % of C_6–C_9 fraction and 2.1 % of C_{29}–C_{36} fraction). Sequence analysis of the fungal isolates detected on oil tank bottom sludge supplemented BH media agar plate showed significant similarities, suggesting that they belonged to the same microorganism. This fungus was putatively identified as *Scedosporium* sp (100 % similarity). Members of this genus are known to degrade both short-chain and aromatic hydrocarbons as well as polychlorinated biphenyls (Prenafeta-Boldu et al. 2006; Shennan 2006; Tigini et al. 2009). Oil tank bottom sludge largely consists of sedimented hydrocarbons (heavy fractions) such as aromatic compounds (Bojes and Pope 2007). Cutter fluids such as diesel are usually used to "solubilize" the sludge before removal from the tank leading to the introduction of aliphatic fractions into the sludge. Fungal groups such as *Scedosporium* would therefore be expected to play important roles in the degradation of both the aromatic and aliphatic fractions of the sludge.

Microbial deterioration of stored crude oil is a major economic and environmental problem in the oil industry (Yemashova et al. 2007), but exploiting this ability to degrade oil tank bottom sludge is desirable. This ability in hydrocarbonoclastic bacterial consortium and bacterial products (surfactants) has been successfully used to degrade oil tank bottom sludge (Deka et al. 2005; Gallego et al. 2007; Zhang et al. 2010). Fungi can degrade hydrocarbon fractions including complex aromatic compounds (Atagana, 1996; Li et al. 2008; Wu et al. 2008; Atagana, 2009; Haritash and Kaushik 2009; Arun and Eyini 2011) by a variety of mechanisms (Prenafeta-Boldu et al. 2006). However, this ability has been less readily exploited (compared to bacteria) for oil tank bottom sludge treatment. We therefore compared the hydrocarbon-degrading abilities of the fungus isolated in this study to those of hydrocarbonoclastic bacteria using laboratory-based oil viscosity assays and soil microcosms. The hydrocarbonoclastic bacterial consortium used in this study was obtained from hydrocarbon-contaminated environments. The sequence identities and the hydrocarbon-degrading capacities of this bacterial consortium are already described (Bird et al. 2012).

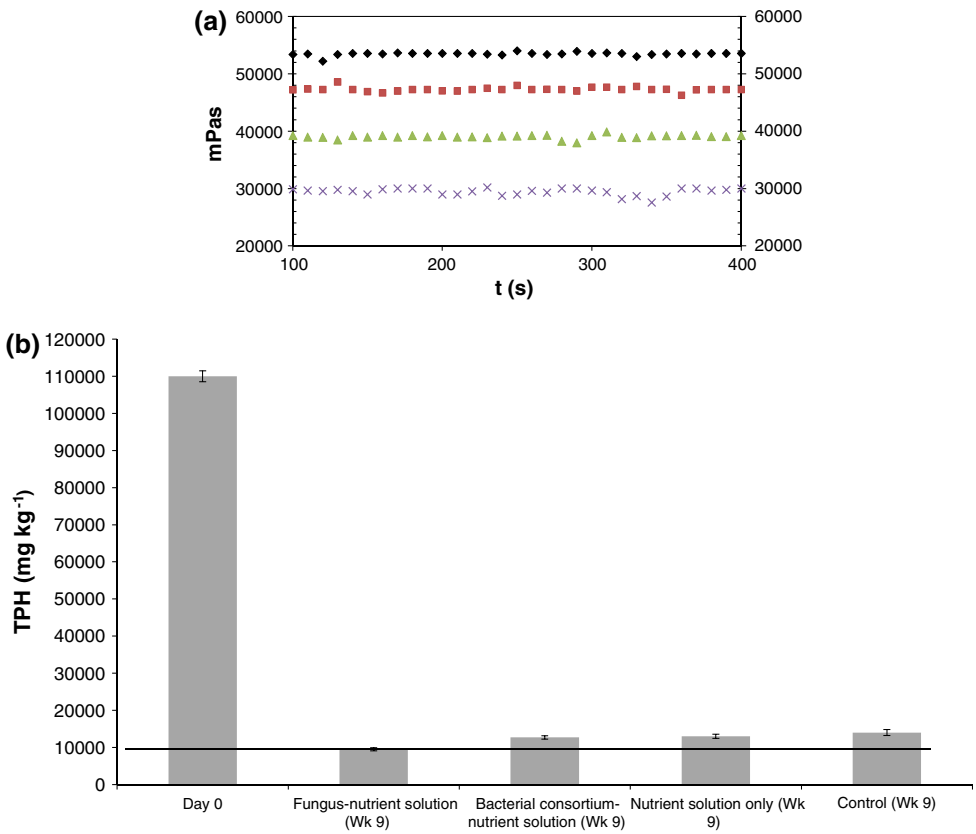

Fig. 1 Changes in oil viscosity (**a**) and soil TPH levels in laboratory-based microcosms (**b**). *Note* For (**a**), *cross symbol*, *triangle*, *square* and *diamond* refer to microcosms with fungus, bacterial consortium, control at day 7 and control at day 0, respectively

Laboratory-based investigations showed that the addition of *Scedosporium* to the nutrient formulation substantially reduced the oil viscosity by 44 % (30,000 mPas) after 7 days compared to the initial 53,580 mPas at day 0. This reduction was better than the 26.5 % (39,355 mPas) reduction observed with the bacterial consortium and the 11.8 % (47,270 mPas) reduction in the control. This shows the beneficial effect of fungal addition to oil degradation (Fig. 1a). The extent of TPH reduction was also greater in laboratory-based soil microcosms with the fungus in a nutrient solution compared to nutrient only microcosms or those with hydrocarbonoclastic isolates (Fig. 1b). From an initial 110,000 mg kg^{-1}, the TPH level in fungal-nutrient-supplemented soil–sludge microcosms was reduced to 9,800 mg kg^{-1} in 9 weeks compared to 12,696 and 13,011 mg kg^{-1} in microcosms with hydrocarbonoclastic consortium and only nutrients, respectively (Fig. 1b). The beneficial effects of nutrient addition to hydrocarbon degradation are well known, but the additional beneficial effect of this fungus on hydrocarbon removal may be related to the source of this isolate (from the oil tank bottom being treated). It was possible that this isolate had adapted (during the years of sludge accumulation) to the toxic hydrocarbon components of the oil tank bottom sludge and could therefore degrade it better. Autochthonous microorganisms are sometimes more efficient degraders of complex hydrocarbons, (due to prior adaptation) than non-indigenous microorganisms (Li et al. 2002; Vitte et al. 2011). As the soil–sludge amended with a fungal nutrient solution was the first to fall below 10,000 mg kg^{-1} (legislated level in Australia for landfill disposal of waste soil), this formulation was used for field-based treatment of sludge-contaminated soils.

Field-based studies

The use of a fungal nutrient formulation in this study was beneficial to hydrocarbon degradation in field-based studies between days 0 and 49. This was because the addition of *Scedosporium* BH mixture resulted in a significant soil TPH reduction of 54 % (from 109,100 ± 14,557 to 49,757 ± 4,598 mg kg^{-1}) compared to 22 % reduction in naturally attenuated soils (from 92,567 ± 3,663 to 71,897 ± 8,837 mg kg^{-1}) (t test, $P < 0.05$) (Fig. 2). However, the rate of TPH reduction slowed down considerably after 49 days with a final value of 9,575 ± 1,425 mg kg^{-1} (cumulative reduction of 91.2 %) for the treated pile and 17,000 ± 500 mg kg^{-1} for the control pile (82 % cumulative reduction) at day 182.

This study therefore demonstrates the beneficial effects of nutrient addition (alongside bio-augmentation) to the degradation of oil tank bottom sludge over 49 days. This

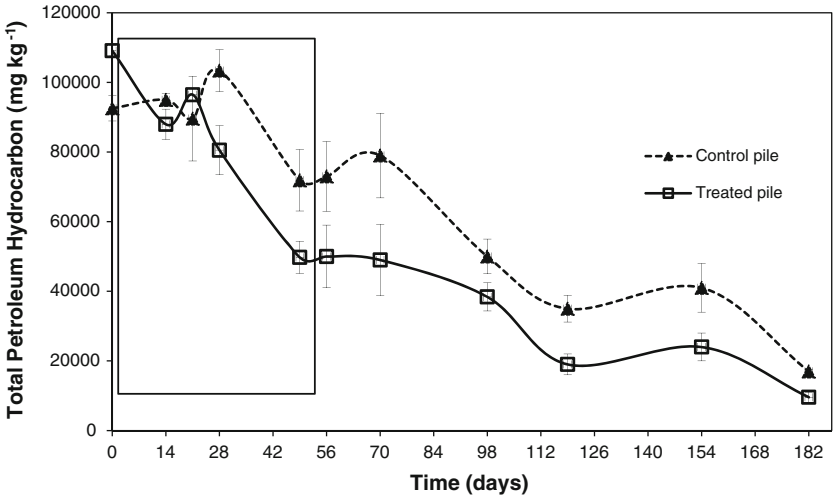

Fig. 2 Total petroleum hydrocarbon reduction in treated and control soil piles over 182 days. *Boxed area* corresponds to the time frame of highest TPH removal in treated soil pile

beneficial effect on hydrocarbon pollutant removal in soils has been reported in other studies (Stallwood et al. 2005; Mancera-Lopez et al. 2008; Coulon et al. 2010). As oil tank bottom sludge contains a variety of microorganisms, it could be a more appropriate source of hydrocarbonoclastic microorganisms (as used in this study) for treating that oil tank bottom waste than microorganisms from other sources. This point is crucial for the management and treatment of waste oil tank bottom sludge. Similar beneficial effects of fungi nutrient combinations on TPH reduction in hydrocarbon-contaminated soils have been reported using indigenous fungal isolates from the same polluted soils (Mancera-Lopez et al. 2008). The initial accelerated reduction in soil TPH could have been due to the beneficial effects or actions of the supplied fungus and nutrients on other unidentified microbial groups in the sludge and waste soil (Li et al. 2008). The substantial hydrocarbon degradation observed could also have been due to the fungus syntrophically promoting hydrocarbon degradation in soil alongside other indigenous hydrocarbon-degrading bacteria in waste soil and sludge. However, this was not investigated in this study.

Microbial community analyses

Focussing on the period of the greatest TPH reduction (day 0–49), DGGE based microbial analysis showed that the bacterial communities in both treated and control soil microcosms were highly diverse (Fig. 3a). The bacterial community diversity increased from day 0 to day 14 and thereafter decreased till day 49. However, the Shannon diversity values of the treated samples were not significantly different from those of control samples at each time frame (ANOVA $P > 0.05$) (Table 2). There was no detectable shift in bacterial community cluster patterns as a result of the addition of the fungus-nutrient formulation over this period (Fig. 3a). Pareto–Lorenz analysis also showed no substantial treatment effect on bacterial community evenness and functional organization (45–55 %) (Fig. 3b). The absence of a substantial shift in bacterial community cluster patterns and PL distribution curves associated with the period of accelerated TPH removal suggested that the changes observed in the bacterial community were related to incubation periods rather than to treatments. A similar trend was reported by Makadia et al. (2011), showing that soil TPH reduction may not always be accompanied by changes in bacterial communities. The mid-range PL value (45–55 %) observed in this community coupled with minimal alterations in the community evenness can be reflective of an adapted microbial community with sufficient functional redundancies (Marzorati et al. 2008). This was likely the case in this study as the oil tank bottom sludge had accumulated over a number of years allowing the indigenous microbial community to adapt to the presence of the various hydrocarbon components of the sludge.

Analysis of the fungal community over the same period, however, showed comparatively greater treatment effects. UPGMA analysis showed that unlike in bacterial communities, the fungal community in treated samples formed a "cluster" (except on day 21) which was different from that of control samples (Fig. 4a). However, the Shannon diversity trend was similar to that observed in the bacterial community analysis with no significant differences between treated and control samples (Table 2). Analysis of the fungal community evenness and functional organization showed greater variability (52–70 %), with the treated samples having higher Pareto–Lorenz values than control samples on most days. However, the fungal community cluster analysis only showed some treatment effects on days 14 and 49. This could have accounted for a higher mid-range PL value range (52–70 %) and less evenness

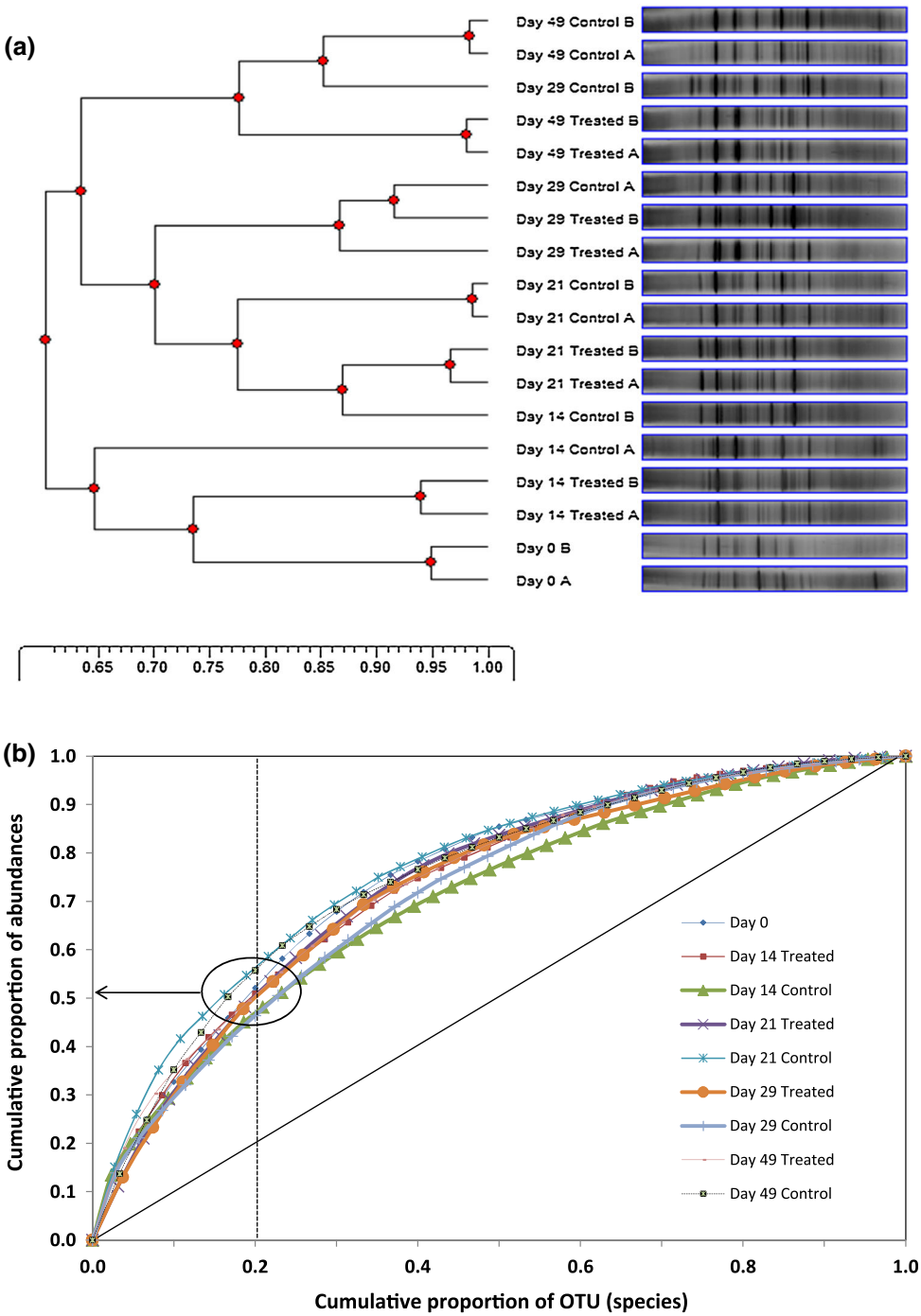

Fig. 3 UPGMA dendrogram (**a**), and Pareto–Lorenz distribution curves (**b**) of bacterial communities in treated and control soil piles. *Note* For (**a**), *letters A* and *B* with the same number are duplicates. Treated—samples with fungus-nutrient solution. Control—no fungus-nutrient solution added. For (**b**), the 45-degree diagonal represents perfect community evenness

observed in treated samples on those days. However, given the absence of large-scale changes in both bacterial and fungal community diversity at this phase of accelerated hydrocarbon removal, further analyses of samples after 49 days were not carried out.

Waste soils (previously bioremediated soils)

The choice of soil which is mixed with oil tank bottom sludge is important. Using previously bioremediated soils which usually have enhanced microbial degrading capacity should be beneficial to the oil tank bottom degradation. Recent reports (Makadia et al. 2011; Sheppard et al. 2011) have shown that under conditions of monitored natural attenuation, such soils were as equally effective as the application of microbe-nutrient formulation for TPH reduction in contaminated soils. The use of the fungus nutrient formulation and previously bioremediated (treated) waste soil was beneficial to TPH reduction in this study especially between days 0 and 49. However, the

Table 2 Shannon diversity values of bacterial and fungal communities in naturally attenuated (control) and treated soils in field-based studies over 49 days

Days	Control pile	Treated pile
Bacteria		
0	2.86 ± 0.00	2.86 ± 0.00
14	3.16 ± 0.03	3.12 ± 0.03
21	3.10 ± 0.05	3.03 ± 0.04
28	2.99 ± 0.02	2.89 ± 0.02
49	2.93 ± 0.06	2.83 ± 0.01
Fungi		
0	3.29 ± 0.00	3.29 ± 0.03
14	3.48 ± 0.09	3.12 ± 0.36
21	3.44 ± 0.04	3.43 ± 0.03
28	2.86 ± 0.51	2.80 ± 0.15
49	3.09 ± 0.32	2.81 ± 0.11

Statistical analyses showed no significant difference in bacterial and fungal communities between treated and control piles at each time frame ($P > 0.05$) ($n = 2$)

beneficial effects of this amendment substantially reduced afterwards. This was because it took a further 133 days (day 182) for the TPH level in field-based studies to reach 9,500 mg kg^{-1} in treated samples which was below the 10,000 mg kg^{-1} legislated TPH level required for landfill disposal in Australia (NEPC 1999). The monitored naturally attenuated control pile was at 17,000 mg kg^{-1} at the same period (day 182). The initial microbial activities which had benefitted (from nutrient and fungal supply) in the amended soil might have ensured that the legislated TPH threshold was reached faster in the treated pile (91 % reduction). However, the occurrence of substantial TPH reduction in the monitored natural attenuation microcosm (82 % reduction) indicated that the waste soil's enhanced hydrocarbon-degrading potential (stimulated by aeration and addition of water) can also lead to significant TPH removal. This offers a cheaper (but with a longer degradation time frame) alternative of oil tank bottom sludge treatment in cases of limited economic resources.

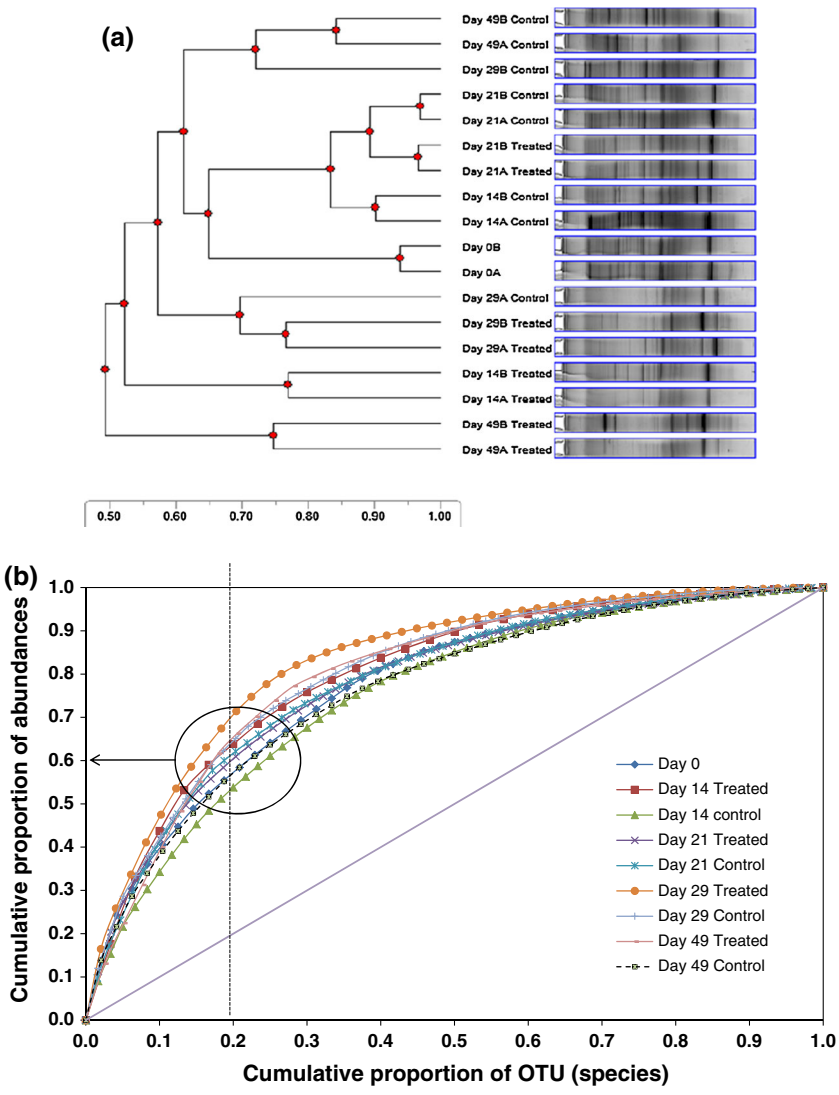

Fig. 4 UPGMA dendrogram (**a**) and Pareto–Lorenz distribution curves (**b**) of fungal communities in treated and control soil piles. *Note* For (**a**), *letters A* and *B* with the same number are duplicates. Treated—samples with fungus-nutrient solution. Control—no fungus-nutrient solution added. For (**b**), the 45-degree diagonal represents perfect community evenness

Depletion of the supplied nutrients could have occurred over the experimental period in field-based studies and might have contributed to the drop in TPH reduction rates after 49 days. Therefore, it was possible that the initial accelerated rate of hydrocarbon degradation in treated samples (pile) might have been maintained if doses of microbe-nutrient formulation were added at periodic intervals (especially after day 49). Bioavailability of hydrocarbon decreases over time and has a negative impact on soil hydrocarbon removal. Surfactants (which can enhance hydrocarbon availability) could also have been added (Cheng et al. 2008). Their addition might have ensured that the high TPH removal levels observed initially between day 0 and 49 continued till the end of the experimental period. Crucially, the accelerated rate of TPH reduction observed in the treated pile showed that oil tank bottom sludge can be rapidly degraded under the right nutrient and microbial resources. This process could be a viable bioremediation option in situations when accelerated soil TPH removal is desired within a relatively short period of time. This could be in countries where maximum contaminant levels (MCLs) are more stringent and are required to be met within a limited period of time.

Conclusion

This study has shown that oil tank bottom sludge should be used as a source of microbial isolates for biological treatment of waste oil sludge. This is because the fungal isolate obtained in this study from oil tank bottom sludge caused greater reduction in TPH levels in the same sludge than isolates from other sources. We therefore suggest that isolation work should be carried out on oil tank bottom sludge with a view of using the obtained isolates for subsequent bio-treatment of waste sludge. The use of this fungus in a nutrient formulation also resulted in considerable reduction in soil TPH compared to naturally attenuated samples especially within 49 days and higher cumulative TPH reduction by the end of the experimental period. The development of a fungal nutrient formulation for oil tank bottom rather than bacterial nutrient formulation represents another method of exploiting inherent fungal degradation potential for waste treatment. This approach offers a sustainable use of waste soil (by mixing with oil tank bottom sludge) and an environmentally friendly approach for waste oil tank bottom sludge treatment.

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