Enhanced bioremediation of crude oil in polluted beach sand by the combination of bioaugmentation and biodiesel

Tiejun Li, Hongmei Hu, Lei Jin, Bin Xue, Yurong Zhang and Yuanming Guo

ABSTRACT

Biodiesel produced from rapeseed oil was used as a cost-effective and sustainable agent to enhance crude oil biodegradation in sand microcosms. The initial concentration of crude oil and total petroleum hydrocarbon (TPH) was 20,000 and 18,750 mg/kg, respectively. The mass ratio of biodiesel to crude oil was 0 (designated T1), 1:10 (designated T2), 1:4 (designated T3), and 1:2 (designated T4). After 80 days of incubation, the total removals of TPH and PAHs were 68.6 and 61.5% in T1, 78.0 and 67.3% in T2, 86.3 and 76.2% in T3, 72.2 and 57.9% in T4, respectively. Higher amounts of biodiesel reduced TPH biodegradation due to the decreased transfer of substrates caused by dilution effect. The addition of biodiesel stimulated bacterial growth during the initial period but the petroleum hydrocarbon degradation is not always correlated with the density of bacteria in the presence of biodiesel. Dehydrogenase activity (DHA) and polyphenol oxidase (PPO) activity increased greatly after the beginning of incubation. From then on, DHA continuously decreased with time. T3 had the highest DHA and PPO activity from day 30 to the end of the experiment. The lowest toxicity was observed in T3 at day 80, and T3 showed the highest degradation rate constant.

Key words | biodiesel, bioremediation, enzyme activity, oil spills, petroleum hydrocarbons

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INTRODUCTION

Marine oil spills frequently occur in nearshore locations or in ports due to incidents, leakage, and careless disposal practices, causing severe damages to the sea and shorelines. During the period 1970 to 2007 more than 5.6 million tonnes of oil was released into the sea worldwide (Nikolopoulou & Kalogerakis 2009).

The spilled oil may persist in coastal regions over long periods, be transported through food chains, and exhibit long-term toxicity to organisms and humans (Xia *et al.* 2015). Various abiotic processes make the residual oil increasingly weathered and sticky, leading to difficulty in removal of the oil by physical methods due to its tight absorption on sands (Pereira & Mudge 2004). The use of surfactants can dissolve and release oil from sands to the surrounding environment, but traditional surfactants have higher costs and toxicity. These surfactants are difficult to biodegrade when used to wash highly sticky oil residue (Edwards *et al.* 2003). Aggressive shoreline clean-up actions can exert further deleterious effects on oil-impacted shorelines (Pereira & Mudge 2004). Therefore, there is considerable interest in low cost-effective technologies that accelerate oil removal from intertidal beaches without causing equivalent or greater damage.

Opposed to the conventional physical/chemical clean-up means like hot water flushing and chemical washing using organic solvents and/or surfactants (Gallego *et al.* 2007), bioremediation may be a more environmentally acceptable and cost-effective alternative for removal of fuel oil from shore rocks and sands. Some studies have been conducted to evaluate the effectiveness of bioremediation on oil spills in simulated or real environments (Kim *et al.* 2005;

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Fernández-Álvarez *et al.* 2006; Pontes *et al.* 2013). However, microbial density in beach sands is generally much lower than that in soils. Moreover, weathered oil attached to sands has low water solubility, resulting in low bioavailability of oil. Thus, bioremediation of oil spill in beach sands is limited under actual field conditions (Prince 2010).

Biodiesel is cheap, non-toxic and readily biodegradable, and has good dissolution ability towards petroleum. Recently, some studies have been conducted on decontamination of petroleum-polluted beaches using biodiesel in laboratory and field tests. For example, Pereira & Mudge (2004) used biodiesel as a solvent to clean oiled shorelines and they found that the cleaning efficiency was positively correlated with the dosage of biodiesel. However, such an operation would leave a large amount of biodiesel–petroleum mixture in the field, and the consumption of biodiesel was too high. In a field test, Fernández-Álvarez *et al.* (2007) found that the addition of biodiesel apparently accelerated the degradation of aliphatic and aromatic fractions of the residual fuel oil, but showed no improvement on polycyclic aromatic hydrocarbons (PAHs) degradation. Xia *et al.* (2015) used the combination of biodiesel and petroleum degrading bacteria to eliminate crude oil from beach pebbles in simulated systems. The biodegradation of petrodiesel was enhanced by 12.8–19.4% with the addition of different sources of biodiesel at a dosage of 20–80% (v/v) (Ng *et al.* 2015). Despite several promising publications, however, the current research concerning the application of biodiesel to clean oil-polluted shorelines showed some limitations, such as high demand of biodiesel and high amount of residue.

Biosurfactants have been widely reported to improve petroleum hydrocarbons' biodegradation in wastewater, contaminated soil, and marine environments (Souza *et al.* 2014). Biosurfactants promote the cracking of hydrocarbons' molecules by micelle formation, increasing their mobility, bioavailability, and exposure to bacteria, thus favoring hydrocarbon biodegradation (Souza *et al.* 2014). However, the production of biosurfactants generally requires light hydrocarbon, which is scarce in weathered oil. Thus, the application of biosurfactants for enhancing oil removal *in situ* is limited.

The purpose of this work was to develop a bioremediation process for crude oil-polluted shoreline in a simulated system that was based on the use of a combination of seeded bacterial degraders, nutrients, biosurfactant producer, and biodiesel.

MATERIALS AND METHODS

Materials

Sea sand (diameter 0.16–0.08 mm) was collected from Hangzhou Bay, China. The physicochemical characteristics of the sand on a dry weight basis were as follows: gravel 0.16%, sand 89.52%, silt/clay 10.32%, total N 3.15 mg/kg, total P 0.73 mg/kg, and total K 54.2 mg/kg. The water holding capacity of the sand was 20.3%. The sand was sequentially soaked in 1 M NaOH for 20 min, 0.5 M HCl for 10 min, rinsed with sterile water, and then autoclaved (121 °C for 20 min) before use.

Crude oil was obtained from Shengli Oilfield, China. The density, kinetic viscosity and API (American Petroleum Institute) value of the oil at 20 °C was 0.963 g/cm^3, 93.5 mm^2/s, and 12.4, respectively. The oil was weathered at 45 °C for 4 weeks in a fume hood before use, losing 12.3% of its initial weight.

Biodiesel, produced from rapeseed oil, was purchased from a local supplier in China. The density and kinetic viscosity at 20 °C were 0.862 g/cm^3 and 5.9 mm^2/s, respectively.

Microorganisms

Bacterial consortium, assigned W16, had been previously isolated from soil polluted with crude oil under aerobic conditions using crude oil as the sole carbon and energy source. Briefly, 5 g crude oil-contaminated soil was added to 100 mL of mineral salt medium (MSM) with 1 g crude oil as the sole carbon source, supplemented with 0.1% (w/v) yeast powder. The MSM was prepared according to Bao *et al.* (2012). After 2 weeks of incubation, 5 mL of the supernatant were transferred to 100 mL of fresh medium with 1 g crude oil, incubated for another 2 weeks. All flasks were incubated at 30 °C with a shaking rate of 150 rpm on a rotary shaker. This procedure was repeated three times and then the consortium was obtained.

The bacterial species in the consortium were identified using 454 pyrosequencing and gene clone library

approaches following the description of Wang *et al.* (2014). The consortium mainly contained species belonging to the following bacterial groups: *Alcanivorax* spp., *Oleispira* spp., *Cycloclasticus* spp., *Achromobacter* sp., *Alcaligenes* sp., *Citrobacter* sp., *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Variovorax* sp., *Comamonadaceae*, *Rhodococcus* sp., *Sphingomonas yanoikuyae*, *Caulobacter* sp.

The consortium was stored at −80 °C in 30% (v/v) glycerol. To activate the microbes and prepare an inoculum, stock suspension (1 mL) was transferred to a 250 mL Erlenmeyer flask containing 50 mL MSM and diesel oil (0.5%, v/v). The flask was incubated for 2 days at 28 °C at 150 rpm. Then, 1 mL aliquot of the cell suspension was transferred to new medium and the culture was grown for 4 days under the same conditions. This step was repeated three times and cells from the last culture were harvested by centrifugation at 12,250 *g* for 5 min at 4 °C. The pellet was washed twice with mineral medium and resuspended in mineral medium to reach an initial cell density equaling about 1.6×10^8 colony-forming units (CFU) per mL of medium. In all procedures aerobic conditions were applied.

Experimental setup

To prepare oil-polluted sand, 2.0 g weathered crude oil was dissolved in 25 mL petroleum ether, and then added into 100 g of dry sand. The mixture was stirred using a glass rod until no liquid could be observed, and then dried for 3 days in a fume hood.

Each 1-L glass jar was charged with the following materials at the onset of the experiment: 100 g of oil-polluted sand, biodiesel, nutrients and/or inoculum at their appropriate concentrations. In this study, five microcosms were set up in triplicate as follows: (1) control: autoclaved sand (121 °C for 20 min) spiked with 2 wt.% $HgCl_2$ (designated CK); (2) sand with inoculation (designated T1); (3) sand with inoculation and 0.2 wt.% biodiesel (designated T2); (4) sand with inoculation and 0.5 wt.% biodiesel (designated T3); (5) sand with inoculation and 1.0 wt.% biodiesel (designated T4). Thus, the mass ratio of biodiesel to crude oil was in the range of 0–1:2. To prevent invasion of exogenous microbes, all microcosm jars were closed with sterile gauze. For inoculation cases, the cell suspension of the consortium was added to the sand and mixed thoroughly to reach an initial cell density of about 3.40×10^6 CFU/g sand. Each microcosm received 0.17 g of a commercial slow release fertilizer S200 to attain a final molar ratio of C:N:P equivalent to 120:10:1 (Pontes *et al.* 2013). The fertilizer contains a saturated solution of urea (nitrogen source) in oleic acid with phosphate esters (phosphorus source) (Díez *et al.* 2005).

During 80 days of experimental period, the microcosms were maintained at controlled temperature (28 ± 2 °C) and shielded from light. The microcosms were stirred every day with a glass rod to homogenize the systems. The microcosms were watered with sterile water every 4 days to maintain sand moisture at 20–22% by weighing. To lessen heterogeneity as much as possible, the sands in each jar were homogenized every 2 days with a nickel spatula in a superclean bench.

Oil extraction and analysis

For oil analysis, the sand in each jar was homogenized and then 10 g of sample was taken at regular interval. The samples were mixed with 2 g of anhydrous sodium sulphate to absorb the residual water. A solvent mixture (20 mL) of 1:1 of hexane:dichloromethane was added to extract petroleum hydrocarbons assisted by an ultrasonic bath for 60 min at room temperature (Bravo-Linares *et al.* 2013). The extracts were dried under nitrogen to remove solvent, and then oil samples were accurately weighed and dissolved in hexane. Then, total petroleum hydrocarbon (TPH) was determined using an infrared spectrometer oil analyzer (CY2000, Laoshan Electronic Instrument Company, China). Fractionation into saturated and aromatic fractions was performed by employing a silica gel microcolumn (200 mm × 10 mm i.d.) covered with a layer of anhydrous $MgSO_4$ (30 mg), as described by Lisiecki *et al.* (2014). Briefly, the column was first washed with a 0.2 mL portion of dichloromethane and two 0.2 mL portions of hexane. Afterwards, a 50 μL aliquot of the extract was loaded onto the top of the column. The saturated analytes were eluted with a 300 μL portion of hexane and the aromatics were eluted with a 250 μL portion of hexane/methyl tert-butyl ether mixture (1:3 v/v). The eluent was concentrated with nitrogen and then adjusted to 1.0 mL using dichloromethane.

Characterizations of n-alkanes and PAHs were performed on an Agilent 6890 gas chromatography (GC) system

interfaced to an Agilent 5973 mass spectrometer (MS) equipped with a HP-5 MS capillary column (30 m × 0.25 mm × 0.25 μm). Quantitation of individual compounds was performed using internal standards. $C_{24}D_{50}$ and D_{14}-trichlorodiphenyl were used as the internal standards for the analysis of n-alkanes and PAHs, respectively (Chen *et al.* 2013). Chromatographic conditions were the same as those reported by Yang *et al.* (2015). The weight loss of oil and individual hydrocarbons was calculated according to Equation (1):

$$R = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

where R is the removal ratio, C_0 is the initial concentration, C_t is the residual concentration at time t (day), and t is time.

Biochemical assay

Total aerobic heterotrophs were determined by the spread plate technique in nutrient agar medium after 24 h of incubation at 30 °C. Results were expressed as CFU/g sand.

Dehydrogenases activity (DHA) was measured using triphenyl tetrazolium chloride as an artificial electron acceptor according to the method of Xia *et al.* (2015). Results were expressed as μg triphenyl tetrazolium formazan (TPF)/h/g sand.

Polyphenol oxidase (PPO) activity was assayed with pyrogallic acid as substrate by the colorimetric method of Sheng & Gong (2006). Results were expressed as μg purpurigallin (PPG)/h/g sand.

Microtoxicity tests were conducted using *Photobacterium phosphoreum* in accordance with the method of Robidoux *et al.* (2004). Microtoxicity values were expressed as EC_{50}, defined as the effective nominal concentration of elutriate (volume percent) that reduces the intensity of light emission by 50%.

RESULTS AND DISCUSSION

Biodegradation of TPH

The results of TPH analysis from all five microcosms are plotted in Figure 1. A statistically significant decrease in TPH concentration ($P < 0.05$; one-way ANOVA) was observed in all the four biotic microcosms. However, the extent of reduction over 80 days was higher (72.2–86.3%) in T2, T3, and T4 compared to 68.6% reduction in T1. Moreover, the abiotic control showed negligible TPH reduction (less than 1%) during 80 days, which is attributed to the weathering treatment of oil before spiking. Thus, TPH reductions in the biotic experiments are attributed to biodegradation by the inoculated consortium.

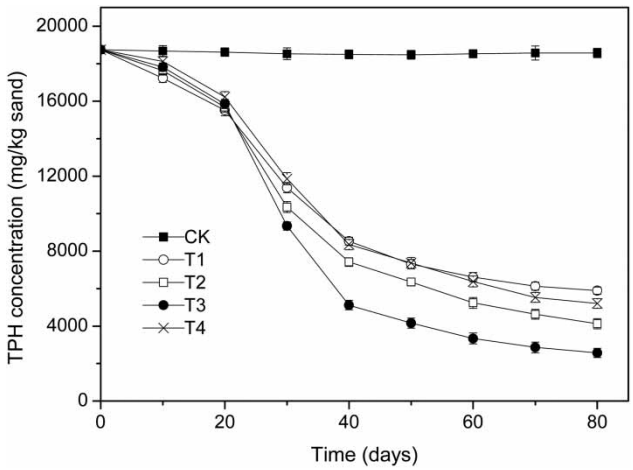

Figure 1 | Evolution of TPH concentration in sand during 80 days of incubation. Error bars represent standard deviations of triplicates.

As shown in Figure 1, the most rapid degradation of TPH across all biotic microcosms was obtained during the initial 40 days of bioremediation, followed by a relatively stable decrease of TPH concentration over time. The addition of biodiesel did not promote TPH biodegradation during the initial period (Figure 1). At 20 days, TPH concentration in sand decreased from an initial value of 18,750 mg/kg to 15,520, 15,680, 15,850, and 16,230 mg/kg in T1, T2, T3, and T4, respectively. Thereafter, however, the difference in TPH removal among various microcosms became more significant. After 80 days of bioremediation, TPH concentration in sand was 5,887, 4,124, 2,569, and 5,213 mg/kg in T1, T2, T3, and T4, respectively, corresponding to total removal of 68.6%, 78.0%, 86.3%, and 72.2%, respectively (Figure 1). These results suggest that the consortium could efficiently degrade petroleum hydrocarbons and an appropriate dosage of biodiesel may enhance TPH biodegradation. From Figure 1, it can also be observed that the degradation rate gradually slowed down after 40 days. This

demonstrates that as the easily biodegradable hydrocarbons were consumed, the remaining hydrocarbon fractions were structurally more complex and therefore of lower bioavailability.

The strains contained in the consortium are known for hydrocarbon-degradation capability. For example, *Alcanivorax* spp., *Oleispira* spp. and *Cycloclasticus* spp. can rapidly degrade many oil constituents in the marine environment (Yakimov *et al.* 2007). *Pseudomonas aeruginosa* can secret biosurfactants and has been successfully used for the degradation of various petroleum products such as gasoline, kerosene, diesel oil, and crude oil (Reis *et al.* 2011). The other strains have also been identified and reported as efficient hydrocarbon degraders under various conditions (Das & Chandran 2011).

The results of the present study (Figure 1) demonstrate that there was a positive synergistic effect where biodegradation was enhanced by blending biodiesel and crude oil. This finding is consistent with the reports of some other similar studies which used different biodiesels (Fernández-Álvarez *et al.* 2007; Ng *et al.* 2015; Xia *et al.* 2015). Solvation effects contributed to the observed enhancements of TPH degradation. As a suitable nutrient source for petroleum hydrocarbons degraders, the presence of biodiesel could enhance the biodegradation rate of petroleum hydrocarbons (Fernández-Álvarez *et al.* 2007; Owsianiak *et al.* 2009; Ng *et al.* 2015). Additionally, biodiesel is able to dissolve and disperse petroleum hydrocarbons, leading to greater contact surface of microbes with oil droplets and thus higher bioavailability of pollutants (Owsianiak *et al.* 2009; Ng *et al.* 2015). Nevertheless, the results of Lisiecki *et al.* (2014) show that biodiesel did not affect long-term (578 days) biodegradation of aliphatic and aromatic hydrocarbons in saturated sand.

Biodegradation of hydrocarbon fractions

To further understand the biodegradative differences in various microcosms, light (C14–C21), heavy (C22–C35), and PAHs fractions in crude oil across all treatments over 80 days were analyzed by gas chromatography–mass spectrometry (GC–MS).

As shown in Figure 2(a), most of the light fraction was consumed with 30 days of treatment, and the addition of biodiesel did not slow down the biodegradation rate. At day 30,

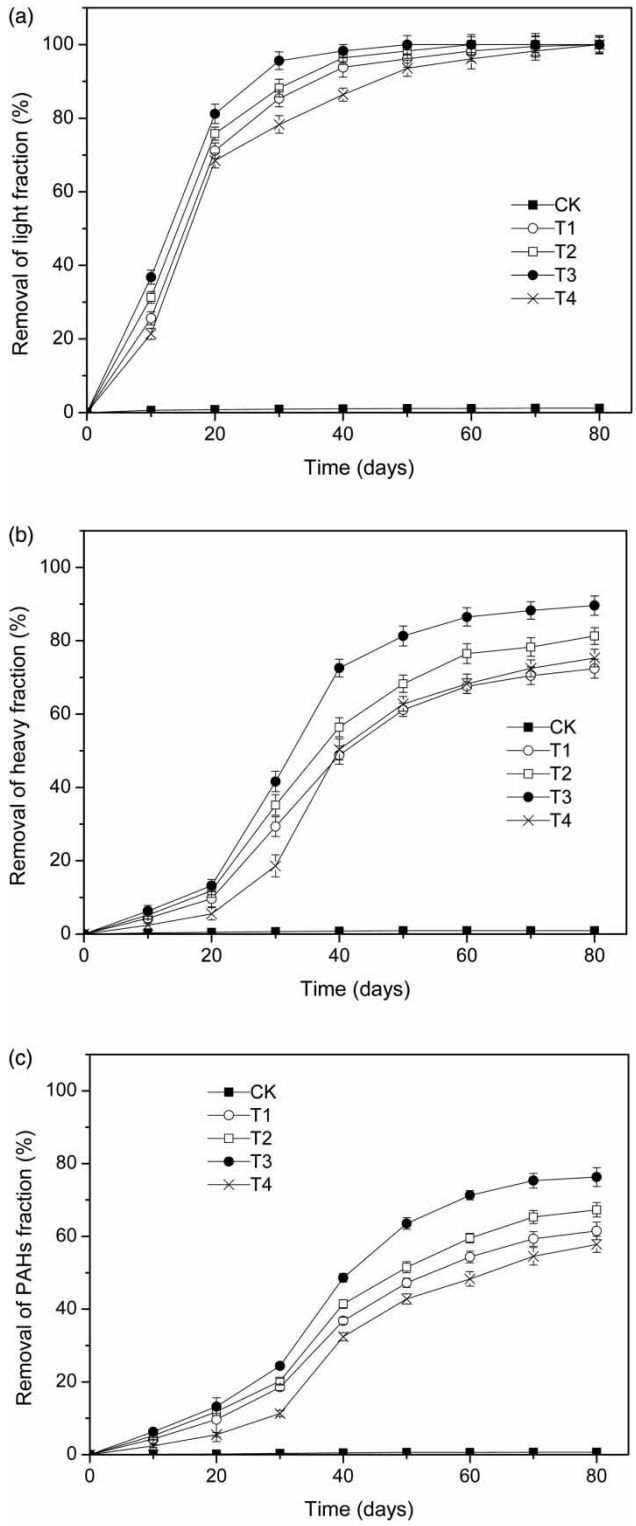

Figure 2 | Cumulative removal efficiency of (a) light (C14–C21), (b) heavy (C22–C31), and (c) PAHs fractions in crude oil during 80 days of incubation. Error bars represent standard deviations of triplicates.

the removal ratio of the light fraction was 85.4, 88.2, 95.6, and 78.3% in T1, T2, T3, and T4, respectively. This indicates that light fraction was degraded together with biodiesel. At day 80, the removal ratio of the light fraction was 99.4, 99.2, 99.6, and 99.3% in T1, T2, T3, and T4, respectively.

At day 20, the removal ratio of the heavy fraction was 9.6, 11.8, 13.2, and 5.5% in T1, T2, T3, and T4, respectively (Figure 2(b)). The degradation of the heavy fraction began to speed up and more than 60% removal was obtained within 50 days (Figure 2(b)). High removal levels of aliphatic compounds were seen with the light fraction relative to heavy ones. This is due to the fact that short- and medium-chain alkanes are generally more easily biodegraded due to their lower hydrophobicity. At day 80, the removal ratio of the heavy fraction was 72.4, 81.3, 89.6, and 75.3% in T1, T2, T3, and T4, respectively (Figure 2(b)).

At day 20, the removal ratio of PAHs was 9.6, 11.8, 13.2, and 5.5% in T1, T2, T3, and T4, respectively (Figure 2(c)). The degradation of PAHs speeded up after 20 days. At day 80, the removal ratio of PAHs was 61.5, 67.3, 76.2, and 57.9% in T1, T2, T3, and T4, respectively (Figure 2(c)). The removal ratios of heavy and PAHs fractions are in agreement with that of TPH. Especially, the addition of appropriate amounts of biodiesel (0.2 and 0.5%) resulted in significant enhancement of heavy and PAHs fractions' degradation. At day 80, the cumulative removal ratios of heavy and PAHs fractions were 89.6% and 76.2%, respectively, in T3, as compared to 72.4% and 61.5%, respectively, in T1. Abiotic control showed negligible removal of various fractions just like the case of TPH.

In the present study, there existed a maximum addition ratio of biodiesel that provided the greatest improvement in the biodegradation of petroleum hydrocarbons (Figures 1 and 2). Pasqualino *et al.* (2006) and Ng *et al.* (2015) also observed a similar phenomenon. The presence of a maximum addition ratio could be ascribed to the differences in solubility and bioavailability of petroleum hydrocarbons for biodegradation at different mixing ratios (Ng *et al.* 2015). The amphiphilic property of biodiesel fatty acid esters allows biodiesel to act as a surfactant to increase the solubility of petroleum hydrocarbons. When biodiesel presents in sufficient amounts, fatty acid esters can form aggregates called micelles, resulting in an increased apparent solubility of hydrocarbons and improved delivery of substrates into microbial cells. However, the increment in the solubility of petroleum hydrocarbons was not always equivalent to an increase in their bioavailability. When mixed with biodiesel, only a fraction of petroleum hydrocarbons dissolved in micelle phase is directly available for microbes, and the other fraction needs to be transferred into the aqueous-dissolved phase for biodegradation (Guha & Jaffe 1996). When the addition amount of biodiesel increases, the substrates would become more diluted. A critical point would appear with increasing the biodiesel concentration. When biodiesel concentration exceeds this critical value, the enhanced bioavailability of the substrates by solubilization cannot offset the decreased transfer of substrates caused by dilution effect. As a result, the overall degradation of petroleum hydrocarbons would be reduced. Moreover, too high a dosage of biodiesel may lower TPH degradation due to competitive inhibition of substrates for microbes.

Microbial growth evaluation

The growth of the degrading microorganisms was assessed by the spread plate technique in the sand bioremediation systems. After the start of treatment, increases in bacterial number were observed over time for all treatments (Figure 3). The increase in microbial populations in the microcosms with crude oil added demonstrates that the initial TPH concentration (18,750 mg/kg of sand) did not

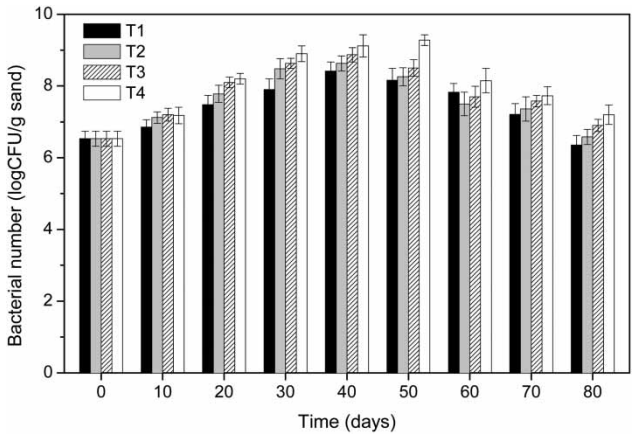

Figure 3 | Variation in bacterial number in bioremediation of oil-contaminated sand during 80 days of incubation. Error bars represent standard deviations of triplicates.

inhibit the population growth. Biodiesel supplementation demonstrated beneficial effects on bacterial population growth (Figure 3). In T1, the density of bacteria rose from an initial 3.40×10^6 to 2.62×10^8 CFU/g at 40 days. Biodiesel addition further increased this count to 4.28×10^8–1.32×10^9 CFU/g on day 40. Obviously, the bacteria grew more rapidly at higher amounts of biodiesel. The stimulation of bacterial growth by biodiesel could be attributed to the dispersion of oil and the supplement of fatty acid esters with good bioavailability and excellent biodegradability derived from biodiesel. In T4 amended with 1% biodiesel, the bacterial density on day 40 was significantly higher (1.32×10^9 CFU/g) than in other microcosms (Figure 3), whereas this microcosm gave a lower TPH removal (Figure 1). This indicates that the petroleum hydrocarbon degradation is not always correlated with the density of available degraders in the presence of additional carbon substrates. At days 30–40, microbial growth peaked and reached a maximum count. During this time, TPH was also significantly reduced (Figure 1). After this period, microbial counts continued to fall until the end of the experiment, because of the gradual depletion of the easily available carbon sources (Figure 3).

Dehydrogenase activity (DHA)

DHA is an index for overall microbial activity such as total oxidative, presenting an accurate measure of microbial capability for petroleum hydrocarbon degradation (Lu *et al.* 2009). As shown in Figure 4, DHA increased substantially after the beginning of incubation. The highest DHA was observed at day 40 in T2 (92.4 μg TPF/h/g sand), T3 (115.0 μg TPF/h/g sand) and T4 (102.2 μg TPF/h/g sand) when the greatest activity occurred at day 30 in T1 (75.3 μg TPF/h/g sand). From then on, DHA continuously decreased with time and even declined to below the initial value (21.0 μg TPF/h/g sand) in T1 at day 80. T3 presented the highest DHA from day 30 to the end of the experiment (Figure 4). A positive correlation was observed between DHA and TPH removal efficiency (Figures 1 and 4). The observed increment in DHA after the start of treatment was related to the increased substrate conversion and mineralization due to nutrient stimulation (Lu *et al.* 2009). Moreover, in this study, the reduction of DHA at the later

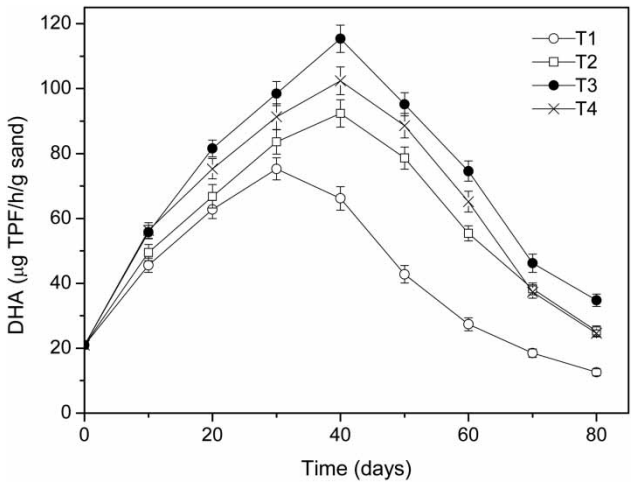

Figure 4 | Variation in DHA in bioremediation of oil-contaminated sand during 80 days of incubation. Error bars represent standard deviations of triplicates.

stage of bioremediation could be due to the accumulation of toxic intermediates, recalcitrant high-branched aromatics, and condensates. It was deemed that DHA indicates the onset of biodegradation but decreases rapidly after the biodegradation rate has declined (Lu *et al.* 2009).

Polyphenol oxidase (PPO) activity

PPO is one of the most important oxidoreductases in soil involved in the conversion of aromatic organic compounds (Sheng & Gong 2006). Figure 5 shows variations in PPO activity over the 80-day period. As shown, the changing

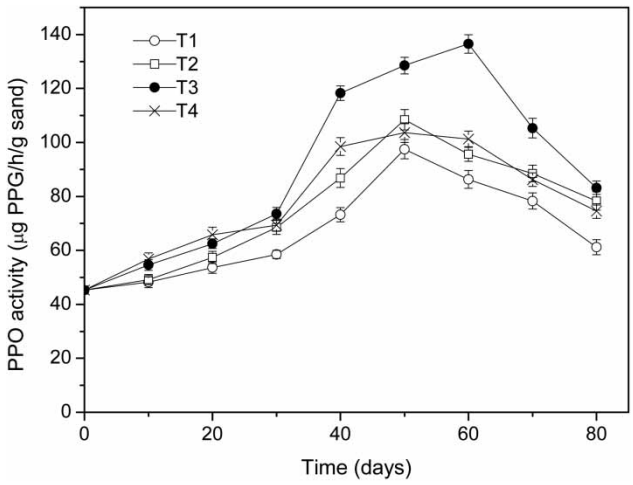

Figure 5 | Variation in PPO activity in bioremediation of oil-contaminated sand during 80 days of incubation. Error bars represent standard deviations of triplicates.

trend of PPO activity was similar to that of PAHs degradation. The initial value of PPO activity was 45.3 μg PPG/h/g sand (Figure 5). PPO activity was relatively lower in the initial period, which increased remarkably after 30 days of incubation. The addition of biodiesel increased PPO activity. PPO activity was highest in T3 at day 60, with the value of around 136.5 PPG/h/g sand and this value was about 1.4 times that in T1. In the later period, PPO activity declined with time in all microcosms. T3 showed higher PPO activity than T4 at most times, although the latter had higher density of degraders than the former. This indicates that PPO activity is not always positively correlated with the number of degraders during bioremediation. In fact, microbial enzyme activity depends on many factors, such as microbial number, substrate induction, inhibitory substances, etc.

Microtoxicity

Microtoxicity assay provides a rapid, economical toxicity measurement to evaluate the response of luminescent bacteria to chemical substances in water, soil, and sediments. Figure 6 shows variation in microtoxicity in bioremediation of oil-contaminated sand during 80 days of incubation. As shown, microtoxicity first remained stable in the initial period, and then increased in the second period of bioremediation, but thereafter decreased gradually. In the second period, microtoxicity was higher but lasted for a shorter time in T3. Higher EC_{50} values in the third period suggested an overall reduction in toxicity for all microcosms. Compared with other treatments, significantly lower toxicity ($P < 0.05$) was observed in T3 at day 80, which corresponded to its highest TPH biodegradation, as shown in Figure 1. The increased microtoxicity was due to an increase in pollutant levels in the bioremediation microcosms. This toxicity assay provided information about the impact of microbial activity for the removal of toxic compounds. Subsequent degradation of the toxic was responsible for the reduced toxicity observed at the third period.

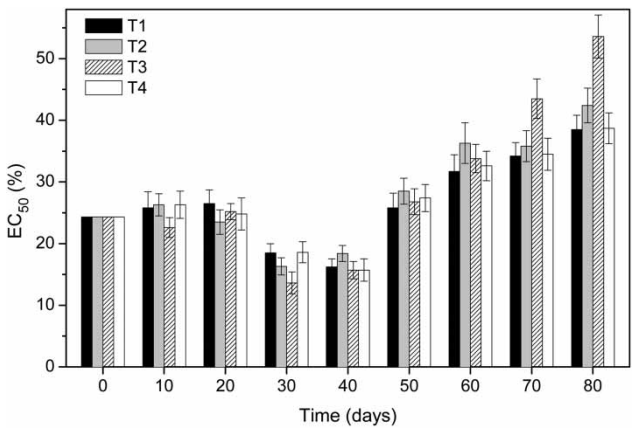

Figure 6 | Variation in microtoxicity (EC_{50} value) in bioremediation of oil-contaminated sand during 80 days of incubation. Error bars represent standard deviations of triplicates.

During biodegradation of petroleum hydrocarbons, some toxic intermediary metabolites such as aldehydes can be formed. These compounds generally have higher hydrophilicity than hydrocarbons and, therefore, can be more efficiently extracted in aqueous solution during microtoxicity assay (Xu & Lu 2010). Moreover, toxicity of crude oil increases with increasing contents of low boiling compounds, unsaturated compounds, and aromatics. Also, aromatics with higher numbers of alkyl substituents have greater toxicity, and toxicity increases along the series alkanes–alkenes–aromatics (Gargouri *et al.* 2014).

Biodegradation kinetics

In general, TPH biodegradation is assumed to be a first-order reaction with respect to TPH concentration, ignoring microbial density (Suja *et al.* 2014; Xia *et al.* 2015). The kinetic expression can be expressed by the following equation:

$$C_t = C_0 e^{-kt} \tag{2}$$

where C_0 and C_t are the initial and residual TPH concentration (mg/kg) at time t (day), respectively; k denotes the observed pseudo first-order rate constant (day^{-1}). In fact, it is difficult to reach 100% biodegradation efficiency of TPH due to various limitations. Therefore, Equation (2) is modified as Equation (3):

$$C_t = C_0 e^{-kt} \times b \tag{3}$$

where b is the variation coefficient to the ideal first-order kinetic (1.0 denotes the ideal first-order kinetic, the larger

the deviation from 1.0, the less the first-order kinetics fits). After integration and rearranging, Equation (3) becomes:

$$\ln\left(\frac{C_0}{C_t}\right) = kt - \ln b \quad (4)$$

Table 1 shows the parameters of the biodegradation kinetic model for different microcosms. As shown, the modified first-order model is passable for modeling the kinetics of the whole 80-day process. The first-order rate constant was in the same order of magnitude when compared with the results of some other authors (Suja *et al.* 2014; Xia *et al.* 2015). The rate constant in T3 was 0.0289 day^{-1} which was significantly ($P < 0.05$) higher than that in other microcosms. This suggests that the addition of biodiesel considerably accelerated the biodegradation rate of crude oil.

CONCLUSIONS

In conclusion, this comparative study conducted on the biodegradation of crude oil in sand microcosms when supplemented with different amounts of biodiesel showed positive synergistic effects. The selected bacterial consortium, together with inorganic nutrients added to the system, was efficient in biodegradation of the oil in sand microcosms. The bioremediation goal was achieved, since high removal efficiency (86.3%) of TPH was obtained for the oil added (2.0 wt.%) with the addition of 0.5 wt.% biodiesel, in comparison to 68.6% removal without biodiesel addition. This positive synergistic effect could be attributed to solvation effects. Interestingly, the biodiesel had a maximum addition amount of biodiesel where biodegradation was enhanced the most. For future work, for application to actual conditions, it will be interesting to evaluate the impact of biodegradation enhancement in seawater.

Table 1 | Kinetic equation and half-life times for different microcosms

Microcosm	Regression equation	R^2	Rate constant k (1/day)	Half-life time $t_{1/2}$ (day)
T1	y = 0.0165x −0.0121	0.9504	0.0165	42.7
T2	y = 0.0212x −0.0686	0.9673	0.0212	35.9
T3	y = 0.0289x −0.1227	0.9488	0.0289	28.2
T4	y = 0.0184x −0.0728	0.9621	0.0184	41.6

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