

*Full Length Research Paper*

# Comparison between different bio-treatments of a hydrocarbon contaminated soil from a landfill site

Ramya Thangarajan<sup>1</sup>, Eric M. Adetutu<sup>1\*</sup>, Robert B. Moore<sup>1</sup>, S. T. Ogunbanwo<sup>2</sup> and Andrew S. Ball<sup>1</sup>

<sup>1</sup>School of Biological Sciences, Flinders University, South Australia.

<sup>2</sup>Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

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We investigated the bio-remediation of a hydrocarbon contaminated soil pile that was slated for landfill disposal, by utilising laboratory based-soil microcosms. The objective was to accelerate the reduction of soil total petroleum hydrocarbon (TPH) to levels that could potentially allow the soil to be used outside a landfill site. Soil TPH content reduced by 57% over a 2 year period from 15,800 to ~6,800 mg kg<sup>-1</sup> in the untreated pile, making the soil eligible for landfill disposal under current Australian legislation. Subsequent bio-remediation (natural attenuation, biostimulation, bioaugmentation and biostimulation-bioaugmentation) resulted in over 74% reduction (~1,800 mg kg<sup>-1</sup>) in soil TPH content over 56 days with most of the reduction occurring in the first 21 days (~60%). Nutrient and microbial amendments did not confer any long-term benefit on the rate of soil TPH reduction with natural attenuation being equally efficient as other bioremediation strategies at day 56. TPH bioavailability assay showed a linear decrease from an initial 84 to 35% by day 56 and could explain the reduced TPH reduction rates observed after day 21. Denaturing gradient gel electrophoresis (DGGE) analyses of 16S rDNA and internal transcribed spacer regions genes revealed diverse and stable (unaffected by amendments) bacterial and fungal communities. Microbial analysis also showed substantial populations of alkane (*alkB*) degrading bacteria in the soils. This study therefore showed that soils slated for landfill disposal possess substantial hydrocarbon degrading capacity, which can be exploited for greater TPH removal through natural attenuation. The soil TPH can be potentially reduced lower than the 1,800 mg kg<sup>-1</sup> obtained in this study provided the contaminant is made more bio-available. This would potentially allow the use of such soils for more productive purposes outside the landfill sites extending the lifespan of these sites.

**Key words:** Bioremediation, denaturing gradient gel electrophoresis (DGGE), 16S rDNA, ITS, *alkB*

## INTRODUCTION

Hydrocarbon pollution of natural environments is of great concern because of health and safety issues. Environmental pollution by hydrocarbons can occur naturally through seepage or from anthropogenic sources via spillage, accidents or improper disposal of petroleum

and related products (Menzie et al., 1992; Golomb et al., 2001). Various hydrocarbon pollutants, such as polycyclic aromatic hydrocarbons (PAHs), which can accumulate in soils, plants and in the food chain (Kipopoulou et al., 1999; Johnsen et al., 2005; Li et al., 2008), are toxic to living systems (Belousova et al., 2001; Haritash and Kaushik, 2009; Arulazhagan et al., 2010). It is therefore important to carry out effective remediation of hydrocarbon polluted environments in order to mitigate their detrimental effects on the environment.

\*Corresponding author. E-mail: [eric.adetutu@flinders.edu.au](mailto:eric.adetutu@flinders.edu.au).  
Tel: +61-882017961. Fax: +61-882013015.

A combination of biological, chemical and physical processes, such as incineration, use of landfills, solidification and biological treatments can be used to treat hydrocarbon contaminated soils (Scragg, 2005; Adams et al., 2009; Yousefi Kebria et al., 2009). Biological treatment (bioremediation) is increasingly becoming important because of its environmentally friendly properties (Adams and Guzman-Osorio, 2008; Fouépé et al., 2009). Bioremediation usually involves the use of biological agents to detoxify a contaminated environment. This process can be carried out using different methods, such as natural attenuation (Makadia et al., 2011), bioaugmentation (Yousefi Kebria et al., 2009; Abdulsalam et al., 2011), biostimulation (Andreoni and Gianfreda, 2007; Gennaro et al., 2009) and a combination of bioaugmentation and biostimulation (Zahed et al., 2010, Sheppard et al., 2011). The role of microorganisms in bioremediation is well documented with bacterial groups capable of degrading different hydrocarbon fractions ranging from straight chain aliphatics (Yuste et al., 2000; El-Gendya and Farah, 2011) to poly-aromatic compounds (Richard and Vogel, 1999; Wu et al., 2008; Wen et al., 2011). Apart from microbial hydrocarbon degrading capabilities, the success of any bioremediation strategy is also dependent on environmental factors, such as temperature, O<sub>2</sub>, soil pH and type, organic pollutant concentration and bioavailability (Jørgensen et al., 2000; Carberry and Wik, 2001; Li et al., 2009; Yang et al., 2009; Sinkkonen et al., 2010; Mohajeri et al., 2010; Nwuche and Ugoji, 2010; Sanscartier et al., 2011).

The aim of these biological and other forms of treatment is to reduce the level of hydrocarbon contaminant so that the soil can be either be safely re-used for other purposes or disposed off in landfill sites. The levels of hydrocarbon contaminant in soils safe for re-use or disposal is usually defined by legislation and may vary from one country to another. For example in Australia, soils with TPH levels below 10,000 mg kg<sup>-1</sup> with the levels of metals and organics below the defined health investigation levels (National Environmental Protection Measure NEPM guidelines) are regarded as being safe for disposal in designated landfill site (NEPC, 1999). Additionally, the Australian Environment Protection Authority (EPA) is changing the old model of waste treatment that relied heavily on landfill disposal as the primary means of waste disposal, and is replacing it with one which minimizes waste generation. This new model is designed to avoid, reduce, reuse, recycle, recover, treat and dispose of wastes with landfill disposal being used only as a last resort ([http://www.epa.sa.gov.au/environmental\\_info/waste](http://www.epa.sa.gov.au/environmental_info/waste)).

While it will take some time before this model becomes fully implemented, current anthropogenic activities generate wastes which still have to be disposed off in a landfill site.

The challenge is to apply bioremediation tools to further

treat soils having TPH levels below 10,000 mg kg<sup>-1</sup> in order to be able to dispose of or even productively use such soils outside landfill sites (example for land filling in construction sites). Although, some disposal sites at receive waste soils with TPH levels above 10,000 mg kg<sup>-1</sup> for treatment, soils deposited there can only be put below ground when the legislated TPH level is reached. Current NEPM guidelines also require amongst other things that the soil C<sub>9</sub> TPH level be ≤ 1,000 mg kg<sup>-1</sup> (*ex-situ* remediation) or the soil C<sub>16</sub>-C<sub>35</sub> level be ≤ 5,600 mg kg<sup>-1</sup> (*in situ* remediation) before it can be re-used (NEPC, 1999; Sheppard et al., 2011). Successful treatment of these soils to below the legislated levels of TPH and remediation of, or compliance with, other parameters such as metal and aromatic hydrocarbon contents (NEPC, 1999) will potentially allow the use of these soils outside the landfill site, creating more space and extending the lifespan of such sites. However, for these objectives to be attained there are crucial questions that need to be answered. Can the TPH of these waste soils be reduced by biological treatments and to what level? As cost is a critical issue, it is also important to determine which bioremediation method is the most efficient and cost effective for waste soil treatment.

In this study, four different bioremediation methods (natural attenuation, bioaugmentation, biostimulation and combined biostimulation-bioaugmentation) were applied to samples from a two year old pile of mixed hydrocarbon contaminated soils at a landfill site in Adelaide, South Australia. The rate of hydrocarbon removal was monitored through Gas Chromatography analysis while changes in both the microbial population were monitored using a PCR-DGGE method. The data generated were then used to determine the most efficient bioremediation method for TPH removal.

## MATERIALS AND METHODS

### Sampling and determination of soil physico-chemical properties

Soil was obtained from a pile comprising a mixture of hydrocarbon contaminated soils at a research station at the Southern Waste Depot, Adelaide, South Australia. This pile of soil (initial TPH level of 15,800 mg kg<sup>-1</sup>) contained soils from different contamination events (waste engine oil, sludge, waste diesel and crude oil) and was subject to natural attenuation for two years. At the start of the present experiment, this pile had a TPH of ~6,800 mg kg<sup>-1</sup>. Soil samples from the pile were mixed and sieved (< 0.25 mm) prior to the start of the microcosm experiments. The moisture content, water holding capacity, pH and elemental composition (Carbon and Nitrogen) of the soil were determined using standard methods (Mishra et al., 2001).

### Microorganism and nutrient amendments

A known hydrocrabanoclastic fungal strain, *Scedosporium apiospermum* (Martin-Gil et al., 2008), was used as a bioaugmenting agent in this study. The fungus stored at -80°C in glycerol was reactivated by culturing it on Potato Dextrose Agar

**Table 1.** Experimental design of soil microcosms.

Amendment	Microcosms			
	Natural attenuation (NA)	Bioaugmentation (BA)	Biostimulation (BS)	Bioaugmentation and biostimulation (BAS)
Fungi	-	+	-	+
Nutrients	-	-	+	+
Water	+	+	+	+

“+” indicates addition of amendment to soil microcosms and “-” indicates absence of amendment in soil microcosms.

(PDA) plates containing 0.015 g/l of tetracycline at 30°C for up to 10 days to produce conidia for spore suspension preparation. Aliquots of prepared spore suspensions were inoculated into sterile Tryptone Soya Broth (TSB) containing tetracycline, and incubated at 37°C for up to 3 days on a shaker at 120 rpm for generation of mycelia. The mycelia were washed in phosphate buffered saline, then filtered and concentrated before being used for subsequent studies. A modified minimal salts nutrient solution based on Bushnell Haas medium (MgSO<sub>4</sub> 0.2 g, CaCl<sub>2</sub> 0.02 g, KH<sub>2</sub>PO<sub>4</sub> 1 g, H<sub>2</sub>NH<sub>4</sub>PO<sub>4</sub> 1 g, KNO<sub>3</sub> 1g, FeCl<sub>3</sub> 0.05 g) (Eriksson et al., 2000) supplemented with urea (resulting in an N:P ratio of 10:1 pH 7.6) was used for biostimulation in this study. Fungal mycelia were added at the desired concentration needed for 200 g of soil by mixing the mycelia either in sterile water (used for adjusting the soil water holding capacity) or in this modified nutrient solution.

### Soil microcosms

Soil microcosms were set up in 1 l flasks (Table 1) as described: (i) Flasks 1-3 (naturally attenuated microcosms) contained 200 g of soil without any amendment; (ii) Flasks 4-6 (bioaugmented microcosms) contained 200 g soil and fungal cultures added at 0.4 mg g<sup>-1</sup> of soil (iii) Flasks 7- 9 (biostimulated microcosms) contained 200 g soil and sterile nutrient solution (25%, w/w); and (iv) Flasks 10-12 (combined bioaugmented and biostimulated microcosms) contained 200 g soil and fungi (0.4 mg g<sup>-1</sup> of soil) and modified nutrient solution (25%, w/w). The water content in all 12 flasks was adjusted to 50% of the soil water holding capacity. The flasks were then covered with perforated aluminum foil and incubated at 30°C for 56 days. Microcosm sampling was carried out from triplicate sets of flasks at predetermined intervals (days 0, 7, 14, 21, 28, 35, 42, 49 and 56). Flask contents were mixed periodically (after sampling) for aeration and water was added when necessary for the maintenance of soil moisture.

### TPH extraction and quantification

TPH was extracted from soil microcosms (day 0 to day 56) by adding 5 ml of acetone and 2 ml of Retention Time Window (RTW) solution to 1 g of soil in Teflon coated tubes. After sonicating the sample for 1 h, the protocol described in ISO/DIS 16703 (ISO 2004) was followed. The extracted TPH was quantified by gas chromatography (GC) on a Varian 3800 gas chromatograph equipped with a Varian 8200 Autosampler, a Flame Ionisation Detector (FID), and a splitless injector valve. The capillary column used was an Alltech EC-5 (30 m × 0.25 mm with 0.25 µm film thickness), with helium as the carrier gas flowing at a rate of 2 ml/min in constant flow mode. A standard calibration curve was constructed from dilutions of a diesel and oil mixture (RTW solution), and the equation from the standard calibration curve was used in conjunction with measurement of the area under the relevant peaks/curve (area between C<sub>10</sub> and C<sub>40</sub>) in each of TPH degradation (reduction) was calculated using the formula:

chromatogram, to determine TPH concentrations. The percentage Percentage of degradation = [(TPH control - TPH treatment)/ TPH control] \*100.

### Bioavailable hydrocarbons

Bioavailable hydrocarbons were extracted from naturally attenuated microcosms by a modified mild extraction procedure (Sabaté, 2006), by adding 15 ml of mild extraction solution (70 mM hydroxypropyl- $\beta$ -cyclodextrin and sodium azide (0.5 g l<sup>-1</sup>) in Milli-Q water) to 1 g of soil in Teflon coated tubes. Tubes were shaken at 150 rpm in a horizontal incubator maintained at 25°C. After 24 h, the soil and aqueous extraction solution were separated by centrifuging the tubes at 2000 rpm for 25 min in a Sigma 3-16PK centrifuge (Sigma Laboratory, Osterode, Germany). Liquid-liquid extraction of the supernatant was carried out three times using 10 ml of chloroform. The organic phase was then concentrated by rotary evaporation and subjected to GC (gas chromatography) analysis for TPH measurements. The TPH level in this extract was then deducted from the initial TPH of the soil (and expressed as a percentage) to get the available (eluted) hydrocarbon content.

### Statistical analysis

Experiments were conducted using three independent replicate microcosms and were subjected to statistical analyses (ANOVA) using Sigma stat 10.0 to determine whether there were significant differences between the samples.

### DNA extraction and PCR

Total genomic DNA from 0.3 g of soil samples (days 0, 7, 14 and 21) was extracted using a Mo Bio soil DNA isolation kit (Mo Bio Laboratories, CA, USA) following the manufacturer's protocol. PCR amplifications were carried out on 16S rDNA genes using universal primers 341FGC and 518R, Internally Transcribed Spacer regions using universal primers ITS1F,1FGC, 2 and 4, and *alkB* genes using *Rhodococcus* based primers R1f438, R1r835and R1r835GC (Muyzer et al., 1993; Anderson et al., 2003; Anderson and Parkin, 2007; Hamamura et al., 2008). Each (48 µl) PCR reaction comprised: 2 µl of forward primer (10 pmol/ µl), 2 µl of reverse primer (10 pmol/ µl), 5 µl of magnesium chloride (25 mM) (Promega, WI, USA), 1 µl of deoxynucleoside triphosphate (dNTP) mixture (10 mM) (Promega), 10 µl of GoTaq Flexi buffer (5 x) (Promega), 0.25 µl of Taq polymerase enzyme (5 U/µl) (Promega) and 27.75 µl of sterile nuclease free water. The desired template DNA (2 µl) was then added. All PCR amplifications were performed using a Bio-Rad DNA Engine<sup>®</sup> Peltier Thermal Cycler (Bio-Rad Laboratories, Mexico, North America) and PCR amplicons were analysed on a 2% agarose gel by electrophoresis. The thermocycling condition used for 341FGC and 518R primer set

was: 1 × 5 min at 95°C, 33 × (95°C 30 s, 55°C 30 s, 72°C 1 min), then 10 min at 72°C.

Fungal *ITS* genes were amplified using a nested PCR approach. The first amplification was carried out with primers ITS 1F (5'-TCC GTA GGT GAA CCT GCG G -3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC -3'). The program for the first amplification consisted of an initial denaturation for 5 min at 95°C, 10 × (95°C 30 s, 65 °C 30 s, 72°C 1 min, touchdown with 1°C decrease per cycle, 1 min at 72°C), 20 × (95°C 30 s, 55°C 30 s, 72 °C 1 min), then 10 min at 72°C. The second amplification was carried out using primers ITS1F-GC, and ITS 2 (5'-GCTGCGTTCTTCATCGATGC-3') using amplicons from the first reaction as template DNA and with the same cycle conditions as the first reaction. The negative control of the ITS1F-4 reaction was also used as a template to eliminate the possibility of carryover contamination.

Amplification of *alkB*, alkane degrading gene of *Rhodococcus* sp. was performed with group-specific primers (R1f438, R1r835GC and R1r835). The first amplification was carried out with primers R1f438 (5'- CGTCGAGCGCTGGTTGTCC- 3') and R1r835 (5'-GACGTAGGAGTCCGTAGTGC - 3') at 95°C for 10 min, 10 × (94°C 45 s, 64°C 45 s (touchdown with 1°C decrease per cycle), then 72°C for 1 min 30 s; 20× (94°C 45 s, 54°C 45 s, 72°C 1 min 30 s); then 72 °C for 7 min. The second amplification was carried out using primers R1f438 and R1r835-GC (with GC clamp) using amplicons (including the negative control) from the first reaction as template DNA. The same cycle conditions as the first reaction were used to generate amplicons suitable for Denaturing Gradient Gel Electrophoresis analysis.

#### Denaturing gradient gel electrophoresis (DGGE)

All PCR products were run on 9% (w/v) polyacrylamide gels (acrylamide-N,N'-methylenebisacrylamide ratio 37:1) (7M Urea) with the appropriate denaturing gradient (16S rDNA; 48-60%, Fungal *ITS*; 50-60% and *Rhodococcus alkB* 53-68%). DGGE was performed in 1.0 X TAE running buffer (40 mmol Tris-HCl, 40 mmol acetic acid, 1 mmol EDTA) at 60 V and 60°C for 20 h using the Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, CA, USA). Gels were stained with silver as described by Benbouza et al. (2006). DGGE gels were incubated in 400 ml of fixing solution (40% methanol/10% acetic acid, v/v) for 2 h and then incubated in 200 ml silver nitrate solution (0.2 g silver nitrate in 200 ml dsH<sub>2</sub>O, w/v) for 20 min. The gels were then placed in 200 ml of developing solution (0.02 g sodium borohydride, 0.8 ml formaldehyde, 3 g sodium hydroxide in 200 ml dsH<sub>2</sub>O) for 20 min and then in 400 ml of fixing solution (10% ethanol/5% acetic acid, v/v) for 10 min. Gels were preserved by soaking in 400 ml preservative solution (125 ml of absolute ethanol, 50 ml glycerol in 325 ml of dsH<sub>2</sub>O, v/v) for 10 min. Stained gels were scanned using an EPSON perfection V 700 Photoscanner (Seiko Epson Corporation, Japan).

#### DGGE gel analysis

Total Lab-120 (TL-120) V2006 F Image software was used to analyse DGGE bands. Unweighted pair groups were generated using arithmetic average (UPGMA) dendrograms constructed via Dice-Sorensen's similarity matching index. TL-120 software was used to generate hierarchical similarity relationships between samples based on the degree of similarity between the bands across the lanes of each DGGE gel. The Shannon Weaver diversity index (*H'*) (Shannon and Weaver, 1949) was calculated using band densities. The Shannon Weaver diversity index is a general diversity value, which increases as the number of species (bands) increases (Krebs, 1999). This was calculated using the formula:

$$H' = -\sum p_i \ln p_i$$

Where, *H'* is the Shannon Weaver diversity index; *p<sub>i</sub>* is the proportion of the community that is made of species *i* (intensity of the band *i*/total intensity of all bands in the lane); and *LNp<sub>i</sub>* is the natural log of *p<sub>i</sub>*.

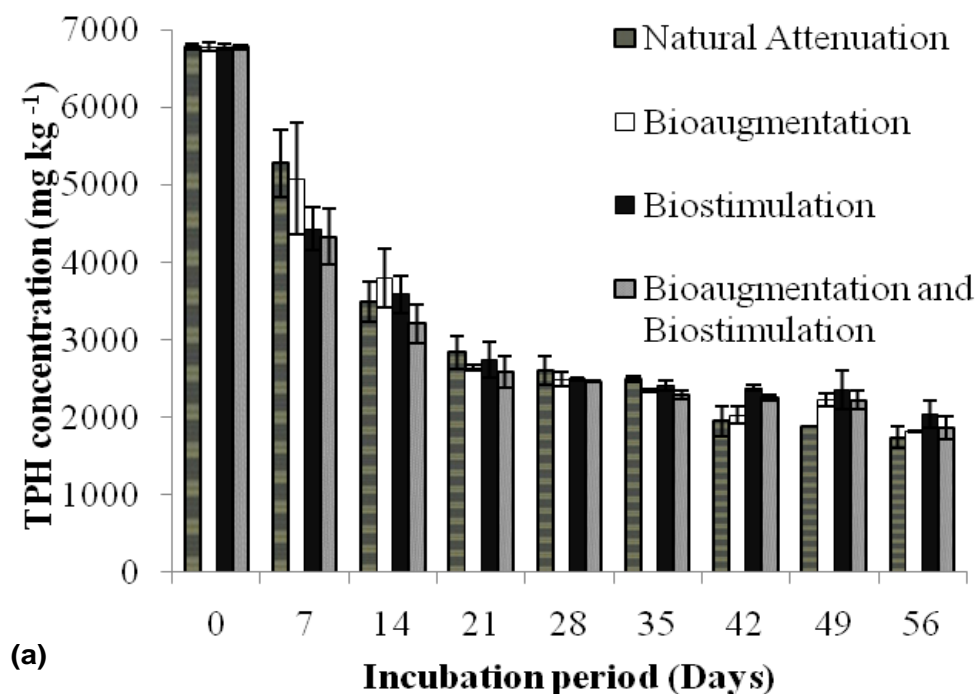
## RESULTS

### Bioremediation treatments and TPH degradation

Gas chromatographic analysis of the petroleum contaminated soil (pH 6.7, moisture content 6.37% and organic carbon content of 15%) showed an initial average TPH concentration of 6791.38 mg kg<sup>-1</sup> at the start of the experiment (Figure 1a). A breakdown of the TPH composition showed that it contained 2.9% of C<sub>10</sub>-C<sub>14</sub> (~197 mg kg<sup>-1</sup>), 55.1% of C<sub>15</sub>-C<sub>28</sub> (~3760 mg kg<sup>-1</sup>), 30% of C<sub>29</sub>-C<sub>36</sub> (~2050 mg kg<sup>-1</sup>) and 12% of C<sub>37</sub>-C<sub>40</sub> (~821 mg kg<sup>-1</sup>). Therefore, the C<sub>15</sub>-C<sub>28</sub> fraction was the dominant hydrocarbon fraction. There was a rapid reduction in soil TPH content from day 0 – 21 in the microcosms, which was then followed by a gradual reduction till day 56. There was a significant reduction in TPH concentrations in all the microcosms by day 7 (ANOVA, *P* < 0.05) with biostimulated and biostimulated-bioaugmented samples also being significantly different from naturally attenuated samples (ANOVA, *P* < 0.05). The highest cumulative TPH reduction of 61.73% was observed at day 21 in the combined bioaugmented-biostimulated microcosms, while naturally attenuated samples had the lowest TPH reduction percentage (58.15%). Further, TPH reductions of approximately 11-16% occurred at day 56 resulting in cumulative TPH reductions of approximately 74.5% (BAS), 74.4% (NA), 72.37 (BA) and 69.8% (BS). There were no significant differences between microcosms at the end of the experimental period on day 56 (ANOVA, *P* > 0.05) (Figures 1a and b) with naturally attenuated samples having the lowest final TPH value of ~1800 mg kg<sup>-1</sup>. At day, 56 the C<sub>10</sub>-C<sub>14</sub> had been completely eliminated (100% degradation), the C<sub>15</sub>-C<sub>28</sub> reduced by 74.6% to 955 mg kg<sup>-1</sup>, C<sub>29</sub>-C<sub>36</sub> by 71.2% to 590 mg kg<sup>-1</sup> and C<sub>37</sub>-C<sub>40</sub> by 76.3% to 194 mg kg<sup>-1</sup> in naturally attenuated samples. Other treatments or microcosm showed a similar trend (data not shown). Consequently, the greatest reduction in TPH was in the C<sub>15</sub>-C<sub>28</sub> fraction. Representative chromatograms showed differences between day 0 and 56, but none between the microcosms at day 56 (Figure 1b).

### Bioavailability quantification

Bioavailable hydrocarbons were extracted from the laboratory microcosm of control soil samples (naturally attenuated) by a mild extraction technique, and were quantified using gas chromatography. The percentage of bioavailable hydrocarbons decreased during the course



i. **Figure 1.** Average residual total petroleum hydrocarbons (TPH) in different laboratory based microcosms incubated for 56 days (a). Representative chromatograms from naturally attenuated microcosms at day 0 (i) and day 56 (ii) compared with a representative chromatogram from bioaugmented and biostimulated microcosms at day 56 (iii) (b). For (a) n=3.

of the experiment (Figure 2). Availability decreased from 81.9% on day 0 to 29.5% at day 56.

### Fingerprinting of the microbial community by DGGE

The effects of the different treatment regimes on bacterial, fungal and rhodococcal communities were determined by analysing PCR amplicons of 0, 14, and 21 day samples (as this was the period during which most of the soil TPH was eliminated) on DGGE. The UPGMA of the 16S rDNA bacterial community in all the samples on day 7 showed they differed from the starting community of day 0 (37% dissimilar). However, clustering over the three week period was largely time dependent and indicated very little comparative difference between treatments (Figure 3). Analysis of banding patterns showed several dominant bands irrespective of treatment or amendment during the experimental period.

In comparison to DGGE of bacterial 16S rDNA, DGGE of the *Rhodococcus alkB* gene showed a similar banding pattern with several dominant bands irrespective of treatment. Clustering was largely time based (Figure 4). The fungal community banding pattern was highly variable and did not show any consistent treatment effect (Figure 5). However, the fungal community was diverse with several dominant bands.

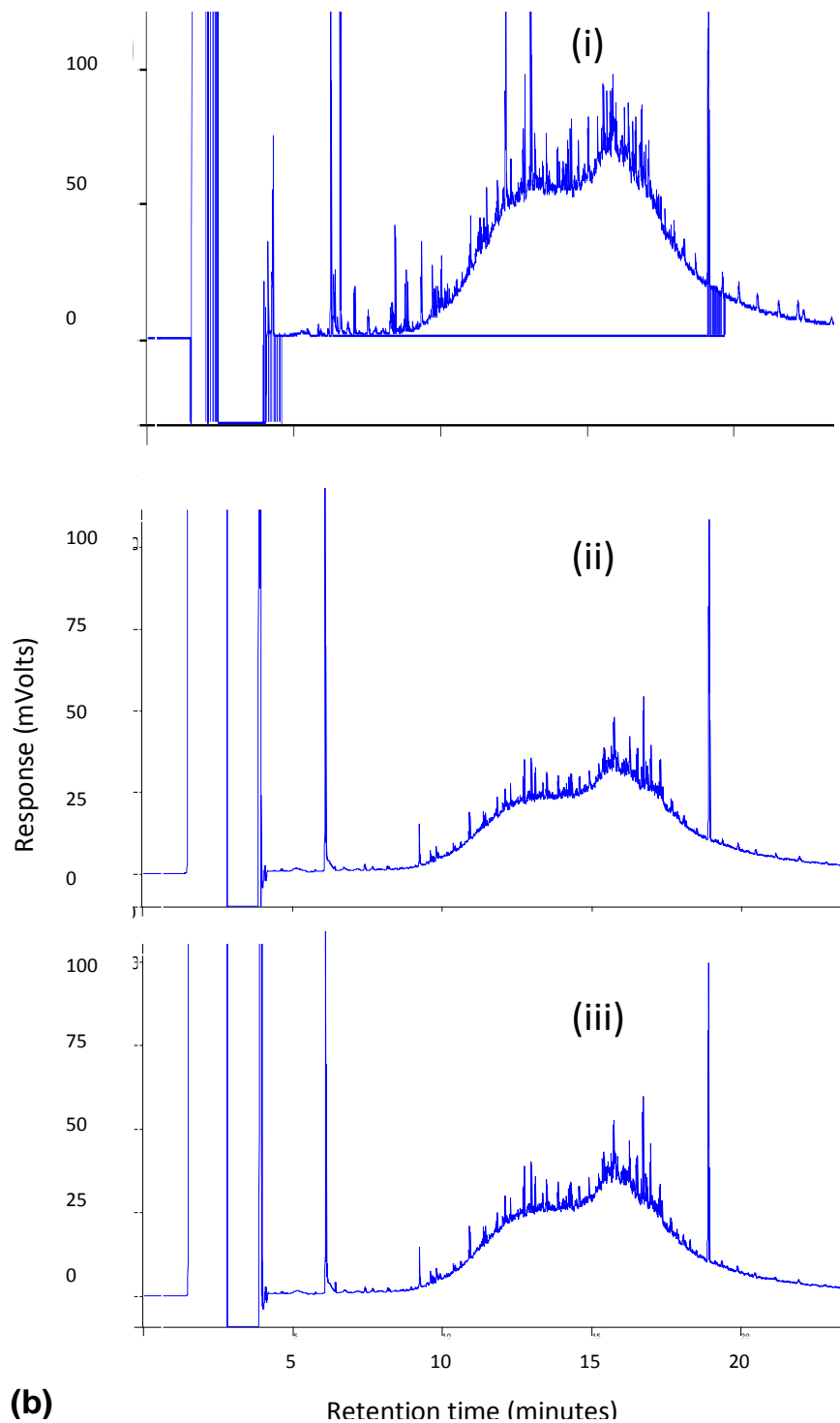
### Microbial diversity

A comparison of the Shannon Weaver diversity indices ( $H'$ ) for all the treatments is shown in Table 2. In bacterial community analyses based on 16S rDNA genes, natural attenuation was observed to have caused an increase in bacterial diversity from day 0 till day 21 with the highest bacterial diversity of 2.29 being obtained at day 21. In other microcosms, bacterial diversity was generally higher at day 21 than at the start of the experiment. The diversities of bacterial groups similar to *Rhodococcus* sp. were higher than those obtained with 16S rDNA genes, the highest  $H'$  values being 2.94 for NA, 2.87 for BA, 2.79 for BS and 2.82 for BAS (Table 2). Analyses of fungal community diversity showed that the community fluctuated over 21 days in the different microcosms (Table 2).

## DISCUSSION

### TPH analysis

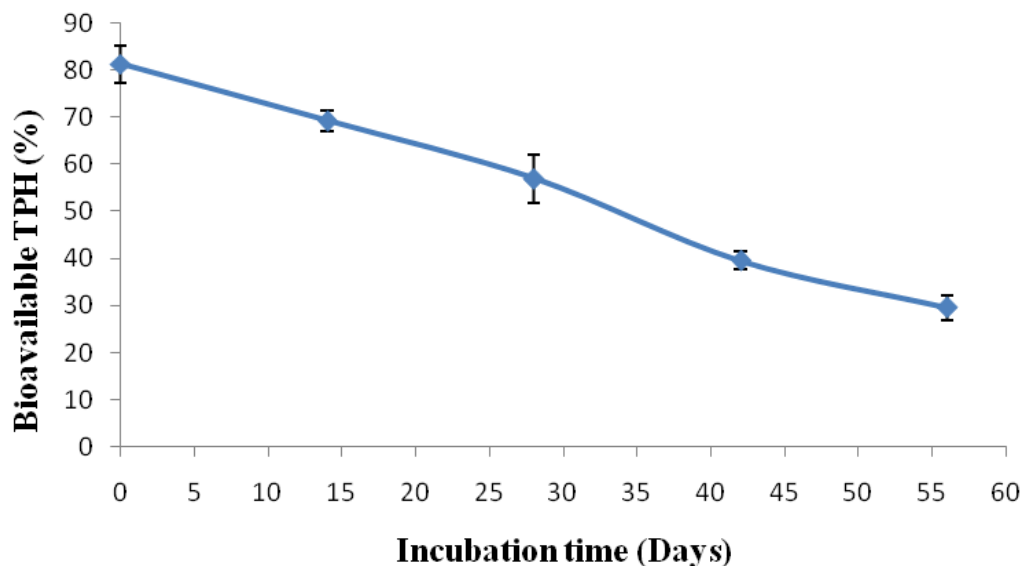
The gas chromatographic (GC) analysis of TPH concentrations in the microcosm experiments showed that the rate of degradation of TPH in all the treatment samples was rapid from day 0 to day 21 (> 50%)



**(b)** Figure 1. Contd.

indicating the beneficial effects of the bio-treatments on the microcosms. Degradation translated to a TPH loss of over  $4000 \text{ mg kg}^{-1}$  over three weeks compared to  $\sim 9000 \text{ mg kg}^{-1}$  lost over the two years (the pile was untreated). At day 7 of the soil microcosm incubation, the TPH level

was significantly reduced ( $P \leq 0.05$ ) in biostimulated (35% reduction) and bioaugmented-biostimulated microcosms (36% reduction) compared to naturally attenuated microcosms (22% reduction). This rapid rate of reduction continued in all the microcosms till day 21. However,



**Figure 2.** Bioavailable total petroleum hydrocarbons (%) in the naturally attenuated soils from the laboratory microcosm (n=3).

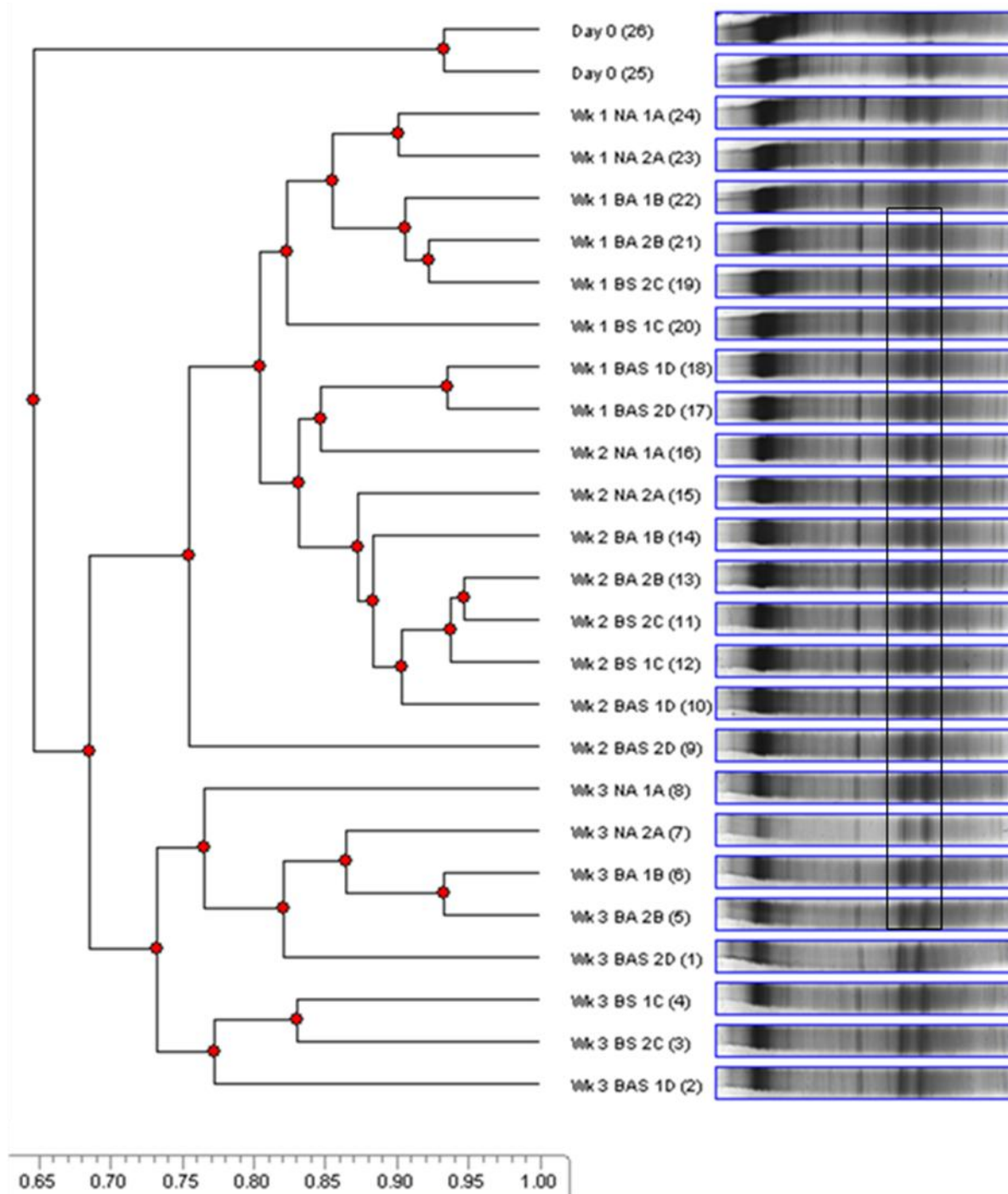
there were no significant differences between the different microcosms in most of the sampling periods after day 7. This indicated that the beneficial effects of fungal and nutrient addition were limited to first week of incubation. Similar trends of an initially rapid TPH reduction during bioremediation especially in the first two weeks of incubation in biostimulation treatment have also been reported by others, such as Ishihara et al. (1995) and Mishra et al. (2001). Bioremediation is known to be enhanced by addition of nutrients like N, P, and/or C (Gallego et al., 2001; Bento et al., 2005; Mohajeri et al., 2005) and there are several reports of the successful bioremediation of hydrocarbon contaminated soils using fungi (Okparanma et al., 2011; Isitua and Ibeh, 2010). It is possible that the addition of nutrients and hydrocarbonoclastic fungus initially boosted the rate of hydrocarbon reduction.

However, the fact that degradation proceeded at largely similar rates in amended and naturally attenuated microcosms after the first week indicated that nutrient and fungal amendments were not of long-term benefit. Hydrocarbon degradation sometimes involves co-metabolic activities by both bacteria and fungi (Vasco et al., 2011) and it is possible that these groups of microorganisms were involved in hydrocarbon degradation in this experiment. Hydrocarbon contamination can lead to an increase in the population of hydrocarbonoclastic organisms (Joo et al., 2008) and the establishment of an adapted microbial community. Bacterial and fungal community analyses of the amended and naturally attenuated microcosms showed that the microbial community was diverse. Assays for the presence of alkane degrading bacteria showed that the soil samples had rich and diverse alkane degraders and it

is likely that these groups would have mediated alkane degradation in the soils. However, there were essentially no shifts in bacterial and fungal communities banding pattern during the period of greatest TPH reduction (day 0 and 21). Dendrogram analysis also showed that nutrient or fungal supplementation had very little effect on bacterial diversity, as clustering from day 0 to day 21 was found to be largely time related rather than treatment related (Figures 3 and 4). This indicates that the bacterial populations, which had presumably adapted to the presence of the contaminant (in years prior to the start of this study), were highly stable and were not affected by perturbations related to amendment. Reduction in TPH levels without a substantial microbial shift is not unusual, as Makadia et al. (2011) for instance have reported that rapid changes in TPH of previously contaminated soils may not always be accompanied by changes in microbial community.

Nutrients can be limiting in hydrocarbon contaminated soils and the addition of nutrients generally benefits soil hydrocarbon degradation (Beolchini et al., 2010; Kauppi et al., 2011). The depletion of the supplied nutrients may have been accelerated after the first week. This could have led to lower than expected TPH reductions being observed in nutrient supplemented microcosms, with the subsequent outcomes being similar to TPH reduction rates across all the microcosms thereafter. However, it was also possible that the bioavailability of hydrocarbons had some influence on TPH degradation rates especially between days 28 and 56. Decreasing or limited bioavailability of hydrocarbon contaminants has been reported to negatively affect hydrocarbon removal from soils (Loser et al., 1999). At day 0, 81% of contaminating hydrocarbons were available for biological activities, yet



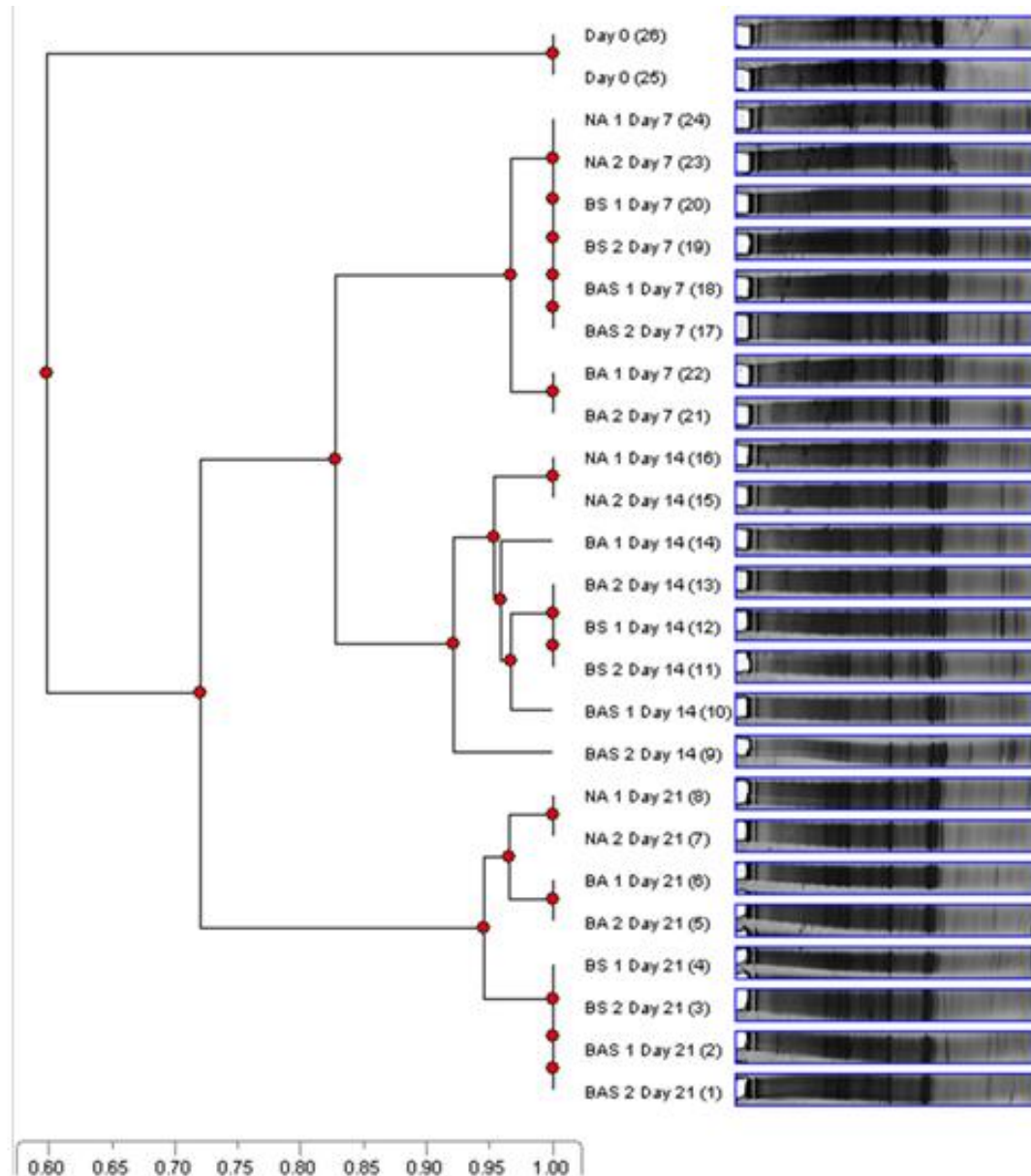


**Figure 3.** UPGMA dendrogram derived from 16S rDNA genes based bacterial community profiles on days 0, 7, 14, and 21. NA- natural attenuation, BA- bioaugmentation, BS- biostimulation and BAS-bioaugmentation and biostimulation. Numbers 1 and 2 with the same letter are duplicate samples of the same treatment and letters A, B, C and D are for NA, BA, BS and BAS respectively. Scale is indicative of similarity levels. Duplicate samples loaded on DGGE gel for ease of analysis. Some examples of common dominant bands are shown in a box.

at day 21, only ~ 61% of hydrocarbons were still available. The bioavailable hydrocarbons continued to reduce less, decreasing to ~30% by day 56, and indicating that the lengthening experimental period may be

correlated with decreasing bioavailability of the hydrocarbon contaminant. Therefore, it is alternately possible that the observed stagnation of TPH reduction between days 21 and 56 was either due to the degradable soil





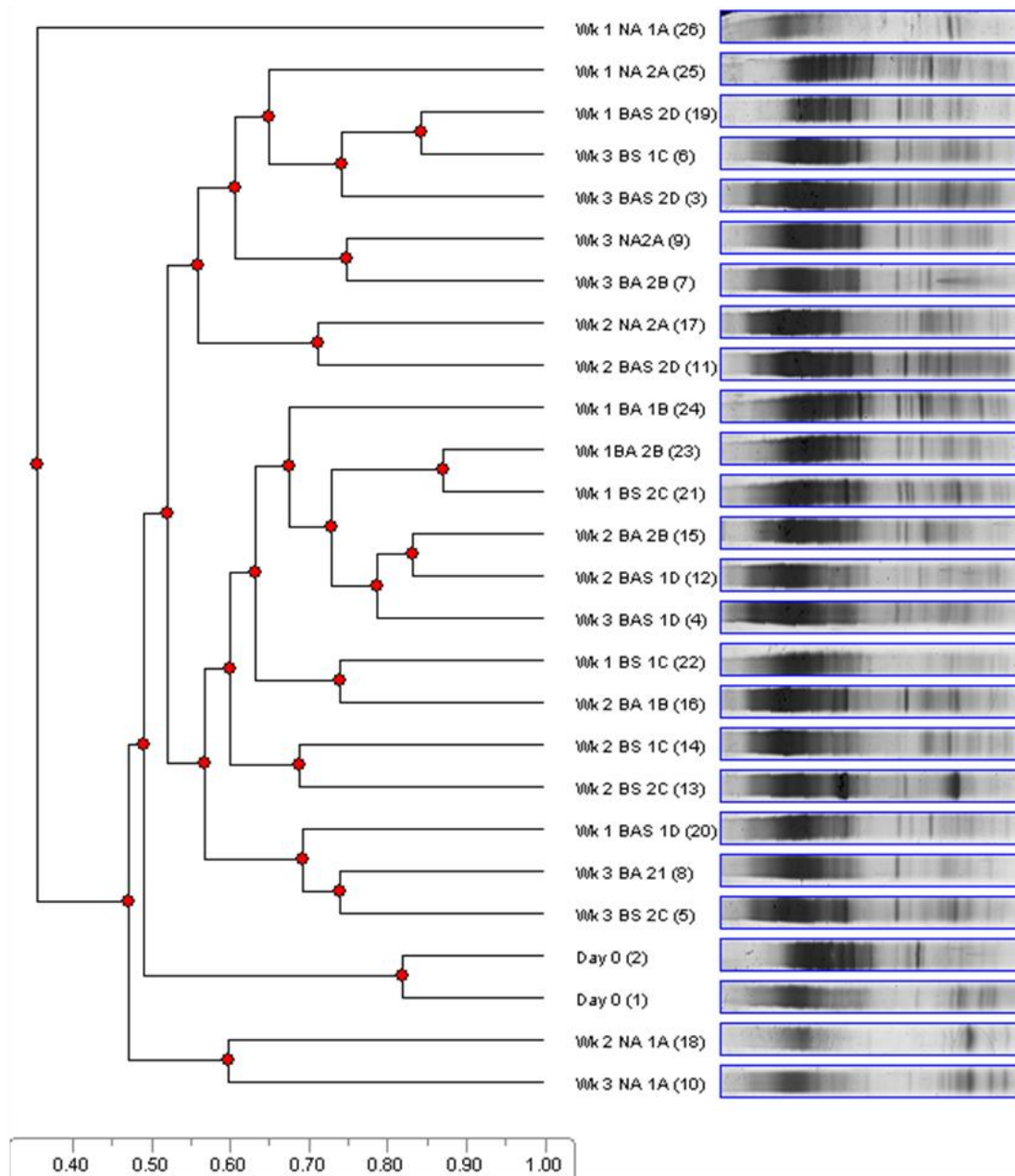
**Figure 4.** UPGMA dendrogram derived from *alkB* genes based bacterial community profiles on days 0, 7, 14, and 21. NA- natural attenuation, BA- bioaugmentation, BS- biostimulation and BAS-combined bioaugmentation and biostimulation. Scale is indicative of similarity levels. Duplicate samples loaded on DGGE gel for ease of analysis.

hydrocarbon having been unavailable or while available could not be degraded by the available microbial capacity.

At the end of the experiment at day 56, there was a substantial reduction in TPH levels, which were down by over 74% in the naturally attenuated samples. Although, this was the highest observed cumulative degradation rate, this was not significantly different from the cumulative degradation rates of other biotreatments. This outcome showed that natural attenuation was just as effective as other treatments over a long time frame

(Margesin and Schinner, 2001). Unlike the original pile, which was left untouched, naturally attenuated samples were mixed regularly ensuring more even distribution of air, contaminants and hydrocarbon degraders, and were watered regularly, as in other studies (Balba et al., 1998). This is likely to have enhanced the activities of the hydrocarbonoclastic microorganisms and leading to the overall extensive TPH reduction observed.

In conclusion, this study has shown that soils with TPH levels of  $\leq 10,000 \text{ mg kg}^{-1}$  destined for landfill disposal can be potentially remediated to lower TPH values of



**Figure 5.** UPGMA dendrogram derived from ITS genes based fungal community profiles on days 0, 7, 14, and 21. NA- natural attenuation, BA- bioaugmentation, BS- biostimulation and BAS-combined bioaugmentation and biostimulation. Scale is indicative of similarity levels. Duplicate samples loaded on DGGE gel for ease of analysis.

about  $1800 \text{ mg kg}^{-1}$ . However, this process can be negatively affected by decreasing TPH bioavailability. Future work should include the addition of surfactants (which are known to substantially improve hydrocarbon solubilization in soil) during the treatment process to improve hydrocarbon bioavailability (Lai et al., 2009; Zhang et al., 2010; Davezza et al., 2011). With the addition of surfactants, it might be possible to reduce the TPH levels to  $\leq 1,000 \text{ mg kg}^{-1}$  (NEPC, 1999) and provided other safety parameters are met, such soils can

be used outside the landfill site. This will create more space at landfill sites, increasing space turnover, and even extending their lifespan. In addition, this study has shown that nutrient addition and or fungal augmentation was of little long-term benefit in the remediation of soils at a landfill site. Simply mixing the soils (to improve aeration) and watering (as practiced in naturally attenuation) was as effective as nutrient and microbial amendments because these soils probably have an already enhanced microbial capacity to degrade hydro-

**Table 2.** Shannon Weaver diversity ( $H'$ ) indices of DGGE profiles generated from PCR-amplified samples of day 0, 14, 21 of all treatments.

Microbial group	Time (days)	NA	BA	BS	BAS
Bacteria (16S rDNA genes)	0	<b>1.79 ± 0.08</b>	<b>1.79 ± 0.08</b>	<b>1.79 ± 0.08</b>	<b>1.79 ± 0.08</b>
	7	<b>2.20 ± 0.07</b>	<b>2.23 ± 0.04</b>	<b>2.08 ± 0.16</b>	<b>2.11 ± 0.4</b>
	14	<b>2.23 ± 0.10</b>	<b>2.11 ± 0.27</b>	<b>2.41 ± 0.07</b>	<b>2.37 ± 0.24</b>
	21	<b>2.29 ± 0.27</b>	<b>2.01 ± 0.03</b>	<b>2.37 ± 0.24</b>	<b>1.81 ± 0.15</b>
<i>Rhodococcus</i> ( <i>alkB</i> genes)	0	<b>2.53 ± 0.07</b>	<b>2.53 ± 0.07</b>	<b>2.53 ± 0.07</b>	<b>2.53 ± 0.07</b>
	7	<b>2.94 ± 0.18</b>	<b>2.82 ± 0.07</b>	<b>2.79 ± 0.13</b>	<b>2.82 ± 0.04</b>
	14	<b>2.76 ± 0.13</b>	<b>2.86 ± 0.06</b>	<b>2.57 ± 0.06</b>	<b>2.71 ± 0.19</b>
	21	<b>2.62 ± 0.2</b>	<b>2.87 ± 0.08</b>	<b>2.75 ± 0.22</b>	<b>2.51 ± 0.1</b>
Fungi ( <i>ITS</i> genes)	0	<b>2.23 ± 0.38</b>	<b>2.23 ± 0.38</b>	<b>2.23 ± 0.38</b>	<b>2.23 ± 0.38</b>
	7	<b>1.67 ± 0.04</b>	<b>1.96 ± 0.77</b>	<b>2.5 ± 0.01</b>	<b>1.86 ± 0.99</b>
	14	<b>2.04 ± 0.16</b>	<b>2.15 ± 0.18</b>	<b>2.3 ± 0.12</b>	<b>1.98 ± 0.85</b>
	21	<b>2.24 ± 0.51</b>	<b>1.86 ± 0.41</b>	<b>2.14 ± 0.15</b>	<b>2.39 ± 0.35</b>

NA- Natural attenuation, BA- bioaugmentation, BS-biostimulation and BAS-combined bioaugmentation and biostimulation.

Data shown in means and standard errors of means. Figures in bold are the highest diversity values obtained for each particular amendment and gene.

carbons. Natural attenuation by mixing and watering may be the cost-efficient and process-efficient bioremediation option for complex hydrocarbon contaminated soils that have been left to stand for long periods unturned.

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