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Harnessing the Hydrocarbon-Degrading Potential of Contaminated Soils for the Bioremediation of Waste Engine Oil

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Abstract Waste engine oil pollution is an endemic problem in African countries as waste oil is often discharged into the environment without adequate treatment because waste oil recycling facilities are not readily available. In this study, laboratory-based microcosms (natural attenuation, biostimulation, bioaugmentation and combined treatment of biostimulation–bioaugmentation) were set up with soils (from old hydrocarbon biopiles) spiked with waste engine oil and monitored for 3 months. Total petroleum hydrocarbon analysis showed that biostimulation and biostimulation–bioaugmentation accelerated hydrocarbon degradation with over 84% reduction (<10,000 mg kg^{-1}) by week 8. It took another 2 weeks for other microcosms to get below this classification of low-level contaminated waste and landfill disposal level. The highest degradation rate of 92% was obtained in biostimulated–bioaugmented microcosms (week 10). However, by week 12, there were no significant differences in hydrocarbon levels in naturally attenuated and treated microcosms. 16S rRNA and ITS-based denaturing gradient gel electrophoresis profiling showed diverse bacterial and fungal communities with some dominant members belonging to hydrocarbon-degrading Proteobacteria, Ascomycetes and Basidiomycetes. This research has therefore shown that hydrocarbon-polluted soils possess substantial microbial hydrocarbon-degrading capacity which was successfully harnessed for degrading engine oil. In developing countries without recycling facilities but readily available hydrocarbon-contaminated soils, using such soils for ex situ monitored natural attenuation could be an effective, low-cost and environment-friendly option for treating waste engine oil.

Keywords Waste engine oil · Bioremediation · Soil · DGGE · 16S rRNA · Internal transcribed spacer regions

1 Introduction

Waste oil consists of oil from a variety of sources such as oils from recreational, mining, manufacturing and automotive industries. Waste oils emanating from the automobile industry such as spent engine oil, lubricating oil and grease from mechanical workshops are important because they contain a mixture of hydrocarbons (*n*-alkanes, polycyclic aromatic hydrocarbons), engine additives such as amines and organometals. These hydrocarbons and engine additives are

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toxic to plants, animals, humans and the environment (Odjegba and Sadiq 2002; Verdin et al. 2004; Mandri and Lin 2007; Wu et al. 2008).

Although waste oils are usually recycled in most developed countries, a significant portion of these oils are often unaccounted for. For example, in Australia (2007–2008), about 270 million litres of waste oils were recycled, but up to 27 million litres of waste oils remained unaccounted for (probably in storage, scrap equipment or illegally dumped; Second Independent Review of the Product Stewardship Discussion Paper 2008). In addition, recycling of oil generates wastes which have to be treated by filtering and dimineralisation, deasphaltining and distillation, requiring substantial time and monetary investment.

The situation is worse in most third world countries in Africa and parts of Asia. In most African countries, waste oil recycling is often poorly practised or unavailable because of the high costs of setting up and maintaining recycling facilities. Unfortunately, increasing dependence on automobiles in these countries has led to the generation of hundreds of millions of litres of spent engine oil which are usually disposed of by dumping untreated oils in landfills, drainage systems or open plots (Odjegba and Sadiq 2002; Garcia-Hernandez et al. 2007; Akoachere et al. 2008). This not only causes substantial environmental damage but also increases the risk of liver and kidney damage and cancer development in people exposed to oil-contaminated environments (Vazquez-Duhalt 1989). Thus, there is a need to find efficient, affordable and more environment-friendly methods of waste engine oil treatment especially in the developing countries. These methods can then be used to treat soils contaminated with waste engine oil or degrade waste engine oil in places where waste oil recycling is not a feasible option and to supplement existing waste recycling treatment technologies.

One popular method of treating hydrocarbon-contaminated soils, which can also be applied to the treatment of engine oil-contaminated soil, is bioremediation. It involves utilising the natural microbial capacity to degrade toxic hydrocarbon contaminants (Bento et al. 2005; Rizzo et al. 2008). Bioremediation methods are well-established, cost-effective and environment-friendly means of cleaning up contaminated land (Jorgensen et al. 2000; Bundy et al. 2004; Wu et al. 2008). It may involve the aeration of contaminated soils (natural attenuation) and addition of organic matter (composting) or nutrients (biostimulation) to stimulate the activities of indigenous or exogenously added microorganisms (bioaugmentation) to speed up the process of contaminant elimination (Bundy et al. 2004).

Investigations have been carried out on the bioremediation of engine oil-contaminated soils using selected bacterial and fungal agents and organic wastes with some success (Ghazali et al. 2004; Adenipekun 2008; Abioye et al. 2010). However, a substantial number of these investigations have been carried out using culture-dependent methods which have yielded limited or biased information as over 90% of soil microorganisms are not culturable (Nannipieri et al. 2003). Consequently, there are very few reports of the application of high-resolution molecular community fingerprinting tools such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism to study engine oil bioremediation, with the impact of engine oil pollution on soil microbial community being poorly qualified. There are also gaps in knowledge of the roles of different microbial groups in engine oil degradation and whether prior soil contact with contaminant will speed up the process of engine oil degradation. In addition to this, the potentially vast microbial engine oil-degrading potential which may be possessed by unculturable soil microbial groups is also poorly studied.

Therefore, the aims of this study were: (1) to assess the ability of natural microbial population in soils with previous hydrocarbon contamination to degrade engine oil to below the 10,000 $mg\,kg^{-1}$ (Australian guideline) limit for landfill disposal over a 3-month period and (2) to assess the impacts of biostimulation and bioaugmentation with a mycoagent on the rate of waste engine degradation and on the soil bacterial and fungal community. These effects would be investigated using a combination of PCR-DGGE sequencing and analytical methods.

2 Methods

2.1 Determination of Soil Physicochemical Parameters

Soil samples used in this study were obtained from an old finished biopile of hydrocarbon-contaminated soil in a waste depot in Adelaide (South Australia). The

soil pH, moisture content, organic matter content and water holding capacity (WHC) were determined according to Rowell (1994).

2.2 Preparation of Inoculum and Microcosm Setup

COM001 which is a hydrocarbonoclastic fungus (Australian provisional patent no. 2009900493) was applied in a unique minimal salt nutrient formulation TankClean (Australian provisional patent no. 2008902645). Prior to its use, COM001 was reactivated in TSB broth (Oxoid^{TM}, Australia) containing tetracycline (0.05 gl^{-1}) at 37°C on an orbital incubator at 130 rpm for at least 48 h, after which the mycelia were washed, concentrated and harvested. COM001 was applied in sterile water to soil samples or in TankClean in concentrations of 4 gl^{-1} (dry weight). The waste engine oil used was obtained from an accredited waste oil depot in Adelaide, South Australia.

Four soil (220 g) microcosms were set up (in triplicate) in sterile 2-l flasks and amended with waste engine oil (where required) at 10% (*w*/*w*, 22 ml) concentration. Soils were amended with waste engine oil and 25% (*w*/*w*) TankClean for biostimulation (BS) studies, whilst bioaugmented (BA) microcosms were amended with waste engine oil and COM001 in sterile water (4 gl^{-1}, Table 1). The combined treatment of biostimulation-bioaugmentation (BS–BA) studies were carried out using soil amended with waste engine oil and COMCLEAN (COM001 in TankClean), and natural attenuated (NA) microcosms were set up with soil amended with only waste engine oil (Table 1). In all microcosms, appropriate amount of sterile water was added to keep them at 50% soil WHC. Microcosms were maintained at 25°C for up to 84 days, mixed regularly for uniform aeration and the soil moisture checked and maintained during the experimental period. Sampling was on a weekly basis for both total petroleum hydrocarbon determination and molecular analysis of microbial community.

2.3 Measurement of TPH

Hydrocarbon was extracted from contaminated soil matrix using a modified standard protocol of determining hydrocarbon content in soil according to the International Standard Organisation (ISO/DIS 16703 GC method) (ISO 2004). Fivemillilitres of acetone was used to extract oil from 1 g of homogenised contaminated soil in a glass tube. Twomillilitres aliquot containing a mixture of standard solutions (Retention Time Window, RTW; consisting of *n*-decane and *n*-tetracontane at 20 and 30 $\mu g/ml^{-1}$, respectively, in heptane) was then added to the contaminated soil, incubated at 37°C at 120 rpm for 60 min, after which the rest of the protocol as described in the ISO/DIS 16703 GC method was followed. GC was performed on a Varian 3800 gas chromatograph equipped with a Varian 8200 Autosampler flame ionisation detector and splitless injector valve. A standard calibration curve was constructed from diesel and waste oil mixture (RTW solution) dilutions, and the equation from the standard calibration curve was now used in conjunction with the area under each chromatogram (area between C_{10} and C_{40}) to determine total petroleum hydrocarbon (TPH) concentrations.

2.4 DNA Extraction and PCR

The total soil DNA was extracted from soil samples (0.25 g) using a PowerSoil^{TM} DNA extraction kit (Mo Bio Laboratories Inc, Carlsbad, CA, USA) according to the manufacturers' instructions. Genomic DNA extraction was carried out on selected samples (day 0, 28, 56 and 84 samples) to evaluate bacterial and fungal community changes over time and in relation to various treatments.

PCR targeting bacteria with 16S rRNA was performed with primer pair 314F GC and 518R (Muyzer et al. 1993). A 50 μl PCR reaction was set up containing nuclease-free water, 2 μl of purified

Table 1 Experimental design of microcosms

Treatments	Soil (g)	TankClean	Com001	Oil
Natural attenuation	220	−	−	+
Biostimulation	220	+	−	+
Bioaugmentation	220	−	+	+
Biostimulation–Bioaugmentation	220	+	+	+

DNA extract from soil, 5 μl of enzyme buffer, 20 pmol μl^{-1} of forward and reverse primers, 4 μM dNTP, 2.5 mM $MgCl_2$ and 1 U of Taq polymerase (Promega, Australia). However, for fungal PCR, touchdown PCR was carried out using Internal transcribed spacer region (ITS) primers, ITS1F and ITS 4 for the first reaction and then ITS1F-GC and ITS 2 for the second nested reaction The initial reactions for ITS1F and ITS 4 were as described in Anderson and Parkin (2007) with a touchdown modification (touchdown started at 68°C with 1°C drop after every cycle for ten cycles), whilst the nested reaction with ITS1F-GC and ITS 2 was carried out using 1 μl of the amplicon from the ITS1-4 reaction and the same thermocycling conditions described for ITS1-4. The negative control of the ITS1-4 reaction was also used as a template to eliminate the possibility of carryover contamination. Bacterial and fungal amplicons were visualised on a 1.5% agarose gel stained with SYBR safe. All PCR were performed on DNA Engine Peltier Thermal Cycler (BioRad, Hercules, USA)

2.5 DGGE and Sequence Analysis

The amplicons from both bacterial 16S rRNA and fungal ITS regions were analysed using a Universal Mutation Detection System (Bio-Rad, CA, USA). DGGE was performed using 9% polyacrylamide gels (the ratio of acrylamide to bisacrylamide was 37:1) at a 45–60% gradient for bacterial analysis and 41–53% for fungal analysis for 20 h at 60°C and 60 V. The DGGE gels were then silver (Girvan et al. 2003) or SYBR Gold stained, scanned, saved as tiff. files with Epson Expression V700 Pro and analysed with Phoretix 1D advanced analysis package (Phoretix Ltd., UK). Bands were excised from SYBR Gold-stained gels for fungal analysis as it was impossible to recover them from silver-stained DGGE gels. Bands of interest on both bacterial and fungal DGGE gels were excised aseptically, incubated in 100 μl of elution buffer either for 4 h at 37°C (McKew et al. 2007). The eluted DNA was then re-amplified with the appropriate primer pair (either 314F-GC-518R or ITS1F-GC-ITS2) and then subject to further rounds of DGGE using a narrower gradient range, band excision and further PCR (as described before) in order to ascertain the purity of the excised bands. Only bands which were determined to be pure were then sequenced. Sequencing was carried out using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI Prism 3730 Genetic analyser under these thermocyling conditions: 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, 60°C for 4 min using 30 cycles and then held at 10°C. The sequence data obtained from the different amplicons were trimmed and aligned with Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor, Michigan, USA) and the consensus sequence data were submitted to GenBank (see Table 2 for accession number). Similarity searches were then generated using BLASTN from GenBank (hhtp://www.ncbi.nlm.nih.gov) to determine putative identities of the bands.

Table 2 Summary of sequence identification of bands excised from the bacterial and fungal DGGE gel

Band no.	Closest match	Phylum/sub-phylum/class	Accession no.	Similarity coefficient (%)
1	Uncultured soil bacterium	–	GQ383866	95
2	*Planomicrobium* sp.	Firmicutes	GU933612	95
3	Uncultured gammaproteobacterium	Gammaproteobacteria	AJ640189	92
4	*Oceanistipes pacificus*	Bacteroidetes/Flavobacteria	EF108216	93
5	Uncultured Acidobacteria	Acidobacteria	FJ713032	89
6	*Alcanivorax* sp.	Proteobacteria/ Gammaproteobacteria	AB435644	90
7	Uncultured cyanobacterium clone	Cyanobacteria	EF662970	90
8	Uncultured proteobacterium clone	Proteobacteria	EF665065	95
9	Uncultured gammaproteobacterium clone	Gammaproteobacteria	AJ640189	90
1F	*Arthrobotrys oligospora*	Ascomycota	EF445989.1	100
2F	*Motierella* sp.	Mucoromycotina	AJ541798.1	100
3F	*Coprinus cordisporus*	Basidiomycota	AY461814.1	98

2.6 Data Analyses

DGGE profiles were analysed using Total Lab-120 with the relatedness of the microbial communities being expressed as similarity coefficients in dendograms generated using unweighted pair group method with mathematical averages. Relative band intensities on DGGE community profile were also determined and used to calculate the Shannon–Weaver diversity (H'). The diversity (H') was calculated using the formula $H'=-\sum p_i \text{ LN } p_i$, where p_i is the proportion of the community that is made of species i (intensity of the band i/total intensity of all bands in the lane). LN p_i is the natural log of p_i (Girvan et al. 2003; Adetutu et al. 2008). Statistical analysis and significance was determined in replicate samples of different treatments by either t test or analysis of variance (ANOVA) and Tukey tests (Sigma Stat 2.03, Systat, London, UK).

3 Results

3.1 TPH Analysis

Figure 1 shows the level of TPH biodegradation in the four laboratory-based soil (sandy loam, pH 8.2, with 29.6% organic carbon, 0.42% total nitrogen and 1.64 mgl^{-1} phosphate) microcosms. There were substantial reductions in TPH within the first 2 weeks of incubation by 53% (31,735 $mgkg^{-1}$; BA), 56% (27,784 $mgkg^{-1}$; NA), 60% (24,480 $mgkg^{-1}$; BA–BS) and 61% (25,510 $mgkg^{-1}$; BS) in the different microcosms. However, by week 8, the TPH levels in BS and BA–BS samples fell below the 10,000-$mgkg^{-1}$ level at 9,651 and 9,835 $mgkg^{-1}$, respectively (shown in box A), making them suitable for landfill disposal by Australian standard. It took BA and NA samples an extra 2 weeks to get below this landfill disposal limit (by week 10). The highest level of engine oil degradation (91.3%, 5,323 $mgkg^{-1}$) was observed in BA–BS samples at week 10 (shown in box B) compared to 88.6% (7,185 $mgkg^{-1}$) in NA, 87.7% (8,346 $mgkg^{-1}$) in BA and 87.3% (8,158 $mgkg^{-1}$) in BS (Fig. 1). Statistical analysis of the TPH reduction levels in the different treatments carried out at weeks 8 and 12 showed that there were no significant differences between the treatments at these times. However, there were significant differences in TPH reduction at week 10 between BA–BS microcosms compared to biostimulated (BS), bioaugmented and naturally attenuated microcosms (t test, $P \leq 0.05$; Fig. 1).

3.2 Microbial Community Analysis

Figure 2a shows the bacterial community response in naturally attenuated and amended microcosms over a 12-week period. Although two major clusters were formed, bacterial community clustering was time-

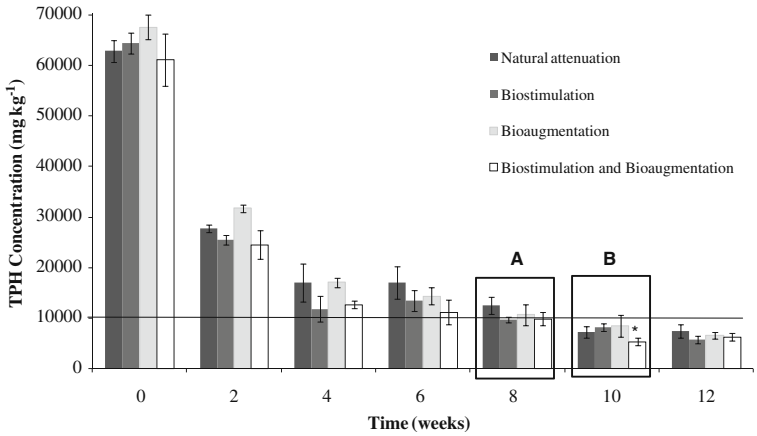
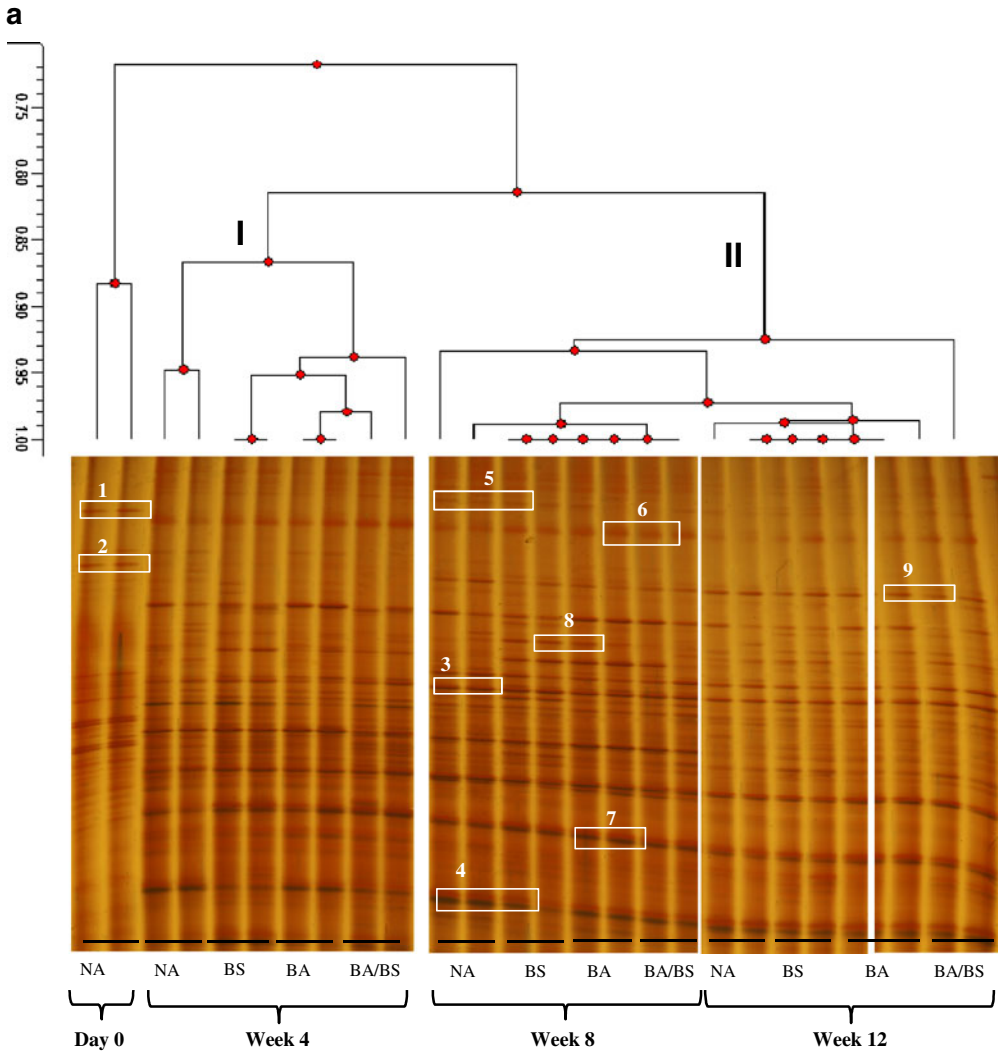


Fig. 1 Level of total petroleum hydrocarbon reduction in engine oil-contaminated soil during bioremediation over a 12-week period. *Box A*; the week when biostimulated and biostimulated–bioaugmented samples' TPH fell below the disposal threshold of 10,000 $mgkg^{-1}$ (shown by a *straight line*). *Box B*; the week when the highest level of TPH reduction was observed in biostimulated–bioaugmented samples. For weeks 8, 10 and 12, samples with *asterisk* are significantly different from other samples in the same sampling period, whilst the absence of *asterisk* indicates no significant difference between the treatments at that time period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II
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Week 4
Week 8
Week 12

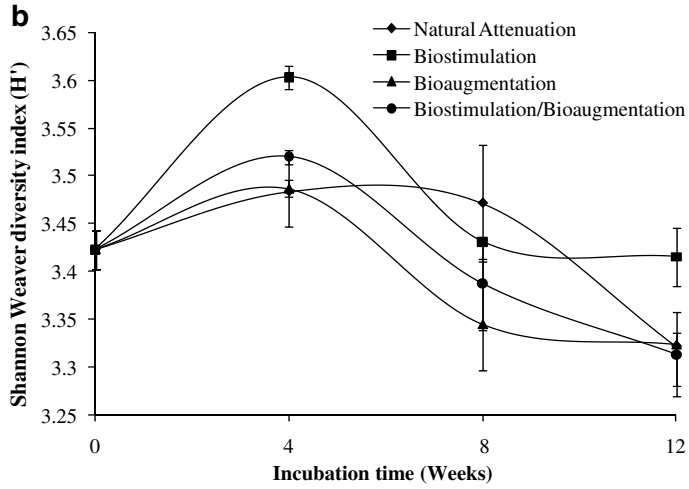

b
Natural Attenuation
Biostimulation
Bioaugmentation
Biostimulation/Bioaugmentation
Shannon Weaver diversity index (H')
3.65
3.6
3.55
3.5
3.45
3.4
3.35
3.3
3.25
0
4
8
12
Incubation time (Weeks)

Fig. 2 Universal bacterial dendrogram and DGGE profile of 16S rRNA gene sequences (**a**) and Shannon–Weaver diversity values (**b**) for the naturally attenuated (*NA*), biostimulated (*BS*), bioaugmented (*BA*) and biostimulated–bioaugmented (*BA/BS*) samples. In **a**, bands shown in *boxes* and *numbered* were excised and sequenced. Major clusters are numbered *I* and *II* and the scale represents the similarity coefficient between the samples

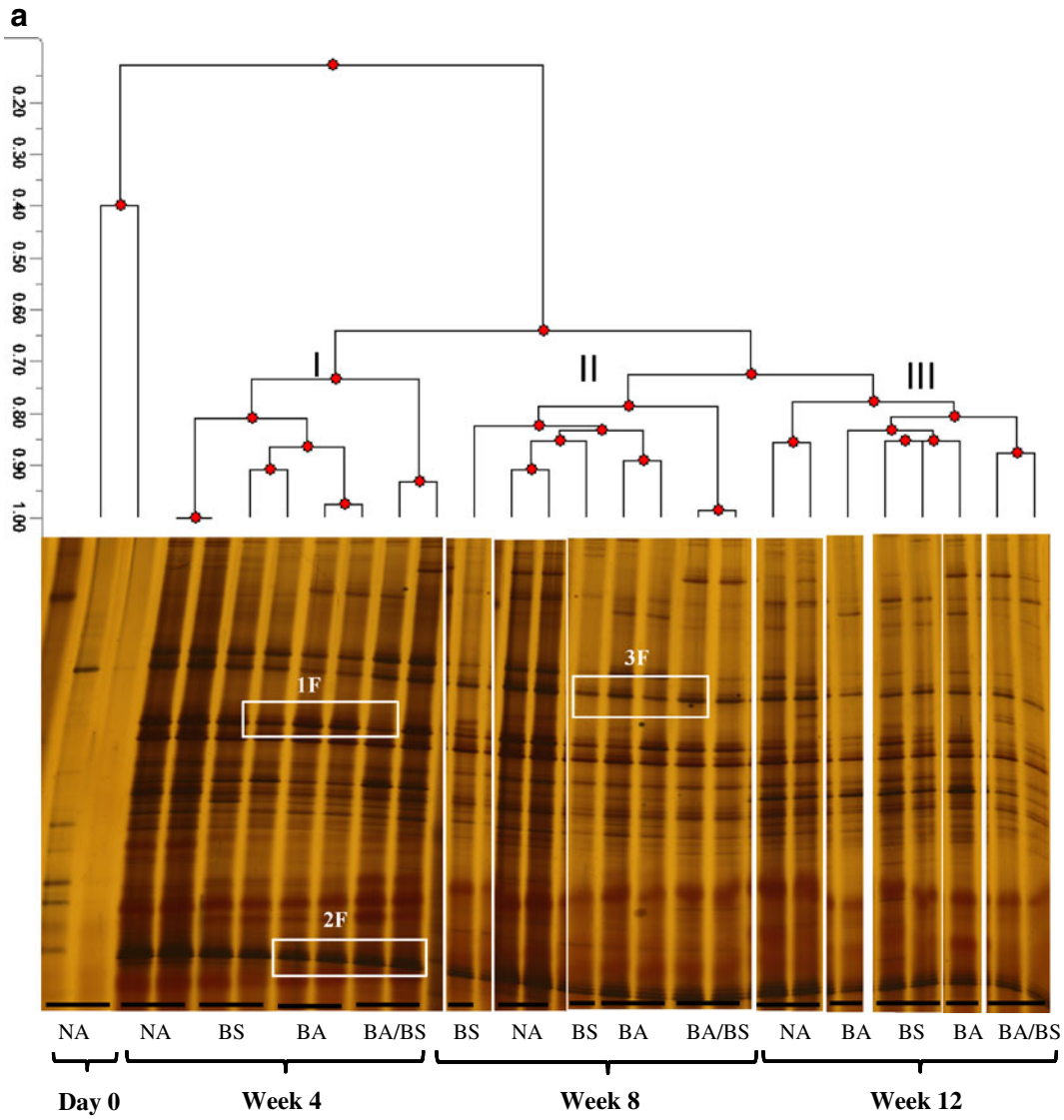
related, with communities in microcosms sampled at the same time being more closely related to one another than to those from other time points. Cluster analysis also showed comparatively greater variation in week 4 microcosms than in weeks 8 and 12. The DGGE profile also showed the presence of dominant bands irrespective of the nutrient or microbial augmentation. A number of the dominant bands (shown in white boxes) were excised and sequenced, with their sequence identities shown in Table 2. The dominant bacterial group amongst the excised bands were Proteobacteria. Shannon–Weaver diversity analysis, however, revealed that the bacterial community diversity largely increased between day 0 and week 4 before decreasing in most of the microcosms. In most of the time points (weeks 4 and 8) analysed, the addition of nutrients caused significant differences in the bacterial community diversity (Fig. 2b). DGGE analysis of the fungal community also showed three time-based clusters. However, unlike in bacterial where there were variations in communities only in week 4, fungal communities showed variation in all the three clusters (Fig. 3a). The identities of a number of the dominant bands excised are also shown in Table 2. Unlike in bacteria, the fungal community diversity remained stable and showed little or no response to nutrient or microbial augmentation (Fig. 3b).

4 Discussion

Biodegradation of engine oil in test soils showed that by week 8, the levels of TPH in biostimulated and biostimulated–bioaugmented samples fell below 10,000 $mgkg^{-1}$, which meant that they could be disposed of as low-level contaminated waste into designated landfills. It took the naturally attenuated and bioaugmented samples a further 2 weeks (week 10) before their levels went below the safe landfill disposal threshold. Nutrients are often limiting in hydrocarbon-contaminated soils, and additions of nutrients such as nitrogen and phosphorus have been reported to accelerate hydrocarbon degradation (Stallwood et al. 2005). It is possible that the 2-week accelerated reduction of the two samples (BS and BA/BS) over other samples was related to the addition of nutrients and COM001.

However, by the end of the experimental period (week 12), the levels of TPH in all the samples were not significantly different from one another (ANOVA, $P>0.05$). This meant that even without any intervention, monitored natural attenuation (NA) led to a substantial reduction (>88%) of the engine oil contaminant in soils. This is in contrast to the findings of Abioye et al. (2010) who showed that amendment of engine oil-contaminated soils with organic matter led to between 22.8% and 54.03% net loss of TPH compared to the naturally attenuated control samples. The reason for the different result obtained in this study is related to the type of soil used. Unlike most studies which used pristine soils, soils from a finished biopile of hydrocarbon-contaminated soils were used in this study. This meant that these soils already have enhanced microbial hydrocarbon-degrading capacity because of prior contact with hydrocarbons (Joo et al. 2008; Kyung-Hwa et al. 2007). This enhanced capacity was probably responsible for the substantial level of engine oil reduction in naturally attenuated soils (although this potential was enhanced by nutrient and nutrient-mycoaugmentation).

This finding is crucial because cost has been a major issue in the treatment of waste engine oil-contaminated environments in developing countries. Most of these countries cannot afford to set up and maintain waste (engine) oil recycling facilities, and this has led to substantial soil contamination with engine oil. Cheaper waste organic matter such as agricultural and brewery wastes, which have been successfully used to treat such soils (Abioye et al. 2010), may not be readily available, but the engine oil-contaminated soils are always available. Hydrocarbon (engine oil) contamination of soils would have led to an enhanced microbial hydrocarbon catabolic capacity. This potential can be cheaply harnessed to successfully carry out ex situ bioremediation of engine oil wastes and contaminated soils by using such soils to set up biopiles (at very little cost), as shown by this study. As engine oil-contaminated soils are easy to find (due to lack of treatment facilities), they can be economically sourced and locally utilised,


a
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Day 0
Week 4
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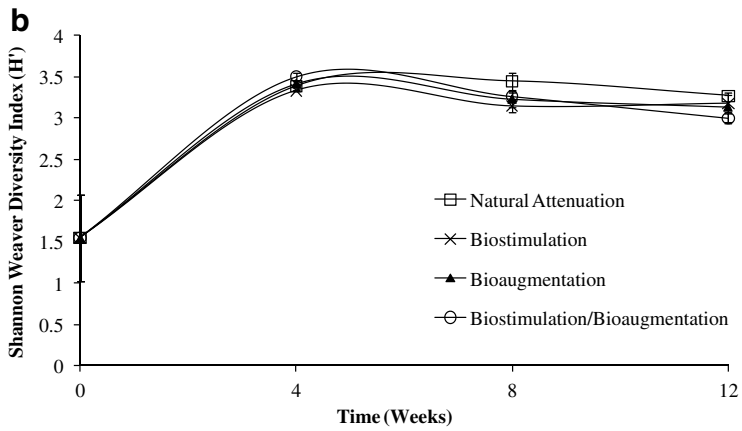
Week 12


b
Shannon Weaver Diversity Index (H')
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
8
12
Time (Weeks)
Natural Attenuation
Biostimulation
Bioaugmentation
Biostimulation/Bioaugmentation

◀ **Fig. 3** Universal fungal dendrogram and DGGE profile of ITS gene sequences (**a**) and Shannon–Weaver diversity values (**b**) for the naturally attenuated (*NA*), biostimulated (*BS*), bioaugmented (*BA*) and biostimulated–bioaugmented (*BA/BS*) samples. In **a**, bands shown in *boxes* and *numbered* were excised and sequenced. Major clusters are numbered *I*, *II* and *III* and the scale represents the similarity coefficient between the samples

requiring very little technology. When economically feasible, nutrients may be added to speed up this process or the pile may simply be left alone (with occasional turning) for natural attenuation to occur.

Evidence of the presence of already enhanced microbial hydrocarbon catabolic capacity is usually shown by the isolation of engine oil-degrading organisms such as *Flavobacterium*, *Acinetobacter*, *Pseudomonas*, *Bacillus* and *Cyanobacteria* from engine oil-polluted soils (Mandri and Lin 2007; Akoachere et al. 2008; Udeani et al. 2009). Some of the dominant bacterial isolates putatively identified from the DGGE profile (irrespective of treatment) were *Acidobacteria*, *Alcanivorax*, *Bacillus* and *Cyanobacteria* (Harayama et al. 2004; Kyung-Hwa et al. 2007). This showed that these engine oil-degrading genera were already present in the soil even before bioremediation started and probably played a substantial role in engine oil degradation during the process. The changes (increase) in the bacterial community diversity especially within the first 4 weeks of incubation, which was also when the greatest TPH reduction occurred, could have been due to the increased activities of these organisms. Fungi were part of the COMclean formulation, and soil fungi have been successfully used to treat engine oil-contaminated soil (Adenipekun 2008). The identification of fungal genera such as *Arthrobytrys*, *Coprinus* and *Mortierella* (Colgan et al. 2004; Di Toro et al. 2008; Mancera-Lopez et al. 2008), which are known hydrocarbon degraders, is also an indication of available fungal hydrocarbon catabolic capacity. However, as the fungal community diversity showed very little fluctuation and differences between the control and the treatments (also reported by Wu et al. 2008), it is possible that fungal role in engine oil reduction was probably not as great as those of bacteria.

This study has shown that the soils with previous contact with hydrocarbons can have enhanced hydrocarbon-degrading capacity because of the presence of hydrocarbonoclastic bacterial and fungal species. This enhanced capacity can be economically utilised for bioremediating waste engine oil or engine oil-contaminated soils (in biopiles) in third world countries via monitored natural attenuation. This is especially important in places where oil recycling facilities are unavailable and could be part of low technological solutions to the endemic problem of engine oil pollution. This enhanced microbial potential can also be harnessed for accelerated treatment (BS/BA) of engine oil-contaminated soils to levels safe for landfill disposal in developed countries

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