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Bioremediation potential of diesel-contaminated Libyan soil

Eman Koshlaf [a,b], Esmaeil Shahsavari [a], Arturo Aburto-Medina [a], Mohamed Taha [a,c], Nagalakshmi Haleyur [a], Tanvi H. Makadia [a], Paul D. Morrison [a], Andrew S. Ball [a,*]

[a] Centre for Environmental Sustainability and Remediation, School of Science, RMIT University, Bundoora, Victoria 3083, Australia
[b] Department of Biology, Faculty of Science Algabal Algarbi University, Gharian, Libya
[c] Department of Biochemistry, Faculty of Agriculture, Benha University, Moshtohor, Toukh 13736, Egypt

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ABSTRACT

Bioremediation is a broadly applied environmentally friendly and economical treatment for the clean-up of sites contaminated by petroleum hydrocarbons. However, the application of this technology to contaminated soil in Libya has not been fully exploited. In this study, the efficacy of different bioremediation processes (necrophytoremediation using pea straw, bioaugmentation and a combination of both treatments) together with natural attenuation were assessed in diesel contaminated Libyan soils. The addition of pea straw was found to be the best bioremediation treatment for cleaning up diesel contaminated Libyan soil after 12 weeks. The greatest TPH degradation, 96.1% (18,239.6 mg kg^{-1}) and 95% (17,991.14 mg kg^{-1}) were obtained when the soil was amended with pea straw alone and in combination with a hydrocarbonoclastic consortium respectively. In contrast, natural attenuation resulted in a significantly lower TPH reduction of 76% (14,444.5 mg kg^{-1}). The presence of pea straw also led to a significant increased recovery of hydrocarbon degraders; 5.7 log CFU g^{-1} dry soil, compared to 4.4 log CFU g^{-1} dry soil for the untreated (natural attenuation) soil. DGGE and Illumina 16S metagenomic analyses confirm shifts in bacterial communities compared with original soil after 12 weeks incubation. In addition, metagenomic analysis showed that original soil contained hydrocarbon degraders (e.g. *Pseudoxanthomonas* spp. and *Alcanivorax* spp.). However, they require a biostimulant (in this case pea straw) to become active. This study is the first to report successful oil bioremediation with pea straw in Libya. It demonstrates the effectiveness of pea straw in enhancing bioremediation of the diesel-contaminated Libyan soil.

1. Introduction

Soil pollution as a result of contamination with petroleum hydrocarbons represents a serious global issue. The extent of hydrocarbon contamination in the environment is not surprising given the amount of oil used and transported around the world. Eighty four million barrels of crude oil are consumed around the world per year, almost 50% of which is transported by sea which leads to the increased chance of oil tanker accidents and in turn large-scale water and soil pollution (Hasan et al., 2010; McKew et al., 2007; Rhodes, 2010).

Diesel is one of the most commonly found hydrocarbons in the environment, consisting of alkanes and aromatic compounds which can be released during storage and transportation (Gallego et al., 2001). According to the Oil and Gas Journal and the Organization of the Petroleum Exporting Countries (OPEC), Libya has the largest proven oil reserves in Africa, with production running at 1.65 million barrels per day of high quality crude oil in 2010 and gross proven oil reserves of 47.1 billion barrels in 2012. Such a large production has environmental consequences too since Libya has already faced one of the largest oil spills in world history when 59 million litres of oil were released in an area southeast of the capital city Tripoli, with the spill covering about 800 km^2 of Libyan soil (O'Rourke and Connolly, 2003).

Traditional physico-chemical methods such as soil washing, soil vapour extraction, incineration, the use of oil booms and solidification have been used for the clean-up of oil contaminated sites; however they are disruptive, labour intensive and relatively expensive processes (Huang et al., 2004; Tang et al., 2010). Over the past twenty years, there has been an increasing global interest in the field of bioremediation due to the limitation of landfills and the growing remediation costs. Among strategies for hydrocarbon contamination management, bioremediation has received considerable attention. One strategy to improve the efficiency of bioremediation processes is the introduction of highly specialized

* Correspondence to: School of Science, RMIT University, Bundoora, Victoria 3083, Australia.
E-mail address: andy.ball@rmit.edu.au (A.S. Ball).

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microorganisms into the polluted environment. Inoculating the soil with contaminant degrading microbes is generally known as bioaugmentation (Barathi and Vasudevan, 2003; Wu et al., 2008; Wu et al., 2011). Bioaugmentation has been successfully employed in the field of environmental pollutant mineralization through inoculation of the affected soil with exogenous hydrocarbon utilizing microbial strains (bacteria or fungi) to enhance the uptake of contaminants. This method is mainly beneficial when the abundance of relevant catabolic genes (such as the *alkB* alkane hydroxylase gene) among the native microbial community is insufficient (Vomberg and Klinner, 2000).

Some of the hydrocarbonoclastic microorganisms reported include members of the *Pseudomonas*, *Acinetobacter*, *Alcaligenes, Brevibacillus* and *Bacillus* genera; all have been listed as among the most important hydrocarbon degraders in marine and soil environments (Leahy and Colwell, 1990). Among the *Bacillus* genera, several *Bacillus* strains have been reported to degrade diesel oil (Bento et al., 2005; Ghazali et al., 2004), crude oil (Das and Mukherjee, 2007), phenanthrene (Doddamani and Ninnekar, 2000), naphthalene (Tuleva et al., 2005) and benzene (Aburto-Medina and Ball, 2015; Dou et al., 2010) among other hydrocarbons (Cooper and Goldenberg, 1987; Menezes Bento et al., 2005, Morán et al., 2000).

Successful application of bioaugmentation is reliant on the subsequent survival and activity of the degrading strains once introduced into the target habitat. An alternative approach, which has also increased the chances of successful bioremediation by maintaining high rates of microbial adaption, persistence and activity, has been the use of plant biomass (Shahsavari et al., 2015, 2013b). Plant residues such as hay and straw are among the cheapest and most plentiful agricultural waste products in the world, with an estimated annual production of more than 2900 million tonnes (Sun et al., 2004) and they have been successful used in the degradation of petroleum hydrocarbons (Barathi and Vasudevan, 2003; Rojas-Avelizapa et al., 2007; Shahsavari et al., 2013a; Zhang et al., 2008).

Often in contaminated soils, nutrients, aside from C are depleted. Therefore in order to increase the efficiency of bioremediation, the addition of nutrients such as nitrates and phosphates to enhance the growth of hydrocarbonoclastic microbes is crucial (Mohan et al., 2008) and this is termed biostimulation (Molina-Barahona et al., 2004). In recent decades, biostimulation together with bioaugmentation, necrophytoremediation and phytoremediation technologies have become valuable alternatives to physical and chemical treatments. The advantages of these biological treatments include low cost, ease of implementation, environmentally friendliness, applicability over large areas, and often results in the complete mineralization of the contaminant (Guo et al., 2014).

The microbial communities in the soil are the main driver for the degradation of contaminants; therefore, assessment of the microbial communities during a bioremediation study is highly desirable. Various methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA) or automated RISA (ARISA) have been developed and used for assessments of microbial communities in soils. Recently next generation sequencing (NGS) has become a popular technique since it provides the largest amount of data (up to one billion short reads per run) with a relatively low cost (Metzker, 2010; Schuster 2007). Therefore, this technique is ideal for the identification of the microbial community.

While several studies have assessed a combination of bioaugmentation and biostimulation worldwide (Calvo et al., 2009; Coulon et al., 2010; Grace Liu et al., 2011; Kauppi et al., 2011, Łebkowska et al., 2011; Sheppard et al., 2011; Zhao et al., 2011), studies in regard to bioremediation technology in Libyan soils are limited and there exists a lack of information (Mansur et al., 2014; Shaieb et al., 2015). In addition, our knowledge about the microbial communities in these types of soils remains low. High-throughput sequencing or NGS not only gives us valuable information about microbial communities in Libyan soils but also underpins any bioremediation process by providing essential information on the diversity and activity of the soil microbial community during the degradation of petroleum hydrocarbons.

Therefore, the aim of this study was to provide an assessment of the potential for cheap and readily available bioremediation technologies for the remediation of petroleum contaminated Libyan soil. Four different approaches including natural attenuation, necrophytoremediation (addition of pea straw), bioaugmentation (addition of a hydrocarbonoclastic bacterial consortium including several *Bacillus*) and a combination of necrophytoremediation and bioaugmentation were evaluated on the degradation of diesel contaminated soil. Moreover the microbial community was elucidated by metagenomic analysis.

2. Materials and methods

2.1. Soil sample collection

The petroleum hydrocarbon contaminated soil used in this study was collected from the top layer (0–15 cm; 20 kg) of a diesel contaminated site in Libya. The original soil contamination resulted from an oil pipeline leak from the main oil reservoirs in Tripoli, Libya. The initial level of contamination, in terms of Total Petroleum Hydrocarbon (TPH) was 18,966 mg kg^{-1} dry soil.

The soil was imported to RMIT University, Melbourne, Australia, coded and stored in a quarantine facility at the university. Prior to use, stones were manually removed from the soil, and the samples were passed through a sieve (4 mm). Plant residue (pea straw) was kindly donated by Johnson's Stockfeed and Horticultural Products Co. (Australia). The straw was chopped using a blender to small pieces (2 mm) prior to use.

2.2. Physico-chemical analysis

The soil was analysed for soil texture, moisture content, pH and water-holding capacity (WHC) using methods previously described (Rayment and Higginson, 1992) (Table 1). The percentage of organic carbon (C), nitrogen (N) and hydrogen (H) was analysed (Chemical Analysis Facility, Macquarie University) using a model LECO TruMac CNS analyser following the manufacturer's instructions. The concentration of different elements including Ca, K, Mg Fe, P, S and Zn in the soil samples were determined using, x-ray fluorescence spectrometry following the method previously described (Norrish and Hutton, 1969) (Table 1).

The concentrations of heavy metals in the tested soil were analysed using inductively coupled plasma mass spectrometry (ICPMS) (Varian Model Spectra AA 220) as per the manufacturer's protocol. Briefly, using a hot block, soil samples were digested with HNO_3 (5 ml, 65–70%) and hydrogen peroxide (5 ml, 30% v/v) at 60 °C for 1 h. Soil samples were further heated at 120 °C for 5 h. The tubes were then cooled at room temperature; the solutions were filtered through No. 1 filter paper.

2.3. Preparation of microbial consortia

A microbial community with previously assessed hydrocarbonoclastic activity was selected for use in this study. The hydrocarbonoclastic microorganisms used in this study were *Bacillus lentus* A5019 ST, *Bacillus megaterium* B 5013, *Bacillus pumilus* C

Table 1
Chemical and physical properties of the contaminated Libyan soil used in the study.

Soil properties	Values
Organic carbon content (%)	0.80
Nitrogen (%)	0.03
Available H (%)	0.08
Soil pH	7.5
Moisture content (%)	12.5
Water holding capacity (%)	51
Organic matter content (%)	8.0
Soil texture	Sandy loam
Sand (%)	80
Clay (%)	2.5
Silt (%)	7.5
Initial TPH (mg kg^{-1})	18,966
Ca (mg kg^{-1})	91,100
K (mg kg^{-1})	49,200
Fe (mg kg^{-1})	31,800
P (mg kg^{-1})	18,300
Mg (mg kg^{-1})	4500
S (mg kg^{-1})	2900
Zn (mg kg^{-1})	230
Heavy metals (mg kg^{-1})	All less than 7

5011S T, *Bacillus cereus* C5014, *Bacillus subtilis* C601/1, *Bacillus cereus* C603, *Bacillus sphaericus* C605 and *Bacillus cereus* C6011. These bacteria were isolated from a biological trickling filter WWTP (biofilter) treating phenol and petroleum hydrocarbon-contaminated wastewater using the method of Zhao et al. (2011). The hydrocarbonoclastic activity of these isolates were confirmed using Biolog MT2 plates as described previously (Mansur et al., 2014). All the isolates were capable of degrading both diesel and pyrene as the sole sources of carbon and energy. However, importantly their combined activity in regard to degradation of hydrocarbons was greater than that of any single isolate.

Individual *Bacillus* strains were streaked onto nutrient agar plates and incubated at 30 °C for 24 h. Following growth, individual colonies were inoculated into nutrient broth and incubated at 30 °C for 48 h. Cultures were then harvested by centrifugation at 4 °C at 5000 rpm for 5 min and washed twice with NaCl (0.85%). Finally, the pellets were re-suspended individually in NaCl (0.85% w/v; 5 ml). Cultures were mixed prior to use in the bioremediation experiments (Cerqueira et al., 2011; Xu and Lu, 2010). OD_{600} was normalised spectrophotometrically (final $OD_{600}=1.4$) and inoculated bacteria was enumerated using a plate counting method.

2.4. Soil microcosm and inoculation conditions

Replicated soil microcosms (n =3) each containing soil contaminated with petroleum hydrocarbons. (250 g) were prepared for four treatments, including natural attenuation and three other bioremediation trials run over 12 weeks. The four treatments included:

I) Bioaugmentation treatment in which hydrocarbonoclastic *Bacilli* (1.8×10^6 CFU g^{-1} dry soil) were added (BA). II) Necrophytoremediation treatment amended with pea straw (3% w/w)(PS).

III) Bioaugmentation and necrophytoremediation treatment containing both the *Bacilli* consortium (1.8×10^6 CFU g^{-1} dry soil) and pea straw (3% w/w) (BAPS).

IV) Natural attenuation (NA).

The water holding capacity (WHC) of all microcosms was adjusted and maintained at 50% throughout the incubation. All microcosms were sampled (20 g) at weeks 0, 4, 8, and 12 for the extraction of DNA and subsequent molecular analysis and determination of the concentration of total petroleum hydrocarbon (TPH). The remaining soil samples were stored at −20 °C for further analyses.

2.5. Total petroleum hydrocarbon analysis

Total petroleum hydrocarbons (TPH) present in the soil was extracted using hexane via a slightly modified solvent extraction method to that previously described (Shang et al., 2014; Tang et al., 2010). Total petroleum hydrocarbons (TPH) analyses were performed during the study in triplicate for each set of microcosms, with results expressed per g of dry soil. Residual TPH was determined at 0, 4, 8 and 12 weeks of treatment. Extracts were analysed using comprehensive two-dimensional gas chromatography (6890 Agilent Technologies, J & W Scientific Products, USA), fitted with a reverse fill/flush differential flow modulator. An aliquot (1 μl) of sample was injected in split mode (10:1) using an Agilent 7683 autosampler. Hydrogen (H_2) was used as a carrier gas; the column flow was 0.3 ml min^{-1} on column number one (Agilent DB-5MS, 10 m long, 100 μm internal diameters with a film thickness of 0.1 μm). The column flow rate was 24 ml min^{-1} on column number two (Supelco-IL100, 4 m long, 250 μm internal diameter with a film thickness of 0.2 μm).

The split/splitless injector and flame ionization detector (FID) temperatures were maintained at 230 °C. The initial oven temperature of 60 °C was held for 0.2 min before ramping to a final temperature of 230 °C at 10 °C /min where it was maintained for 2.8 min. The modulation period was 2 s (1.9 s fill, 0.1 s flush) GC Chemstation (Rev B.04.01) was used for instrument control and data acquisition. The data acquisition rate was 200 samples per second (200 Hz). Time vs. response data was exported from GC Chemstation and imported into GC Image multi-dimensional data analysis software (Ver 2.5).

2.6. Microbial counts and total microbial activity

Microbial cell numbers were estimated using the dilution plate-counting technique as previously described (Olsen and Bakken, 1987). Briefly, soil (1 g) was suspended in 9 ml of sterilized water and vortexed for 30 min. A tenfold dilution of the soil suspension was prepared in distilled water. Samples were homogenized for 30 min on a rotary shaker (250 rpm). Aliquots (0.1 ml samples) from each serial dilution was spread onto nutrient agar plates for bacterial growth and potato dextrose plates for fungi, and subsequently incubated at 37 °C for 2 days (bacteria) and for 15 days (fungi). The number of developed colonies were calculated and expressed as colony forming units (CFU) per gram dry soil.

To estimate the numbers of diesel fuel-degrading microorganisms, a modified five well most probable number (MPN) technique was used. Bushnell–Hass liquid media containing petroleum hydrocarbon (sterilized diesel, 100 μl) as the sole carbon and energy source was added to 24-well microtiter plates (Gaskin and Bentham, 2005; Gaskin et al., 2008).

Total microbial activity of the different soil treatments at different time points were determined using the fluorescein diacetate (FDA) hydrolysis method as previously described (Margesin and Schinner, 2005).

2.7. DGGE analysis

Soil microbial DNA was extracted from contaminated soil samples (0.25 g) from weeks 0, 4, 8 and 12 in triplicate using a Power Soil™ DNA extraction kit (MoBio Laboratories Inc., Carlsbad, California) according to the manufacturers' instructions. The total soil bacterial community was evaluated by PCR using universal primers of 16S rDNA using the primer set 341F (5′CCTACGGGAGGCAGCAG3′) with GC clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG)

and 518R (5′ ATTACCGCGGCTGCTGG3′). These two primers were used in this study based on our previous studies (Makadia et al., 2011; Shahsavari et al., 2013b; Simons et al., 2012).

Denaturing Gel Gradient Electrophoresis (DGGE) analysis was performed using the Universal Mutation Detection System (BioRad) as described by Sheppard et al. (2011). In brief, PCR products were loaded on 9% (w/v) polyacrylamide gels with a denaturing gradient of 45–60% using Universal Mutation Detection System D-code apparatus (Bio-Rad, CA, USA). The gel was run in 1 × TAE running buffer at 60 °C at constant voltage of 60 V for 20 h. After electrophoresis, the DGGE gels were silver stained (Girvan et al., 2003), scanned and digital images saved as TIFF files using an EPSON Expression 1600 V.2.65 E scanner.

2.8. Metagenomic analysis

In regard to metagenomic analysis, the library was prepared using Nextera® XT Index Kit (Illumina, San Diego, CA) as outlined in 16S Metagenomic Sequencing Library Preparation guide (Illumina). The DNA from the library was quantified using Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The samples were pooled and run in a MiSeq platform (Illumina, San Diego, CA) at School of Science, RMIT University.

2.9. Statistical analysis

Multiple data was analysed using a one-way ANOVA sliced by time using IBM SPSS software (version 22). Data were considered significant at P=0.05. Mean values were separated using the Least Significant Difference (LSD) test, where the F-value was significant. All error bars shown in the figures represent one standard deviation. Dendrograms were produced by cluster analysis of DGGE bands on the presence or absence at a specific location on the gel by using Phoretix 1D. The similarity relationship between the different microbial communities were analysed by the Unweighted Paired Group with Arithmetic Averages (UPGMA) method. In regard to metagenomic data, the obtained sequences were quality trimmed, filtered and processed using Quantitative Insights In to Microbial Ecology (QIIME) package available in Illumina Basespace (Caporaso et al., 2010). The output data obtained from QIIME were used for principal coordinate analysis (PCoA) using XLSTAT software.

3. Results and discussion

3.1. Total petroleum hydrocarbon degradation

A summary of the effect of different bioremediation treatments on the degradation of total petroleum hydrocarbon (TPH) is shown in Fig. 1. All microcosms showed a significant decrease in TPH by week 15. However, natural attenuation and microcosms amended with the *Bacillus* were the least effective with an average TPH loss of 14,444 mg kg^{-1} and 14,991 mg kg^{-1} respectively, compared to the initial TPH concentration (18,966 mg kg^{-1}) while the highest degradation was found in microcosms amended with pea straw (18,240 mg kg^{-1}) followed by microcosms amended with pea straw combined with *Bacillus* (17,991 mg kg^{-1}) (Fig. 1).

To the authors best knowledge this is one of the first reports on the potential use of this bioremediation method on Libyan TPH contaminated soil. In terms of TPH reduction, all 4 remediation protocols led to a significant reduction in the levels of soil contamination during the 12 week study. However, there were significant differences in the impact of the treatment on the remediation; pea straw, with and without the addition of the eight

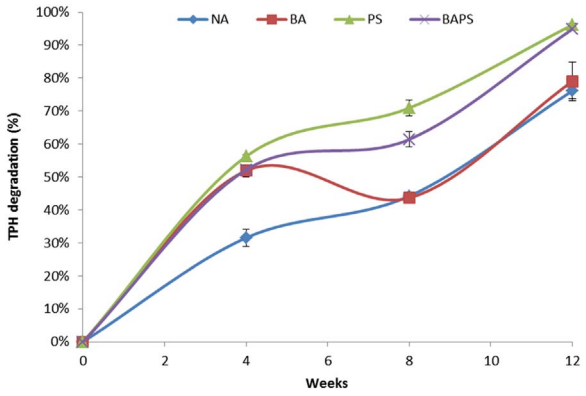

Fig. 1. Reduction of TPH concentration in mesocosms containing contaminated Libyan soil amended with pea straw (PS), a *Bacillus* consortium (BA), pea straw +*Bacillus* consortium (BAPS) and natural attenuation (NA) treatments over 12 weeks incubations (mean ± SD, n=3).

hydrocarbonoclastic *Bacilli* was found to be the most effective bioremediation treatment, resulting in 96.1% degradation of TPH over the 12 week incubation.

Plant residues (dead plant biomass) such as straw represent one of the most inexpensive and plentiful resources in the world. These materials contain microbes that not only can be contaminant degraders but also promote the biodegradation of contaminants in soils by providing essential nutrients to achieve effective bioremediation (Zhang et al., 2008). The reduction in TPH observed in soils amended with pea straw obtained in this study was 96.1% (18,239.6 mg kg^{-1}) and 95% (17,991.14 mg kg^{-1}) for necrophytoremediation and bioaugmentation respectively; these values far exceed some previously reported values, even for other plant residues and support the potential of the approach for the treatment of petroleum-contaminated Libyan soils. Wu et al. (2011) reported that the addition of 5% (v/v) wheat straw to petroleum- contaminated soil led to increased degradation (56%) in contaminated soils augmented with *Enterobacter cloacae* compared to 25% degradation in the control soil after 8 weeks of incubation. Similarly Shahsavari et al. (2013a) showed that phenanthrene and pyrene degradation was significantly accelerated when the PAH-contaminated soil was amended with pea and wheat straws. In addition, the impact of hay, pea, wheat and other types of plant residues on the degradation of aliphatic hydrocarbons was investigated. The results demonstrated that the addition of plant residues to contaminated soil led to statistically significant increases in the degradation of TPHs in the soil compared with those obtained in control soil. For example, 83% and 70% of TPH degradation occurred in soil mixed with pea straw and mixed residues respectively over 90 day compared to only 57% degradation in control soils (Shahsavari et al., 2013b).

3.2. Microbial activity (fluorescein diacetate (FDA) hydrolytic activity) and microbial enumeration

In the current investigation the impact of treatments on the soil microbial community was studied. A most probable number (MPN) technique was used to determine the impact of the bioremediation treatments on the number of hydrocarbonoclastic organisms present in the soil (Fig. 2a). In addition, the overall microbial population including bacteria and fungi were detected using standard plate counts (Fig. 2b and c). MPN results of TPH utilizing microbes illustrated that the application of pea straw led to a ~15 fold increase in the population of petroleum hydrocarbon utilizing microorganisms after 12 weeks of treatment compared to untreated soil. This may account for the high rates of TPH

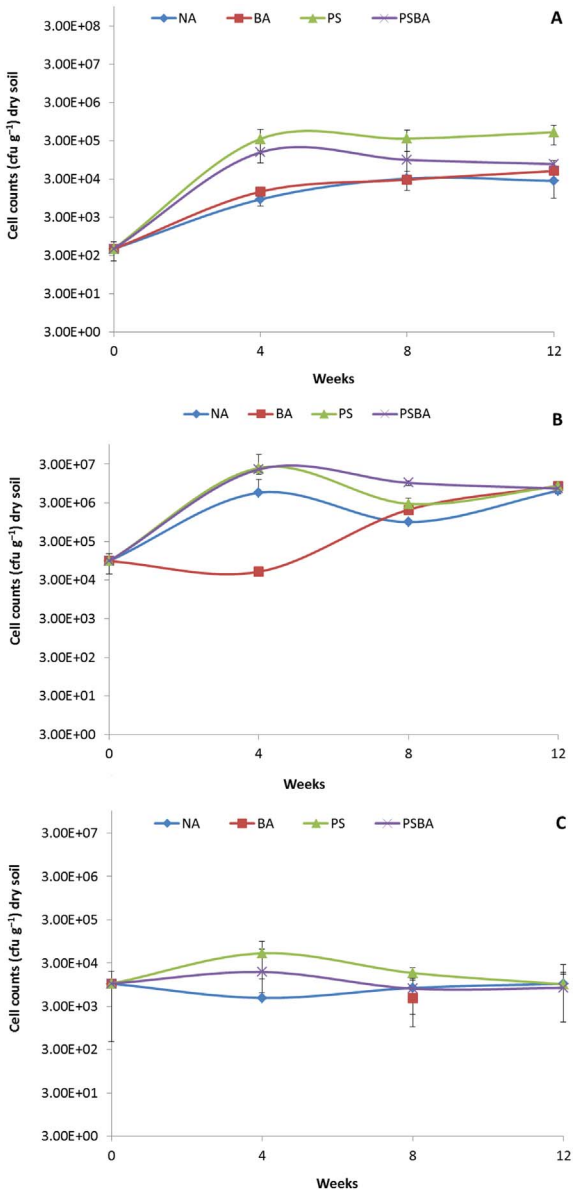

Fig. 2. Microbial abundance (colony-forming units (CFU^{-1} dry soil) of hydrocarbon degraders (a), total bacteria (b), fungi (c) in diesel-contaminated soil amended *Bacillus* consortia (BA), pea straw (PS), a mixture of both (BAPS) and without any addition (NA) during 12 weeks of bioremediation (mean ± SD, n=3).

degradation observed in these soils (Fig. 1). More specifically, the presence of pea straw (3% w/w) enabled an increase in the number of hydrocarbonclastic organisms, 5.7 log CFU g^{-1} dry soil, compared to numbers present in the untreated soil (natural attenuation), 4.4 log CFU g^{-1} dry soil (Fig. 2a). After 4 weeks of incubation, the overall number of bacteria was relatively low in natural attenuation and microcosms amended with the *Bacillus* consortium, compared to the treatments in which pea straw was used as an amendment for the contaminated soil (Fig. 2b).

By the end of the treatment, plate counting showed no significant difference in the total bacterial number between the amended soils (between 6.9 log CFU g^{-1} dry soil and 6.8 log CFU g^{-1}dry soil) and untreated soil (6.7 log CFU g^{-1}dry soil). Similarly, in terms of fungi, there was no significant difference in fungal concentration between the natural attenuation experiment (4 log CFU g^{-1} dry soil) and the amended treatments (3.9 log CFU g^{-1}dry soil) (Fig. 2c).

Fluorescein diacetate [30, 60-diacetylfluorescein (FDA)] activity

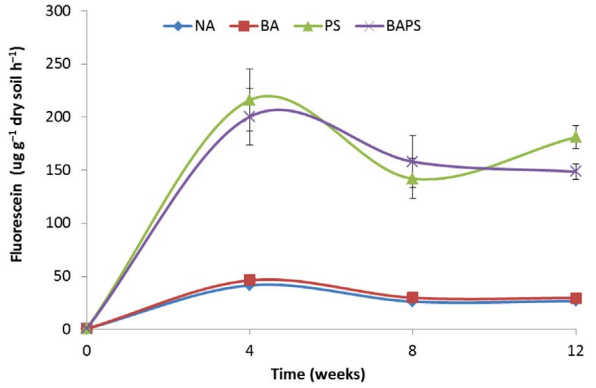

Fig. 3. Fluorescein diacetate (FDA) hydrolytic activity in diesel-contaminated soil amended with the *Bacillus* consortia (BA), pea straw (PS), a mixture of both (BAPS) and without any addition (NA) during the 12 weeks of bioremediation (mean ± SD, n=3).

was used as a biological indicator to monitor the overall activity of the soil microbial community (Fig. 3). FDA hydrolytic activity remained constant and relatively low in NA and BA treatment groups throughout the treatment period (Fig. 3). After 12 weeks of incubation, the highest fluorescein diacetate activity was obtained in microcosms amended with pea straw, (185.2 μg fluorescein g^{-1} dry soil min^{-1}), followed by the microcosm inoculated with the BAPS treatment (148.5 μg fluorescein g^{-1}dry soil min^{-1}). The average enzyme activity of the NA and BA treatments were significantly lower than that observed in PS and BAPS treatments, with an average of, 26.6 and 29.6 μg fluorescein g^{-1}dry soil min^{-1} respectively.

3.3. Evaluation of the bacterial communities

Having observed a significant increase in the hydrocarbonoclastic community in microcosms amended with pea straw, DGGE analysis in combination with 16S metagenomics was conducted to assess and compare the structure and diversity changes of the bacteria community in the treated soil using different amendments. The DGGE profiles of both bacterial communities demonstrate shifts in community structures among the samples collected in 0, 4, 8 and 12 weeks of incubation (Fig. 4).

The DGGE profiles show an increasing order of complexity from natural attenuation and bioaugmentation microcosms through to the microbial communities in microcosms amended with pea straw, with approximately 16 distinct bands found in NA and BA samples and approximately 22 bands found in pea straw amended samples (Fig. 4). UPGMA analysis showed that the initial time (day 0) communities formed a separate cluster distinct from NA, BA and BAPS communities in both bacteria and fungi (Fig. 4). Generally, the dendrograms resulting from bacterial and fungal genes showed that the clusters were largely time related rather than treatment related. For example cluster 1 contained all treatments sampled at week 12 along with the control (NA), while cluster 2 contained all treatments sampled at week 8 along with the control and BAPS treatment for week 4. BA (soil +*Bacillus* strains), PS (soil + pea straw) and natural attenuation treatment formed the third cluster. Cluster 4, original soil showed only 40% similarity to the other samples. Within each cluster, banding patterns revealed that the bacterial communities in microcosms under different treatments were distinct and exhibited no more than 65% similarity to the (NA) community.

Metagenomic analysis also showed significant changes in the bacterial community in all treatments compared to original soils, confirming that incubation or addition of pea straw led to changes in the bacterial communities (Fig. 5). Three main community

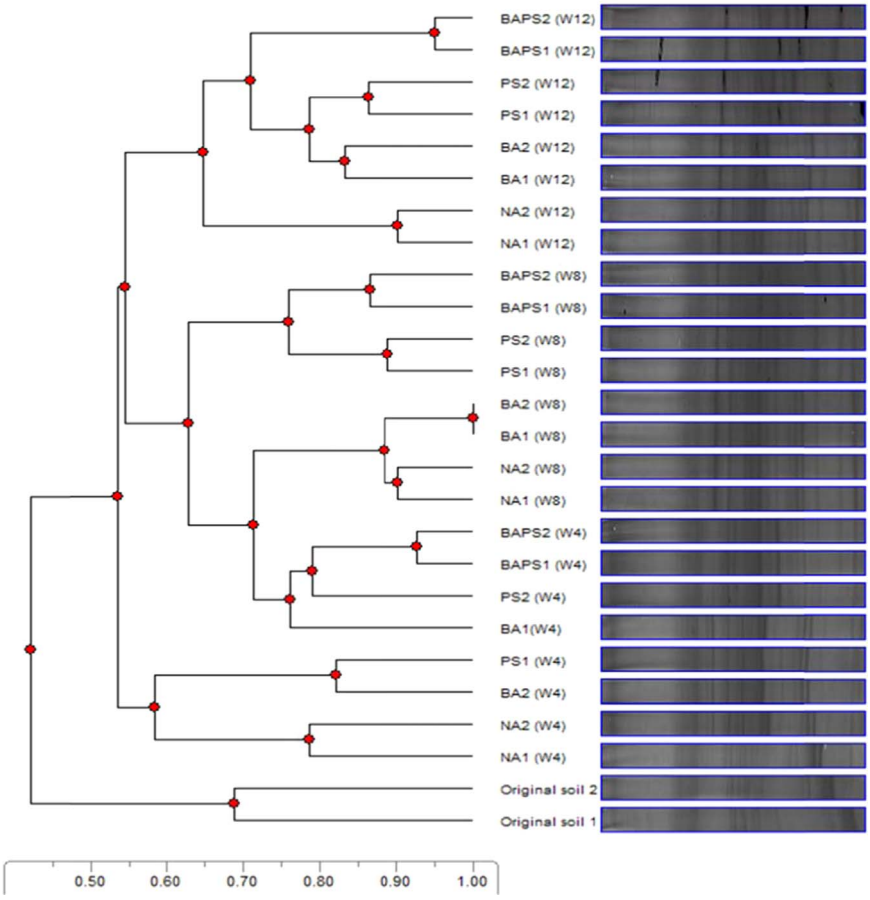

Fig. 4. Cluster analysis of bacterial community using UPGMA method and band location obtained from DGGE profiles. Original soil: (control), NA: natural attenuation, PS: contaminated soil + pea straw, BA: contaminated soil +*Bacillus*, BAPS: contaminated soil + pea straw +*Bacillus*.

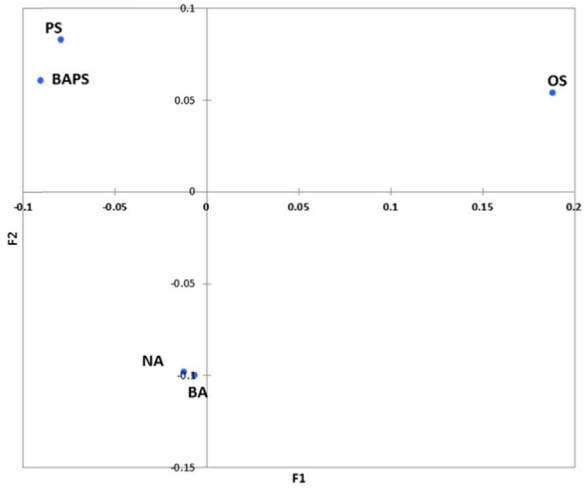

Fig. 5. Principal coordinate analysis (PCoA) of bacterial community assessed with 16S rDNA metagenomic analysis. OS: Original soil, NA: natural attenuation, PS: contaminated soil + pea straw, BA: contaminated soil +*Bacillus*, BAPS: contaminated soil + pea straw +*Bacillus*.

groups were observed, i: original soil, ii: NA and BA treatments, iii: PS and BAPS. Illumina 16S rDNA metagenomic bacterial abundance is shown in Fig. 6. The results indicated that the Gammaproteobacteria were the dominant class in all the treatments and the original soil OS (day 0), especially in NA and BA where they comprise approximately 75% of the total community. Alphaproteobacteria were the second largest group in all the treatments, with a higher proportion in PS and BAPS compared to OS, NA and BA. Betaproteobacteria was the third most abundant class in the OS while the Actinobacteria were present in both the BS and BAPS treatments. Within the Gammaproteobacteria, *Pseudoxanthomonas* was the dominant genus in the OS treatment, followed by *Alcanivorax*, while the latter was the dominant group in BS and BAPS. Although no specific genus was detected for treatments NA and BA they were also dominated by members of the Gammaproteobacteria.

Pseudoxanthomonas mexicana has been observed in BTEX contaminated groundwater (Aburto et al., 2009) and the strain *Pseudoxanthomonas* RN402 has been shown to be able to remove diesel oil (Nopcharoenkul et al., 2012). Moreover, other *Pseudoxanthomonas* strains such as *Pseudoxanthomonas kahosiungensis* that produces extracellular surface activity was also isolated from an oil polluted site (Chang et al., 2005); strain PN04 produces a biosurfactant (Nayak et al., 2009) and strain DMVP2 can degrade phenanthrene (Patel et al., 2012).

Alcanivorax has been found to degrade several alkanes (Dastgheib et al., 2010; Liu et al., 2011; Makadia et al., 2011; Qiao and Shao,ahul et al., 2014). Within the Alphaproteobacteria, the genus *Parvibaculum* dominates in the BS and BABS treatments while members of the Sphingomonadaceae were the dominant microorganisms in OS, BA and NA. *Parvibaculum lavamentivorans* catabolizes linear alkylbenzenesulfonate (Schleheck et al., 2004). Within Betaproteobacteria, only the genus *Candidimonas* was detected in OS and NA. *Candidimonas* has been detected in an alpine hydrocarbon-contaminated site (Hemala et al., 2014).

In the present study, the impact of four bioremediation strategies (bioaugmentation, necrophytoremedation, bioaugmentation +

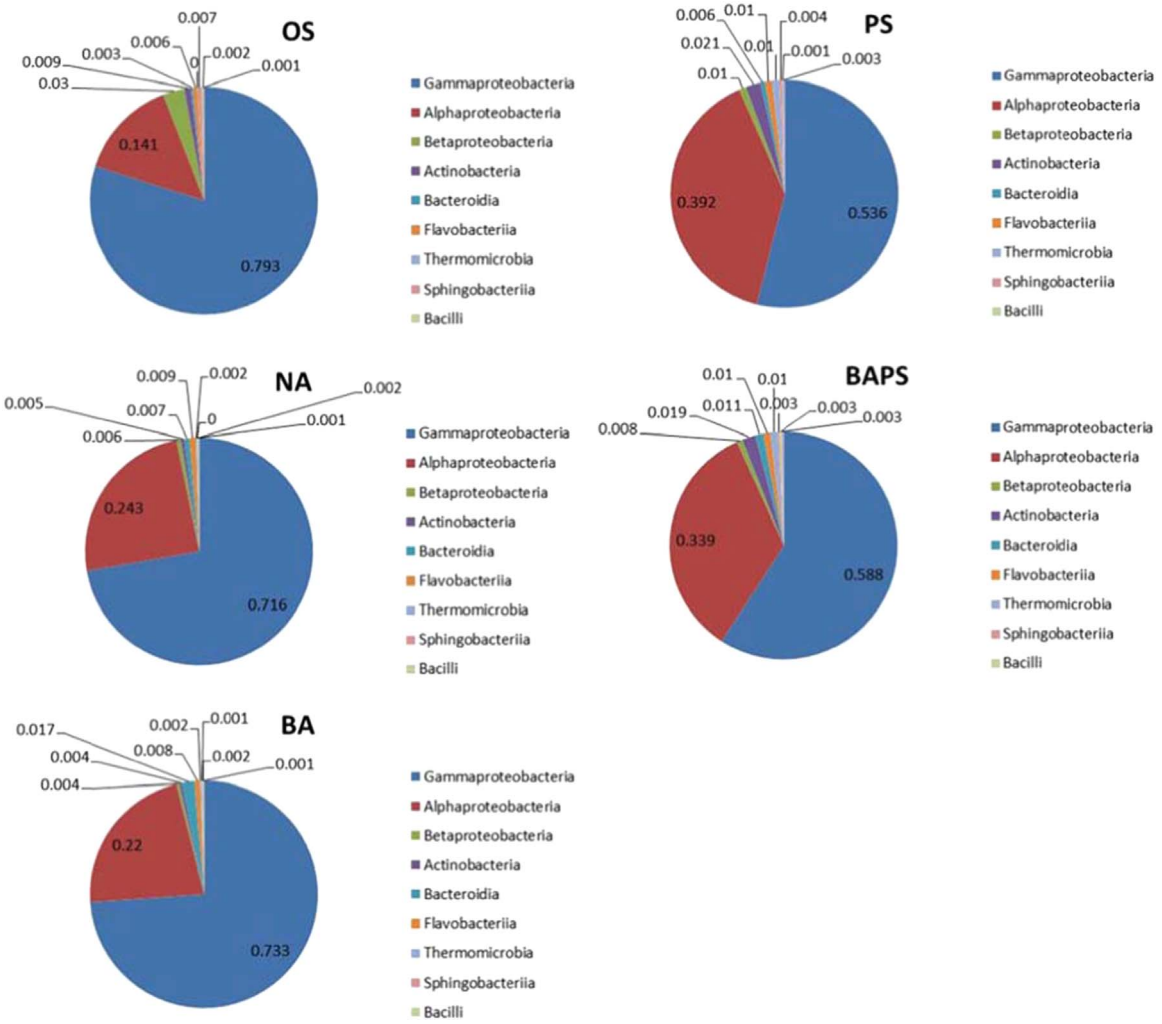

Fig. 6. Class abundance of the soil bacterial community in diesel-contaminated soil in different treatments. OS: Original soil, NA: natural attenuation, PS: contaminated soil + pea straw, BA: contaminated soil +*Bacillus*, BAPS: contaminated soil + pea straw +*Bacillus*.

necrophytoremedation and natural attenuation) on the remediation of petroleum hydrocarbon impacted Libyan soil was investigated together with the effect of the treatments on the activity and diversity of the soil microbial community. Many microorganisms (isolated from bulk soils) including *Pseudomonas*, *Flavobacterium, Achromobacter*, *Corynebacterium, Mycobacterium, Arthrobacter*, *Nocardia, Bacillus, Alcaligenes* and *Micrococcus* have been reported as hydrocarbon degrading bacteria (Germida et al., 2002). While considerable information on the important role of Bacilli in the biodegradation of organic material when applied to the soil as a consortium mixed with other bacterial or fungal species is available in the literature (Ghazali et al., 2004, Łebkowska et al., 2011; Nwaogu et al., 2008), in this study the Bacillus consortia showed a relatively weak response in terms of enhancing biodegradation relative to natural attenuation. This suggests that the activity of the inoculated bacteria in the soil was limited. Metagenomic analysis also confirmed that the abundance of Bacilli species were low in treatments amended with the Bacillus consortia (BA and BAPS).

These findings are not in agreement with those results reported by Kebria et al. (2009). The authors indicated that indigenous Bacilli displayed considerable biodegradation potential when applied to both low (500 ppm) and high concentrations (10,000 ppm) of diesel in soil. In another study, Zhao et al. (2011) tested the effect of a selected consortium including *Bacillus* spp., *Rhizobiales* spp., *Pseudomonas* spp., *Brucella* spp., *Rhodococcus* spp., *Microbacterium* spp. and *Roseomonas* spp.) and the addition of nutrients (N and P) on biodegradation of hydrocarbon-contaminated soil. Bioaugmentation with the consortium significantly enhanced hydrocarbon degradation (> 50% degradation) compared to controls (8–13% degradation). In contrast, the addition of nitrogen and phosphate limited the degradation process (Zhao et al., 2011).

Similar results were obtained by Łebkowska et al. (2011) using bioaugmentation with different bacterial strains and biostimulation using a biosurfactant. The application of isolated bacterial strains including two *Bacillus* species (one producing biosurfactants), *Pseudomonas mendocina and Pseudomonas putida* resulted in 81.98% degradation of diesel oil and engine oil in aged contaminated soil over 25 days (Łebkowska et al., 2011). In another site, bioaugmentation of soil polluted with diesel oil by isolated bacterial strains (two species of *Bacillus, Pseudomonas mendocina* and *Acinetobacterl woffii*) resulted in an overall reduction of about 80.53% of the total diesel hydrocarbons in aged polluted soil after 65 days. At the third site two species of *Bacillus*, *Pseudomonas alcaligenes, Sphingomonas paucimobilis* and *Alcaligenes xylooxidans* were able to remediate an aircraft fuel contaminated aged soil achieving 97.57% degradation rate within 22 days (Łebkowska et al., 2011).

Overall, research on the effect of exogenous microorganisms on the acceleration of petroleum hydrocarbon degradation in contaminated soils has led to variable results. The inoculation of contaminated soil with non-indigenous microbes has resulted in

no additional impact on hydrocarbon degradation (Makadia et al., 2011; Sheppard et al., 2011). Mansur et al. (2015) reported a decrease in TPH of 97% in laboratory studies of Libyan soil inoculated with indigenous bacteria isolated from the contaminated site. In this study, similar rates of TPH reduction were observed by week 12 for the natural attenuation and bioaugmentation treatments. Local microbes are well adjusted to their native habitat. Therefore, the reintroduction of previously adapted indigenous microbes appears to represent the most robust bioaugmentation approach.

Oxygen diffusion in the soil is one of the most important requirements for effective bioremediation (Huesemann and Truex, 1996). Bioremediation is also strongly dependent on the nitrogen and phosphorus availability in soils. The variability of previous bioaugmentation studies may, in part reflect the status of these environmental parameters. Plant residues represent a cheap biomass which may stimulate microbial activity and in turn accelerate bioremediation by promoting soil aeration, and increase porosity and nutrient concentrations (nitrogen and phosphorus) in the soil (Barathi and Vasudevan, 2003).

4. Conclusion

This study has shown that biological treatment of diesel oil polluted soil can lead to a significant improvement in the biodegradation of TPHs in contaminated Libyan soils. It has also demonstrated that the addition of pea straw represents a simple and sustainable treatment leading to increased oil remediation rates. The application of plant residues might be a suitable technique for use in subsequent bioremediation projects which could result in substantial cost savings. In contrast, in this study, inoculation of the soil with a hydrocarbon degrading consortium did not contribute to any substantial changes in the bioremediation efficiency. The findings of the present research indicate that improvement of the bioremediation potential through the application of pea straw and their own associated microbial community with relevant catabolic genes which may contribute to the degradation of hydrocarbons is a valuable, commercial and sustainable strategy for improving the bioremediation efficiency of oil contaminated soils. This is the first report of the application the bioremediation of Libyan contaminated soils.

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