Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil

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ABSTRACT

Nutrient additions can stimulate petroleum hydrocarbon degradation, but little is known about how these additions affect the microbial community involved in that degradation. A microcosm study was conducted to assess the impact of bioaugmentation with *Acinetobacter* SZ-1 strain KF453955 and biostimulation with nutrients nitrogen and phosphorus on petroleum hydrocarbon degradation efficiency and microbial community dynamics during bioremediation of an oil-contaminated soil. Soils were incubated without shaking at room temperature for 10 weeks, and petroleum hydrocarbon degradation efficiency, catalase activity, petroleum hydrocarbon degrader population, and bacterial community diversity were determined. Results showed biostimulation and bioaugmentation, respectively, promoted 60% and 34% degradation of the total petroleum hydrocarbons (TPH) after six weeks of incubation. A degradation plateau occurred in the seventh week. Catalase activity and the populations of oil degraders in soil were generally greater for biostimulation than for bioaugmentation. The inoculants survived into the seventh week for the bioaugmentation treatment, and bacterial diversity did not increase by biostimulation. The populations of TPH-degraders in soil were positively related to TPH degradation efficiency during bioremediation of petroleum-polluted soils.

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1. Introduction

Petroleum hydrocarbons are composed of complicated mixtures of non-aqueous and hydrophobic components such as n-alkane, aromatics, resins and asphaltenes. Due to the adverse impact of these chemicals on human health and the environment, they are classified as priority environmental pollutants by the US Environmental Protection Agency (US EPA, 1986).

Oil pollution accidents have become a common phenomenon and have caused ecological and social catastrophes (Snape et al., 2001). When the oil spill accidents occur on land, degradation of petroleum hydrocarbons by indigenous microorganisms is often a slow process due to low microbial population and activity (Cerqueira et al., 2014; Abed et al., 2015). It is therefore a challenge to find an effective and efficient method to remediate these polluted soils.

Among a variety of remediation methods, bioremediation has been recognized as a cost-effective clean-up technology to treat oil-polluted soils and sediments (Al-Mutairi et al., 2008; Cerqueira et al., 2014). There are two main types of bioremediation technologies-bioaugmentation and biostimulation (Lladó et al., 2012; Simarro et al., 2013). Bioaugmentation involves inoculating exogenous degrading microorganisms to the soil (Ruberto et al., 2003; Maria et al., 2011; Taccari et al., 2012; Wu et al., 2013). Biostimulation stimulates the degrading capacity of the indigenous community by adding nutrients to avoid metabolic limitations (Yu et al., 2005; Kauppi et al., 2011; Sayara et al., 2011; Taccari et al., 2012; Abed et al., 2014). Currently, many reports have shown that bioaugmentation and biostimulation enhance the biodegradation of hydrocarbons in oil-polluted soil (Ruberto et al., 2003; Mancera-López et al., 2008; Tahhan et al., 2011; Taccari et al., 2012; Suja et al., 2014). However, the effects of bioaugmentation and biostimulation

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are case specific, and results are inconsistent and vary with inoculants and nutrients (Mancera-López et al., 2008; Abed et al., 2014; Suja et al., 2014). In general, it is considered that bioaugmentation and biostimulation are very promising methods for remediation of oil-contaminated soil.

Because the process of bioremediation is carried out by various microorganisms in soil, understanding how bioaugmentation and biostimulation influence the populations of hydrocarbon degraders, the diversity and activity of the microbial community, and the ability of inoculants to adapt to new environmental conditions are very important in ensuring effective bioremediation of petroleum-contaminated soil (Kaplan and Kitts, 2004; Kauppi et al., 2011; Liu et al., 2012; Taccari et al., 2012). However, little research has been conducted to investigate the relationship between petroleum hydrocarbon degradation efficiency and microbial community dynamics and microbial activity (Ruberto et al., 2003; Colombo et al., 2011; Taccari et al., 2012; Hassanshahian et al., 2014; Abed et al., 2015).

The objectives in this study were (1) to assess the efficiency of bioaugmentation with *Acinetobacter* SZ-1 strain KF453955 and biostimulation with addition of nutrients nitrogen and phosphorus, for petroleum hydrocarbon degradation and (2) to explore the relationship between hydrocarbon degradation efficiency and microbial community dynamics in petroleum-polluted soil.

2. Materials and methods

2.1. Soil analysis

A petroleum-contaminated soil was collected from the immediate area surrounding an oil well in Zichang county, Yan'an city, Shaanxi province, China. The site has a history of petroleum contamination over a period of years. The basic chemical and microbiological properties of this initial soil (IS) are shown in Table 1.

2.2. Experiment design for the microcosm study

The petroleum-contaminated soil was air-dried, ground and sieved through a 2-mm sieve prior to use. Afterwards, the soil was subjected to three different treatments. (1) CK, sterile water was added to soil to maintain 20% water content. (2) BA; bioaugmentation with *Acinetobacter* SZ-1 strain KF453955, a TPH-degrader, which was isolated from a petroleum-polluted soil by Yang et al. (2014). This strains was inoculated into soil to achieve a density of 10^8 cfu g^{-1} of soil as suggested by Abalos et al. (2004). Sterile water was added to soil to maintain 20% water content. (3) BS; biostimulation with $(NH_4)_2SO_4$ and KH_2PO_4 that were added to

Table 1
Selected chemical and microbiological characteristics of the petroleum-contaminated soil.

Main characteristics	Values
Total petroleum hydrocarbons (TPH) (mg/kg)	44,600
pH	7.5
Total carbon (g/kg)	789
Total nitrogen (mg/kg)	102
Total phosphorus (mg/kg)	160
Total bacterial population (cfu/g)	1.0×10^8
TPH degrader population (MPN[a]/g)	1.2×10^5
Alkane degrader population (MPN/g)	4.6×10^4
PAH[b] degrader population (MPN/g)	ND[c]

[a] MPN: Most probable number.
[b] PAH: polycyclic aromatic hydrocarbon.
[c] ND: Not detected.

soil to achieve a C:N:P ratio of 100:10:1. Sterile water was added to soil to maintain 20% water content.

For each treatment, three microcosms (three replicates) were prepared, each containing 500 g soil, 100 g sterile water (20% water content) plus the bioaugmentation or biostimulation treatment materials in a 10 × 10 × 10 cm plastic box without a lid. The microcosms were then incubated at room temperature for 10 weeks without shaking to mimic an oil spill situation. Soil moisture was periodically monitored and water was supplied to the microcosms to keep the 20% water content. The soils inside the microcosms were stirred weekly to ensure sufficient air and oxygen.

2.3. Total petroleum hydrocarbon (TPH) degradation

A 5 g soil sample was collected weekly from each microcosm for 10 weeks for TPH analysis. The samples were dried for 24 h at room temperature in a fume hood immediately after sampling. Each soil sample was then extracted three times in an ultrasonic ice-bath (15 min for each extraction) with 15 mL of carbon tetrachloride and then centrifuged at 6000 rpm for 15 min. The extracts were combined to obtain the total organic extract. After filtration, the extracts were dried over Na_2SO_4 and adjusted to a 50 mL volume. The TPH in the extracts was analyzed using an Infrared Photometer Oil Content Analyzer (OCMA-350, Japan).

2.4. Catalase activity determination

Catalase activity was measured by the method of Lin et al. (2009). Briefly, 1 g air-dried soil was suspended in 40 mL distilled water and shaken for 30 min on a rotary mixer at 30 rpm, and then 5 mL of 0.3% H_2O_2 was added. The mixture was reacted with shaking for a further 10 min at 20 ± 2 °C, and then 5 mL of 3 M H_2SO_4 was added to stabilize the undecomposed H_2O_2. Finally, the mixture was filtered and titrated using 0.02 M $KMnO_4$. Catalase activity was expressed as ml $KMnO_4$ g^{-1} dry soil h^{-1}.

2.5. Determination of TPH-, alkane- and PAH- degrading microbial populations

The TPH-, alkane- and PAH-degrading microbial populations in soils collected from the microcosms at weeks 0, 1, 2, 6, and 7 of incubation were enumerated by a modified most probable number (MPN) procedure as described by Wrenn and Venosa (1996). Briefly, one gram fresh soil was homogenized in 9 mL PBS buffer (0.27 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.8 g NaCl, 0.2 g KCl, 1 L distilled water, pH 7). Then 0.2 mL of the soil solution was transferred to a 5-mL-snap-cap culture tube containing 1.8 mL Bushnell-Haas medium containing 2% NaCl and the specific selective growth substrate. For TPH-degrading microbial population, the growth medium contained 56 μL standard petroleum hydrocarbons as the selective growth substrate. For alkane-degrading microbial population, the growth medium contained 56 μL n-hexadecane as the selective growth substrate. For PAH-degrading microbial population, the growth medium contained three PAHs (800 μg anthracene, 800 μg phenanthrene, and 400 μg pyrene) as the selective growth substrates. Tenfold serial dilutions were made until a dilution occurred where microbial growth was no longer evident.

The alkane- and TPH- degrader cultures were incubated by shaking at 180 rpm at room temperature (25 °C) for 1 week, and the PAH-degrader cultures were incubated for 3 weeks. For enumerating the alkane and TPH-degrader populations, iodonitrototnzolium violet (INT) was used to identify positive cultures. After 1 week of incubation, 100 μL of filter sterilized INT (3 g L^{-1}) was added to each culture tube. If red precipitate occurred due to INT reduction, the culture tube was positive. For PAH-degrader

populations, positive tubes were identified by culture turning yellow or brown owing to the accumulation of partial oxidation products of the aromatic substrates. The TPH, alkane, and PAH-degrading microbial populations were determined by referring to an appropriate MPN table and expressed as log10 MPN g^{-1} dry soil.

2.6. Bacterial community diversity determination

Total DNA was extracted and purified from soil using the Power Soil DNA Kit (MoBio Laboratories, USA) following manufacturer's recommendations. A polymerase chain reaction (PCR) was conducted using a set of universal bacterial primers, PRBA-338F and PRUN-518R. These primers amplify the 338 to 518 region of the 16S rRNA gene of bacteria. For the PCR reactions, the 50 μL of final mixture volume contained 2 μM of each primer, 25 μL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 μL DNA template, and 20 μL of ddH$_2$O. The PCR reactions were performed using an automated thermal cycler (Mastercycler pro, Eppendorf, Germany). The temperature program for the PCR reaction started with a 94 °C denaturation step for 5 min. Then 30 cycles were conducted in which each cycle included a denaturing step of 94 °C for 30 s, an annealing step of 57 °C for 30 s and an extension step of 72 °C for 30 s. The last step in the PCR program was a final extension at 72 °C for 7 min (Mehdi et al., 2012). The DNA content of the PCR products was measured by using Nanodrop 2000 instrument (ND-2000, Thermo Fisher Scientific, USA). DNA concentrations were adjusted to 3–10 ng mL^{-1} before further treatments.

Denaturing gradient gel electrophoresis (DGGE) analysis was used for separation of PCR products obtained as described above. 5 μL of purified PCR products was loaded onto 10% (wt/vol) poly-acrylamide gels, 16 cm by 16 cm, with denaturing chemical gradients of formamide and urea ranging from 40% to 60%. DGGE was performed using the BioRad Dcode Universal Mutation Detection System (Bio-Rad Laboratories, USA). The gel was loaded and run in 1 × TAE buffer (20 mM tris—HCl, 10 mM acetate, 0.5 mM Na$_2$EDTA) at 60 °C for a total of 900 V h (constant voltage of 60 V for 15 h). The gels were then stained for 45 min in 1 × TAE buffer containing GelredTM Nucleic Acid gel stain (Biotium, US) and visualized by UV illumination (Jacqueline et al., 2009; Ji et al., 2014).

Images of the DGGE gels were digitalized, and the DGGE bands were processed using the Quantity-one image analysis software, version 4.1 (Bio-Rad Laboratories) and manually corrected. The Shannon—Weiner (H) diversity index was calculated using the DGGE data according to Dunbar et al. (Dunbar et al., 2000) as follows: H = −Σ(pi) (log$_2$ pi), where p is the proportion of an individual peak height relative to the sum of all peak heights.

2.7. Sequencing analysis

Predominant DGGE bands were excised with a sterile razor blade, resuspended in 300 μL sterilized MilliQ water and stored at −20 °C overnight. After being recovered by thawing at room temperature, the supernatant was used to reamplify the DGGE bands with primers 338F/518R under the same amplification conditions described in 2.6.

Four μL of the band-PCR product were cloned into a pMD®19-T Simple Vector system (Takara, Japan). Single-clone colonies of ampicillin-resistant *Escherichia coli* transformant were selected to perform colony PCR using the M13-47 specific primer set to check whether the target DNA had been inserted. Positive transformants were transferred to Luria—Bertani (LB) broth medium and incubated at 37 °C overnight. The plasmids extracted from the transformants were sent to Sangon Biotech Co., Ltd (Shanghai, China) for sequence determination. After checking the sequence chromatograms with Chromas software (version 2.31) for errors, the

sequences were obtained. Homology searches were conducted using the GenBank server of the National Centre for Biotechnology Information (NCBI), China and the Basic Local Alignment Search Tool (BLAST) algorithm.

2.8. Statistical analysis

All experiments were performed in triplicate and results, with the exception of the diversity indices, are reported as the mean ± one standard deviation (SD). The experimental results were also statistically analyzed using two-way ANOVA (Statistical Prediction for Social Science (SPSS), version 19.0, China). When means were found to be significantly affected ($p \leq 0.05$) by treatment, means were separated using least significant difference (LSD) test.

3. Results

3.1. The basic chemical and microbiological properties of the initial soil (IS)

The chemical and microbiological characteristics of the petroleum-polluted soil revealed the C:N:P ratio in the initial contaminated soil was 438:1.0:1.6 (Table 1). Also, this soil had low populations of TPH-, alkane- and PAH- degrading microorganisms.

3.2. Degradation of TPH by biostimulation and bioaugmentation

After 10 weeks of incubation, TPH was reduced from 46,600 mg kg^{-1} soil to 18,400 and 29,300 mg kg^{-1} soil by the biostimulation (BS) and biaugmentation (BA) treatments, respectively (Fig. 1). This represents a TPH degradation efficiency of 60% and 34%. The TPH in the CK treatment was reduced to 35,400 mg kg^{-1} soil and represents a 16% depletion of the TPH content. Both biostimulation and bioaugmentation significantly enhanced the biodegradation of TPH, compared to the CK treatment, but the biostimulation treatment was the more effective of
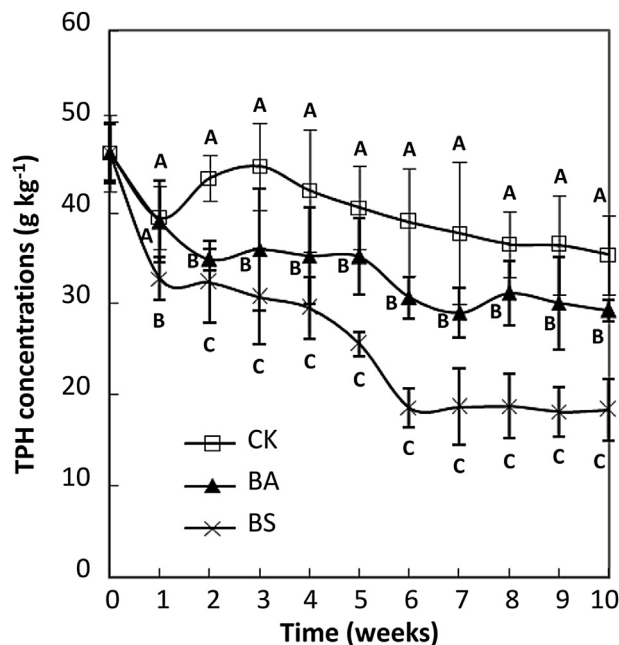

Fig. 1. Degradation of TPH by biostimulation and bioaugmentation in oil-contaminated soil. Errors bars indicate ±SD of triplicate samples. Different letters in the same week represent a significant difference at $P \leq 0.05$.

the two treatments. This indicates that supplying nutrients for the indigenous microorganisms is an effective method to enhance petroleum hydrocarbon degradation in soil.

For both the BA and BS treatments, TPH concentrations in the microcosm soils decreased for the first six weeks. After 6 weeks of incubation, however, TPH concentrations were not further decreased and degradation plateaus occurred.

3.3. Effects of biostimulation and bioaugmentation on catalase activity in oil-contaminated soil

Catalase activity increased in all treatments in the first six weeks of incubation (Fig. 2), and then decreased rapidly in the seventh week. Catalase activity was usually greater for the BS treatment than for the BA treatment after six and seven weeks of incubation. The results suggest that catalase activity may be an important indicator for assessing the extent of bioremediation of oil-contaminated soil.

3.4. Effects of biostimulation and bioaugmentation on TPH-, alkane-, and PAH- degrading microbial populations in oil-contaminated soil

The TPH-, alkane-, and PAH-degrading microbial populations in microcosm soils of different treatments at weeks 0, 1, 2, 6 and 7 of incubation are presented in Fig. 3. For all three types of degraders, the initial levels (i.e. at time zero) were not different from each other as affected by the CK, BA or BS treatments.

TPH degrading microbial populations reached the maximum value in the second week after incubation, and maintained that level through the seventh week. Highest levels of TPH degraders were achieved for the BS treatment. Alkane-degrading microbial populations increased after only one week for the BS treatment. This treatment then was consistent in having the highest levels of alkane degraders through seven weeks. The BA treatment also stimulated alkane degraders after two weeks, and this was sustained through seven weeks. The PAH-degrading microbial populations increased at weeks 2 and 6 and then decreased at week 7. The BA and BS treatments were higher than the CK treatment after
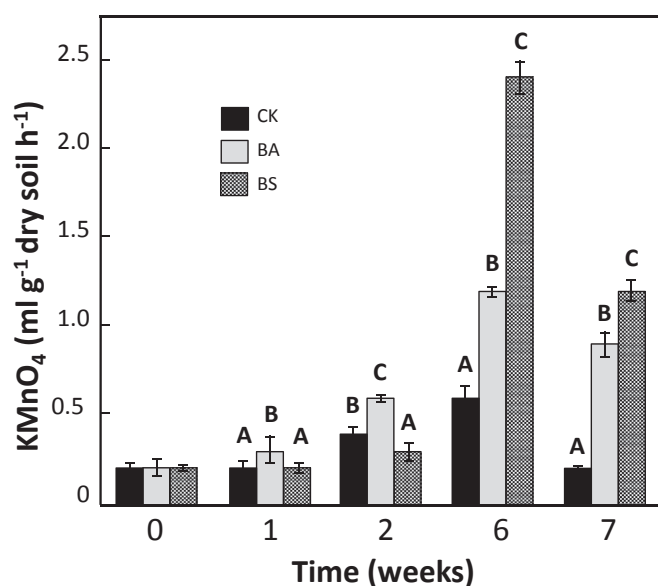
Fig. 3. Effects of biostimulation and bioaugmentation on TPH-, alkane-, and PAH-degrading microbial populations in oil-contaminated soil. Errors bars indicate $\pm$SD of triplicate samples. Different letters over each bar in the same week represent a significant difference at $P \leq 0.05$.
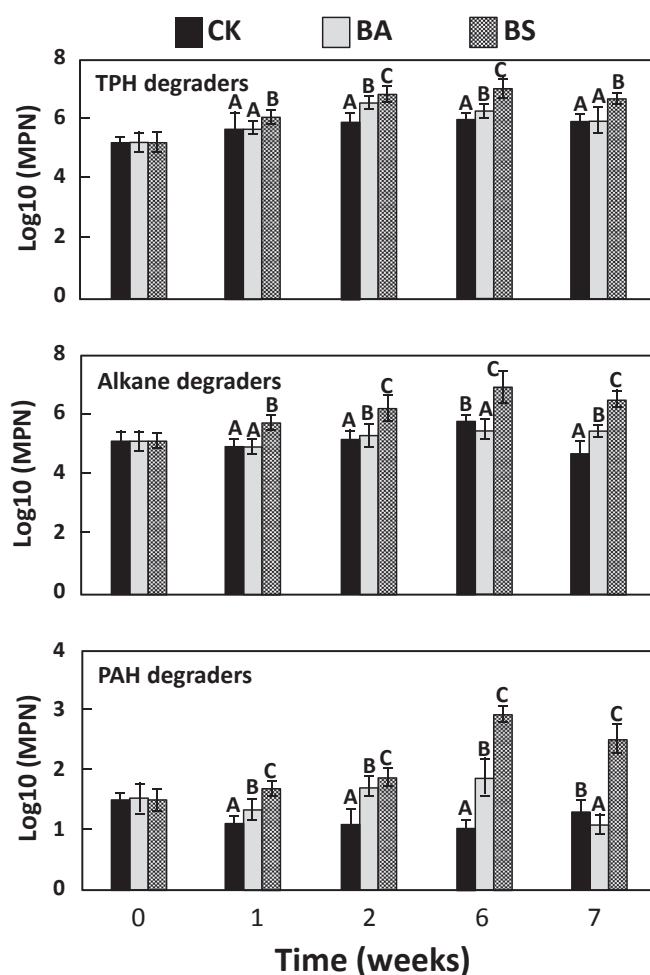
1, 2 and 6 weeks of incubation. However, at week 7, the BA treatment actually had a slightly lower number of PAH degraders than the CK treatment.

Among the three treatments, the BS soils generally showed the greatest degrading microbial populations. The TPH-, alkane- and PAH- degrading microbial populations in the sixth week were, respectively, 1.2×10^7, 9.2×10^6, and 9.2×10^2 cells g^{-1} soil. However, they decreased in the seventh week when the degradation plateau occurred.

3.5. Effects of biostimulation and bioaugmentation on bacterial diversity in oil-contaminated soil

Denaturing gradient gel electrophoresis (DGGE) analysis of the microbial consortia in the microcosm soils of the three different treatments at weeks 1, 2, 6, and 7 was conducted (Fig. 4). A total of 23 distinguishable bands were randomly selected for sequence analysis. The 23 nucleotide sequences (DGGE bands 1—23 in Fig. 4) identified in this study were deposited in the GenBank database under accession numbers KJ481836 to KJ481858 and bacterial species in these microcosms are listed in Table 2. Band 2 in Fig. 4 belonged to *Acinetobacter* sp (Table 2), suggesting it was likely that band 2 was the *Acinetobacter* SZ-1 strain KF453955 used as the inoculants for the bioaugmentation (BA) treatment.

Fig. 2. Effects of biostimulation and bioaugmentation on catalase activity in oil-contaminated soil. Errors bars indicate $\pm$SD of triplicate samples. Different letters over each bar in the same week represent a significant difference at $P \leq 0.05$.
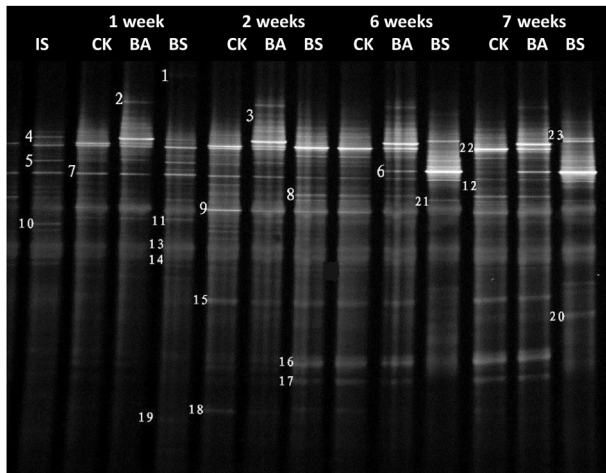
Fig. 4. Denaturing gradient gel electrophoresis (DGGE) analysis of bacterial community structure in soil to which different treatments have been applied. Lane IS is the initial soil before treatment, CK is the untreated soil, BA is the soil bioaugmented with *Acinetobacter* SZ-1 strain KF453955, and BS is the soil biostimulated with N and P.

Among the three treatments, the highest Shannon—Weiner diversity index was generally in the BA soil during the seven weeks of incubation (Table 3). The Shannon—Weiner diversity index reached maximum values in the sixth week for the CK and BA treatments and then decreased in the seventh week. The Shannon—Weiner index was mostly unchanged for the BS treatment, and then decreased in the sixth and seventh weeks. In general, the Shannon—Weiner diversity index decreased between the sixth and seventh week when a degradation plateau occurred.

4. Discussion

A review of the chemical and microbiological characteristics of the petroleum-polluted soil revealed the C:N:P ratio in the contaminated soil was 438:1.0:1.6. It is generally consider that the

Table 3
The Shannon—Weiner diversity indices of the microbial communities in the petroleum-contaminated soils with different treatments. These indices were calculated from the DGGE bacterial profile of 16S rRNA gene fragments.

	Time (weeks)				
Treatments	0	1	2	6	7
CK	2.86 b	2.42 A a	2.82 A b	3.21 AB c	3.00 B bc
BA	2.86 b	2.95 B ab	3.24 B bc	3.53 B c	3.33 C bc
BS	2.86 a	3.02 B b	3.05 AB b	2.80 A ab	2.56 A a

Different capital letters in the same column represent a significant difference *at P ≤ 0.05*. Different lowercase letters in the same row represent a significant difference at *P ≤ 0.05*.

appropriate C:N:P ratio in soil for bioremediation operations is 100:10:1, which is equivalent to the nutrients required for an active microbial population (US EPA, 2002). Also, the initial or time zero soil had lower populations of TPH-, alkane- and PAH- degrading microorganisms after the BS and BA treatment (Fig. 3). Thus supplying appropriate amount of N and P nutrients for biostimulation and inoculating suitable petroleum hydrocarbon degraders for bioaugmentation clearly can impact the numbers of relevant degraders in an oil polluted soil, leading to more effective TPH degradation.

The BS and BA treatments, respectively, degraded 60% and 34% of the added petroleum hydrocarbons (TPH). A greater degradation efficiency for the BS treatment, compared to the BA treatment, seems due to adjusting the C:N:P ratio to a more appropriate level in soil. This stimulates the growth and degrading activity of the indigenous microorganisms (Atlas and Hazen, 2011).

The performance of the BA treatment was consistent with existing studies showing that bioaugmentation can be helpful in promoting degradation during the early stage of remediation. Our results also suggest that the decreases of hydrocarbon degrading microbial populations, including that of the inoculants, may be one of the reasons for the degradation plateau that occurred after six weeks of incubation.

Wu et al. (2013) reported that a consortium composed of many different bacterial species was required to efficiently degrade

Table 2
Identity of selected 16S rRNA gene sequences of excised bands of the DGGE gel. Comparisons were made to sequences in the database of the National Center for Biotechnical Information (NCBI), China.

Band No.[a]	Accession number	Closest match in Genbank database	Similarity (100%) (%)
1	KJ481836	*Bacillus lentus* KF378647	100
2	KJ481837	*Acinetobacter* sp. KF453955	94
3	KJ481838	*Methanogenic prokaryote* KC821450	98
4	KJ481839	*Pseudomonas* sp. JX624259	100
5	KJ481840	*Uncultured proteobacterium* JN409106	91
6	KJ481841	*Uncultured Moraxellaceae bacterium* JN038240	99
7	KJ481842	*Sphingomonas*sp. KF830230	100
8	KJ481843	*Uncultured Salinimicrobium sp.* KF859581	99
9	KJ481844	*Uncultured Pseudomonas sp.* EU755103	98
10	KJ481845	*Uncultured Gemmatimonas sp.* JQ400417	96
11	KJ481846	*Pseudomonas* sp. AB628746	98
12	KJ481847	*Acinetobacter* sp. KF453955	100
13	KJ481848	*Geodermatophilus* sp. X92363	99
14	KJ481849	*Uncultured Acinetobacter sp.* AB908751	99
15	KJ481850	*Paracoccus marcusii* KF856725	100
16	KJ481851	*Sphingosinicella* sp. KF877719	100
17	KJ481852	*Sphingomonas* sp. KF777648	99
18	KJ481853	*Mycobacterium* sp. KF663791	99
19	KJ481854	*Uncultured actinobacterium* JX442917	95
20	KJ481855	*Uncultured prokaryote* KC337096	98
21	KJ481856	*Acinetobacter* sp. KF663060	100
22	KJ481857	*Uncultured Moraxellaceae bacterium* JN038240	99
23	KJ481858	*Acinetobacter* sp. KF792192	99

[a] Band numbers are those identified in Fig. 4.

polycyclic aromatic hydrocarbons. No single microbial species had the ability to metabolize more than two classes of compounds typically found in crude oil. Only inoculating one bacterial species, as was done in this study, could be the major reason for lower degradation efficiency (34%) for the BA treatment compared to the BS treatment.

Our results were consistent with many previous studies indicating that biostimulation was more efficient than bioaugmentation for bioremediation of oil-contaminated soil (Kauppi et al., 2011; Sayara et al., 2011; Abed et al., 2014). Furthermore, many reports have reveled that the best treatment method for hydrocarbon degradation in oil-contaminated soil is a combination of bioaugmentation and biostimulation (Mancera-López et al., 2008; Taccari et al., 2012; Suja et al., 2014).

Band 2 in the DGGE gel (Fig. 4) was present only in lanes that represented DNA from the BA treatment soil. DNA sequencing showed this band belonged to *Acinetobacter* sp (Table 2) and it is the *Acinetobacter* SZ-1 strain KF453955 used as the inoculants for the BA treatment. This strain persisted throughout the seven weeks. Also, this meant the *Acinetobacter* SZ-1 strain KF453955 could survive in the petroleum-polluted soil and coexist with indigenous microorganisms. Together the combined activity of the inoculant strain and the indigenous were responsible for the effectiveness of the bioaugmentation (BA) treatment.

After an initial period of bioremediation, hydrocarbon concentrations stabilized and further degradation was limited (Liu et al., 2013; Maria et al., 2013; Simarro et al., 2013). In this study, the degradation plateau began between the sixth and seventh week of incubation. Our results were consistent with many previous reports that a contaminant degradation plateau was often observed during 35–40 days of bioremediation (Liu et al., 2011, 2013).

When the degradation plateau was formed, the TPH residue concentration was still 18,600 mg kg^{-1} in the BS soil and 29,300 in the BA soil. These levels are higher than the residual concentrations reported in other studies which were approximately 2000 mg kg^{-1} (Liu et al., 2013; Maria et al., 2013). In our experiment, the initial TPH concentration was 44,600 mg kg^{-1} and was higher compared to the previous studies. However, the degradation efficiency of 60% for the BS treatment was similar to that reported for other studies (Liu et al., 2011, 2012).

Regarding the residual concentration or degradation plateau, one hypothesis is that the pollutant diffusion process is retarded by repeated sorption and desorption. These processes are controlled by both soil characteristics and contamination aging (Williamson et al., 1997; Nocentini et al., 2000; Chaineau et al., 2003) and Liu et al. (2013) emphasized that the presence of high proportions of organic matter and clay in soil can affect the extent of biodegradation. Venosa et al. (1997) and Chaineau et al. (2003) considered the degradation plateau was a result of the accumulation of metabolites, some of which could inhibit the initial step of degradation. Other researchers believe that formation and accumulation of high molecular weight aromatic and polar hydrocarbon fractions due to condensation reactions in soil due of the TPH and its metabolites, can lead to low biodegradation rates during bioremediation (Chaillan et al., 2006; Lee et al., 2007).

Generally, the larger the TPH degrading microbial population, the more TPH degradation occurs (Krutz et al., 2005; Wu et al., 2013). Miya and Firestone (2001) reported that hydrocarbon degrader enrichment by slender oat root exudates and root debris enhanced biodegradation of phenanthrene in soil. In this study, TPH-degrading microbial populations were greater in BS soil than in BA soil, and TPH degradation efficiency was 60% for BS treatment and 34% for BA treatment. This result also suggested supports the existence of a positive relationship between hydrocarbon degrader population and hydrocarbon degradation. Thus one way to achieve

more effective remediation, but either the BA or BS treatment, is to focus attention on achieving the highest possible levels of TPH degraders. This could be done by enhancing the inoculum levels or by enhancing the TPH degraders of the indigenous population. These efforts could also increase the time of active TPH degradation before the plateau effect is reached.

Kaplan and Kitts (2004) reported that the largest shift in bacterial community structure occurred at the end of the initial stage of TPH biodegradation, and a similar result was observed in this study. In addition, the Shannon–Weiner diversity index indicated that the number of species was greater in the inoculated soil (BA) than in the un-inoculated soil (CK and BS). Therefore, hydrocarbon degradation is not directly associated with the number of microbial species in oil-polluted soil, but more to the number of individual microorganisms that can promote degradation. Ruberto et al. (2003) also reported that bacterial diversity was reduced in contaminated soil by biostimulation with N and P, but they did not report on the effect of the N and P treatments on the total numbers of TPH degraders.

Catalase is produced by microorganisms present in soil. Soil catalase activity is the total activity of the enzyme stabilized in the soil matrix and the activity of the current viable microbial population in the soil. The role of catalase is decompose hydrogen peroxide and is often an indication of the size or activity of the soil microbial population. Lin et al. (2009) reported that the catalase activity in the contaminated soil decreased with increasing oil concentration. The activity of catalase improved after the bioremediation. The catalase activity was sensible to the oil and could be alternative to monitor the bioremediation process. Our study verified this observation by Lin et al. and suggests that catalase activity could be used to monitor the bioremediation extant of oil-contaminated soil.

5. Conclusions

The TPH degradation in the oil-polluted soil was enhanced by bioaugmentation with *Acinetobacter* SZ-1 strain KF453955 and biostimulation with nutrients nitrogen and phosphorus. A degradation plateau occurred between the sixth and seventh weeks of incubation during bioremediation. The TPH-, alkane-, and PAH-degrading microbial populations in the polluted soil were positively related to TPH degradation efficiency during bioremediation. Supplying nutrients such as N and P to promote the growth of indigenous microorganisms in oil-polluted soil is an effective and efficient method to remediate petroleum-contaminated soil.

Acknowledgments

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