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ORIGINAL PAPER

Diesel degradation in soil catalyzed by the addition of a bioagent

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Abstract The supernatant harvested from a mesophilic, molasses-fed, non-methanogenic bioreactor, which is rich in nitrogen, phosphorous, metals, free amino acids, *Sporolactobacillus* sp., *Prevotella* sp., and *Clostridium* sp., is diluted with tap water and tested as the bioagent to catalyze diesel degradation in soil. Outdoor experiments are performed under the following conditions to assess the effectiveness of the bioagent: diesel doses: 9–15 mg TPH/g soil; bioagent concentrations and dose: 1–5 % at 60 ml/day; soil sample size: 600 g; ambient temperatures: 28–32 °C, and relative humidity: 40–82 % (TPH: total petroleum hydrocarbons). Diesel degradation in soil treated with 3 % bioagent, which proceeds at the rates of 1.04–1.55 mg TPH/g soil-day, is completed in about a week with up to 83 % efficiencies. In contrast, diesel degradation in soil sprinkled with water (60 ml/day) proceeds at the rate of 0.3 mg TPH/g soil-day that achieves 15–22 % degradation efficiencies. The addition of 3 % bioagent yields desired soil moisture content (10–15 %), soil pH (6.8–8.2), and nutrition inputs. Both *Sporolactobacillus* sp. and *Prevotella* sp. grown on molasses are robust, tolerant high diesel doses, able to utilize hydrophobic hydrocarbons, and readily adaptable to the soil environment. Most notably, prior acclimatization is not required to enable these properties.

Keywords Diesel degradation · Bioagent · *Sporolactobacillus* sp. · *Prevotella* sp. · *Clostridium* sp. · Soil

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Introduction

The release of hydrocarbon-based fuels into the environment is a widespread problem that has significant environmental, ecological, and health consequences (Abioye et al. 2010; Abioye 2011; Kvenvolden and Cooper 2003; Nadim et al. 2000). It is estimated that close to 900,000 metric tons of crude oil are released into the environment annually by either accidents or human activities (Abioye 2011). The remediation of contaminated sites is therefore an urgent task that requires better understanding of contamination phenomena and rational risk-mitigation assessments, since certain components in hydrocarbon-based fuels are toxic and/or carcinogenic (Abioye 2011). Among many remediation technologies and practices that have been studied and developed to handle the problems, in situ bioremediation of contaminated sites that utilize microorganisms (e.g., indigenous and/or exogenic) as the agents to render the hazardous compounds harmless appear to be versatile, environmentally benign, and cost-effective (Abioye 2011; Bento et al. 2005; Choi et al. 2002; Gentry et al. 2004; Grishchenkov et al. 2000; Kim et al. 2005; Kostka et al. 2011; Vidali 2001; Wasilkowski et al. 2012).

Numerous microbial species are capable of utilizing hydrocarbons as the carbon and energy sources, and Table 1 summarizes some bacterial species which are capable of degrading specific hydrocarbons (Abioye 2011;

Table 1 Examples of bacterial species capable of degrading hydrocarbons

Hydrocarbon	Bacterial species
n-Alkanes (C_{10}–C_{40})	*Acinetobacter* sp. (Britton 1984)
	Brevibacillus sp., *Brevibacterium erythrogenes* (*n*-pentadecane), and *Pseudomonas* sp. are able to achieve 20–25 % degradations of *n*-Alkanes (C_{10}–C_{40}) under the nitrate-reducing conditions (Grishchenkov et al. 2000)
Asphaltenes	The bacterial consortium consisting of *Bacillus* sp., *Brevibacillus* sp., *Corynebacterium* sp., and *Staphylococcus* sp. The consortium can mineralize asphaltenes under the aerobic conditions at the room temperature, salinity of 100 ppm, and pH of 7.4 (Pineda et al. 2004)
BTX	*Acinetobacter calcoaceticus* (benzene), *Acinetobacter* sp. (toluene), *Corynebacterium* sp. (*o*-xylene), and *Pseudomonas* sp. (toluene) (Britton 1984)
	The bacterial consortium consisting of *Burkholderia kururiensis*, *Cellulomonas hominis*, *Ralstonia insidiosa*, and *Serratia marcescensis*, which is isolated from the rhizosphere of *Cyperus* sp. grown in a petroleum-contaminated area, is capable of degrading BTX (30 mg/l of each) in 14 days at 28 °C (Ortega-González et al. 2013)
Diesel and Kerosene	Bacillus subtilis SPB1 is cultivated on the orbital shaker (150 RPM) at 30 °C. When dosed with 2 % of either diesel or kerosene, the bacterial strain achieves the following degradation performance at the end of 28 days: 88 and 72 % for diesel and kerosene, respectively with the addition of 0.1 % yeast extract (Mnif et al. 2014)
	The bacterial consortium consisting of *Pseudomonas desmolyticum* NCIM 2112 and *Nocardia hydrocarbonoxydans* NCIM 2386 is capable of achieving 95 % degradation of diesel in Bushnell Hass medium (10 % diesel concentration) in 7 weeks. The consortium is cultivated on the orbital shaker (120 RPM) at 30 °C (Kalme et al. 2008)
Naphthalene	*Pseudomonas putida G7* (Lee et al. 2003)
2-Nitrotoluene	*Micrococcus* sp. strain SMN-1 (Mulla et al. 2013)
PAH	*Arthrobacter* sp. strain Sphe3 is capable of achieving 90 % removal of PAH (400 mg/l) in 4 days at 30 °C in the shaking flask (180 RPM) (Kallimanis et al. 2007)
Phenanthrene	*Pseudomonas vesicularis* achieves 87 % degradation of phenanthrene (100 mg/l) in 8 days at the temperature of 30 °C and pH of 7.0 (Masakorala et al. 2013)

Gentry et al. 2004; Grishchenkov et al. 2000; Lee et al. 2003; Ortega-González et al. 2013; Pirnik et al. 1974; Shen et al. 1998; Vidali 2001). Moreover, certain microbial species (e.g., *Bacillus subtilis*, *Candida antarctica*, and *Pseudomonas aeruginosa*) are able to produce bio-surfactants to emulsify the hydrophobic hydrocarbons and thereby, rendering these compounds more susceptible to biodegradation than would otherwise be possible (Banat et al. 2000; Mnif et al. 2014; Mulligan 2005; Rahman et al. 2002). In situ bioremediation causes minimal disturbances at the contamination site, and it is less expensive to implement than many conventional remediation technologies (Vidali 2001). In situ bioremediation of hydrocarbons can take different forms depending on the Exploitation of the naturally occurring processes in soil (Korda et al. 1997). Natural attenuation is the process in which hydrocarbons in soil are degraded by the indigenous microorganisms without human interventions (Korda et al. 1997). The concurrent abiotic elimination (e.g., volatilization) and natural attenuation processes in soil often enhance the overall effectiveness of hydrocarbon remediation(Korda et al. 1997). Biostimulation also utilizes indigenous microorganisms to carry out the desired remediation reactions, but with the addition of nutrients, trace metals, and other substances (e.g., sulfur and fertilizer) to catalyze the natural attenuation processes(Abioye et al. 2010; Choi et al. 2002). Bioaugmentation involves the introduction of exogenic microorganisms in conjunction with biostimulation to boost the indigenous ones to yield further and better degradation of target compounds. The exogenic microorganisms are usually cultivated outside the soil environment using specifically designed engineering devices such as the bioreactor systems (Chou et al. 2011; Shen et al. 1998). Sometimes genetically modified microorganisms may be used (Wasilkowski et al. 2012).

The biologically mediated remediation reactions can proceed under the aerobic respiration conditions when free oxygen is readily available in soil. Alternate electron acceptors (e.g., nitrate and sulfate) can be used by certain microorganisms to carry out the remediation reactions under the anaerobic respiration conditions when free oxygen is absent. Anaerobic fermentation reactions occur when hydrocarbons are used as the terminal electron acceptors. Other factors that affect the effectiveness of in situ bioremediation include pH, temperature, and moisture content. The acceptable pH range is from 6.0 to 8.0, and the preferred moisture content is between 18 and 30 % by weight (Vidali 2001). In addition, nutrients, oxygen (or alternate electron acceptors), and water should be made readily available to microorganisms to avoid creating the pockets in soil that are devoid of microbial activities (Vidali 2001). Although the favorable temperature for in situ biodegradation was from 20 to 40 °C, however, a recent Canadian study reported that bioremediation of the

hydrocarbon-contaminated soils could occur at the temperature as low as 5 °C (Paudyn et al. 2008). The potential of oil bioremediation in cold terrestrial and marine ecosystems has also been reported (Margesin and Schinner 2001).

The supernatant harvested from a mesophilic (40 ± 1 °C), molasses-fed, anaerobic bioreactor without methanogenesis, which is rich in nitrogen, phosphorous, metals, free amino acids, *Sporolactobacillus* sp., *Prevotella* sp., and *Clostridium* sp., has been used to cure tree root rotting problems caused by *Phellinus noxius* (Lay 2013) as well as to enhance cellulosic fermentation in digested sludge (Han et al. 2015). The results suggest that the supernatant offers the combined benefits of biostimulation and bioaugmentation which are key to the success of biodegradation of complex compounds. It was therefore the primary objective of this study to investigate the feasibility of utilizing the diluted supernatant as a bioagent to catalyze diesel degradation in soil. Outdoor experiments were performed over a range of diesel doses and bioagent concentrations to assess the effectiveness of the bioagent for the intended applications.

Materials and methods

Preparation of bioagent

The supernatant harvested from a pilot-scale, mesophilic (40 ± 1 °C) anaerobic bioreactor system, which has been operated for a number of years to produce H_2 and volatile organic acids from molasses, was diluted with tap water to prepare the bioagent (e.g., 1 l 1 % bioagent was prepared by diluting 10 ml supernatant with 990-ml tap water). Table 2 summarizes the average composition of the clarified supernatant from which the bioagents were prepared.

Table 2 Supernatant composition

Component	Concentration
N	0.07 %
P	0.08 %
K	0.8 %
Ca	1.2 %
Mg	0.05 %
Fe	50 mg/l
Mn	5 mg/l
Free amino acids	270 mg/l
Sporolactobacillus sp.	1.05×10^{15} copy number/ml
Prevotella sp.	5.59×10^{13} copy number/ml
Clostridium sp.	3.35×10^{11} copy number/ml

The PCR (polymerase chain reaction) data revealed that three predominant bacterial species were identified in the supernatant when methanogenesis was absent in the anaerobic bioreactor (Chang et al. 2008; Chou et al. 2011): *Sporolactobacillus* sp., *Prevotella* sp., and *Clostridium* sp. It is noteworthy that, in addition to supplying the bacterial inoculations with diverse characteristics, the bioagent also contained many essential ingredients needed to support healthy bacterial growth (i.e., nutrients, metallic ions, and free amino acids).

Preparation of soil samples

Soil taken outside the laboratory that housed the anaerobic bioreactor system was sieved through a 40-mesh sieve to removed coarse materials. The sieved soil was divided into a number of 600-g test samples, and each sample was transferred to a glass jar. The ultra-grade diesel obtained from the China Petroleum Corporation was added to attain a specific dose, and the jar was then capped. The glass jars were placed on a roller-mixer and mixed at 240 rpm for 24 h to ensure a uniform adsorption of the diesel on soil grains. The diesel fuel-soaked soil sample was removed from the glass jar and transferred to and spread in an aluminum pan (20 cm × 11 cm × 5 cm) to form a uniform layer (thickness: 4 cm, bulk volume: 880 ml). The average bacterial count in sieved soil was 6.0×10^3 CFU/g soil (CFU: colony forming unit).

Experimental design

Three diesel doses, i.e., 9, 12, and 15 mg TPH/g soil (TPH: total petroleum hydrocarbons), were used to assess the effectiveness of bioagents that were prepared with concentrations ranging from 1 to 5 %. The soil aluminum pans were placed outside on the patio. Each test sample was sprinkled with 60 ml bioagent daily, whereas water was used in lieu of the bioagent for the untreated (or control) samples. Triplicate soil samples were prepared for a specific pair diesel dose and bioagent concentration. The ambient temperature and relative humidity during the experimental period were from 28 to 32 °C and 40 to 82 %, respectively.

A daily sample was taken from each pan during the 1st week, followed by weekly samples taken between week 2 and week 7. A designated stainless soil sampling tube (inner diameter: 2 cm, length: 10 cm) was used to take 4-g soil samples from each pan at the spots that were randomly chosen. Only one sample was taken at the same spot to ascertain the accuracy of the data. The tube was thoroughly washed with water and then cleaned with *n*-pentane between uses.

Analytical methods

The Soxhlet extraction method (NIEA M165.00C 2013) was used to extract diesel from soil grains. Two grams of soil and 2 grams of anhydrous Na_2SO_4 were added to a cylindrical extraction thimble and thoroughly mixed. The extraction thimble was then placed inside an extraction tube which sat on a flask containing 200 ml reagent-grade CH_2Cl_2 with a condenser inserted into its top. The extraction was performed for 16 h at 60 °C. After the extract was removed from the flask, the Soxhlet extractor was cleaned using a mixture of CH_2Cl_2 and C_6H_{14}. The cleansing solvents were mixed with the extract, and the mixture was then concentrated to a final volume of 1 ml. The samples were preserved at −20 °C prior to GC analyses.

An Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a DB-1 capillary column (0.32 mm × 30 m) was used to analyze the TPH in the extracts (NIEA S703.62B 2013). H_2 was used as the carrier gas at 30 ml/min, and the injection sample size was 1 µl. The temperatures of the injector and FID were 325 and 300 °C, respectively. The oven temperature was programmed as follows: at 50 °C for 6 min, increased at 15 °C/min to 310 °C, and then at 310 °C for 10 min. The ultra-grade diesel was serially diluted using CH_2Cl_2 to prepare the TPH standards.

The colony forming unit (CFU) was calculated from the dilution plating results and used to estimate the total number of bacterial cells in soil (NIEA E203.53B 2013). One gram soil sample was added to 100 ml water in a flask (both water and flask were sterilized beforehand), and the sample was mixed at 200 rpm on an orbital shaker for 30 min to dislodge the bacterial cells. A series of diluted samples were prepared from the water sample, and each test sample (0.2 ml) was spread across the surface of an agar plate and cultivated at 35 ± 1 °C for 48 h. The plates with countable bacterial colonies were used to calculate CFUs and then converted to CFU/g soil. In addition, PCR and real-time PCR (RT-PCR) analyses were performed on the soil sample that was dosed with 9 mg TPH/g soil and treated with 3 % bioagent in order to delineate the growth and/or decay patterns of *Sporolactobcillus* sp., *Prevotella*, sp., and *Clostridium* sp. as the diesel biodegradation progressed in soil. The procedures for PCR and RT-PCR analyses as well as the primer sets used are described in the Appendix 1 (Chou et al. 2011; Lay 2013).

Results and discussion

The bioagent was evaluated under the non-ideal experimental conditions in order to gain better insight into its effectiveness for real-world diesel degradation applications. First, three primary bacterial cultures identified in the bioagent (i.e., *Bacillus* sp., *Prevotella* sp., and *Clostridium* sp.), which were grown on easily degradable molasses, were not acclimatized to diesel prior to being applied to the soil samples. Second, it was likely that anaerobic bacterial cultures in the bioagent might have limited tolerance toward the ambient oxygen present in the soil matrix prepared for the experiments (i.e., soil layer thickness: 4 cm). Therefore, important questions concerning the ability of these bacterial cultures to adjust physiologically when compelled to utilize recalcitrant hydrocarbons (e.g., paraffins and naphthalenes) as carbon and energy sources in a unfavorable reaction environment could be addressed. Finally, the adsorption of diesel to soil grains would render some hydrophobic hydrocarbons less accessible to bacteria for biodegradation. Nevertheless, surfactants were not used to increase solubility, mobility, and bioavailability of these compounds. As a result of the experimental design adopted, therefore, useful data could be collected for devising desirable bioagent-based approaches to ensure the success of the endeavor in the field.

Bioagent effectiveness

Figure 1a shows examples of TPH concentrations in soil as a function of time for both untreated (or control) samples and treated samples (3 % bioagents at 60 ml/day) that were dosed with 9 mg TPH/g soil. Figure 1b shows the overall diesel degradation efficiencies in both treated and untreated soil samples. The path of diesel degradation in untreated soil was slow, and it took longer than 2 weeks to achieve about 22, 17, and 15 % degradations at the doses of 9, 12, and 15 mg TPH/g soil, respectively. Diesel degradation occurred in untreated soil could be attributable to concurrent abiotic elimination (e.g., volatilization of light hydrocarbons) and natural attenuation carried out by the microorganisms that were indigenous to soil. In contrast to untreated soil, accelerated diesel degradation in treated soil was observed to occur without delays at the doses as high as 15 mg TPH/g soil, and it was completed about a week after the startup of the experiments. Figure 1b shows that, when treated with 3 % bioagent, diesel degradation in soil could be increased to about 83, 74, and 70 % at the respective diesel doses, a clear indication of the benefits offered by the bioagent treatment. It is noteworthy that biologically enabled diesel degradation was fast and effective without prior acclimation, despite issues related to hydrophobic and recalcitrant hydrocarbons as well as the exposure of anaerobic bacterial cultures to an unfavorable reaction environment.

Figure 2 shows the chromatographic profiles of three extracts prepared from the soil sample that was dosed with 9 mg TPH/g soil and treated with 1 % bioagent. The ultra-

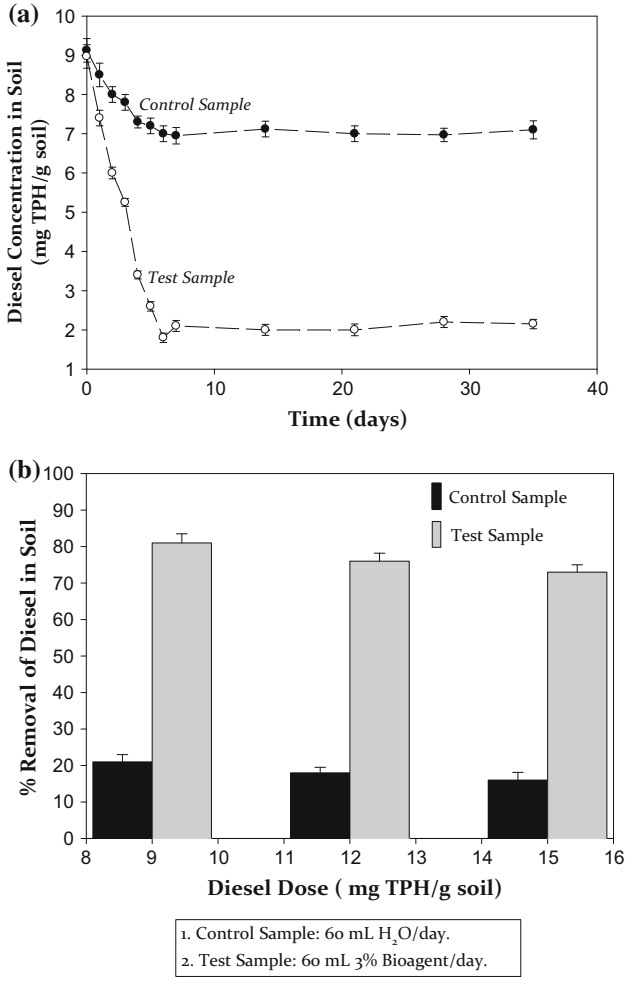

Fig. 1 **a** Diesel concentration in soil plotted as a function of time. **b** % removal of diesel in soil as a function of diesel dose

grade diesel used is a mixture of carbon chains that contain between 1 and 18 carbons per molecule, with a majority of components containing between 3 and 10 carbons per molecule. Also, the percentage of mass of the components with carbon numbers $\geq$13 was low [Fig. 2a]. Figure 2b shows that, 1 week after the startup of the experiment, the components with carbon numbers $\leq$3 and $\geq$13 were largely degraded. Moreover, the extent of degradation of components between these two groups, which accounted for the majority of TPH measured, was also significant. Figure 2c shows the chromatographic profile of the extract measured 7 weeks after the startup of the experiment. It is seen that only components with carbon numbers between 6 and 10 were detected at low concentrations.

Diesel degradation rates

The rate at which diesel was degraded in soil, R_{Diesel}, could be estimated during the period when active diesel degradation occurred. The data illustrated in Fig. 1 showed that the TPH concentration in soil, in terms of mg TPH/g soil, decreased linearly with time during the 1st week, indicating heightened biological activities during this period of time. As a result, R_{Diesel} could be calculated from the slope of the linear segment of the TPH concentration versus time curve:

$$R_{\text{Diesel}} = -\frac{\left(\frac{x}{m}\right)_2 - \left(\frac{x}{m}\right)_1}{t_2 - t_1} \quad (1)$$

where R_{Diesel} is the diesel degradation rate in soil, mg TPH/g soil-day; $\left(\frac{x}{m}\right)_1$ is the amount of TPH adsorbed per unit weight of soil measured at time t_1, mg TPH/g soil; and $\left(\frac{x}{m}\right)_2$ is the amount of TPH adsorbed per unit weight of soil measured at time t_2, mg TPH/g soil. Both t_1 and t_2 are in days. Equation (1) was derived by assuming a uniform adsorption of diesel on soil grains which was valid because of the manner the soil samples were prepared. Figure 3 illustrates the R_{Diesel} data plotted as a function of bioagent concentration. The degradation rates of untreated soil samples were included for comparison.

Diesel degradation in untreated soil yielded a fairly constant rate at about 0.3 mg TPH/g soil-day over the range of doses tested. This rate was indicative of the extent of concurrent abiotic elimination and natural attenuation processes that could be sustained at the ambient temperature (28–32 °C) and relative humidity (40–82 %) encountered. Enhanced degradation rates ranging from 1.04 to 1.55 mg TPH/g soil-day were achieved in treated soil samples depending on the doses applied. On the basis of the results illustrated in Fig. 3, the addition of 3 % bioagent at 60 ml/day would increase diesel degradation by as much as 250–400 % beyond that was achievable in untreated soil. It also is evident from Fig. 3 that the biologically mediated diesel degradation could be increased in response to increases in diesel availability. Moreover, the data illustrated in Fig. 3 reveal that increases in R_{Diesel} became marginal as the bioagent concentration was increased to beyond 3 %. Therefore, it appears that the amounts of inorganic nutrients (i.e., nitrogen and phosphorous), metals, and growth factor (i.e., free amino acids) added daily via the application of 3 % bioagent at 60 ml/day were sufficient to satisfy the bacterial requirements for carrying out diesel degradation in soil at doses as high as 15 mg TPH/g soil. Similar observations were also reported elsewhere that showed diesel degradation carried out by *B. subtilis* could benefit from the addition of either glucose (Youssef et al. 2007) or yeast extract (Mnif et al. 2014).

It is worth noting that with the ambient temperature at 28–32 °C and the relative humidity at 40–82 %, the absolute humidity of air would vary from 9.5 to 24.5 g

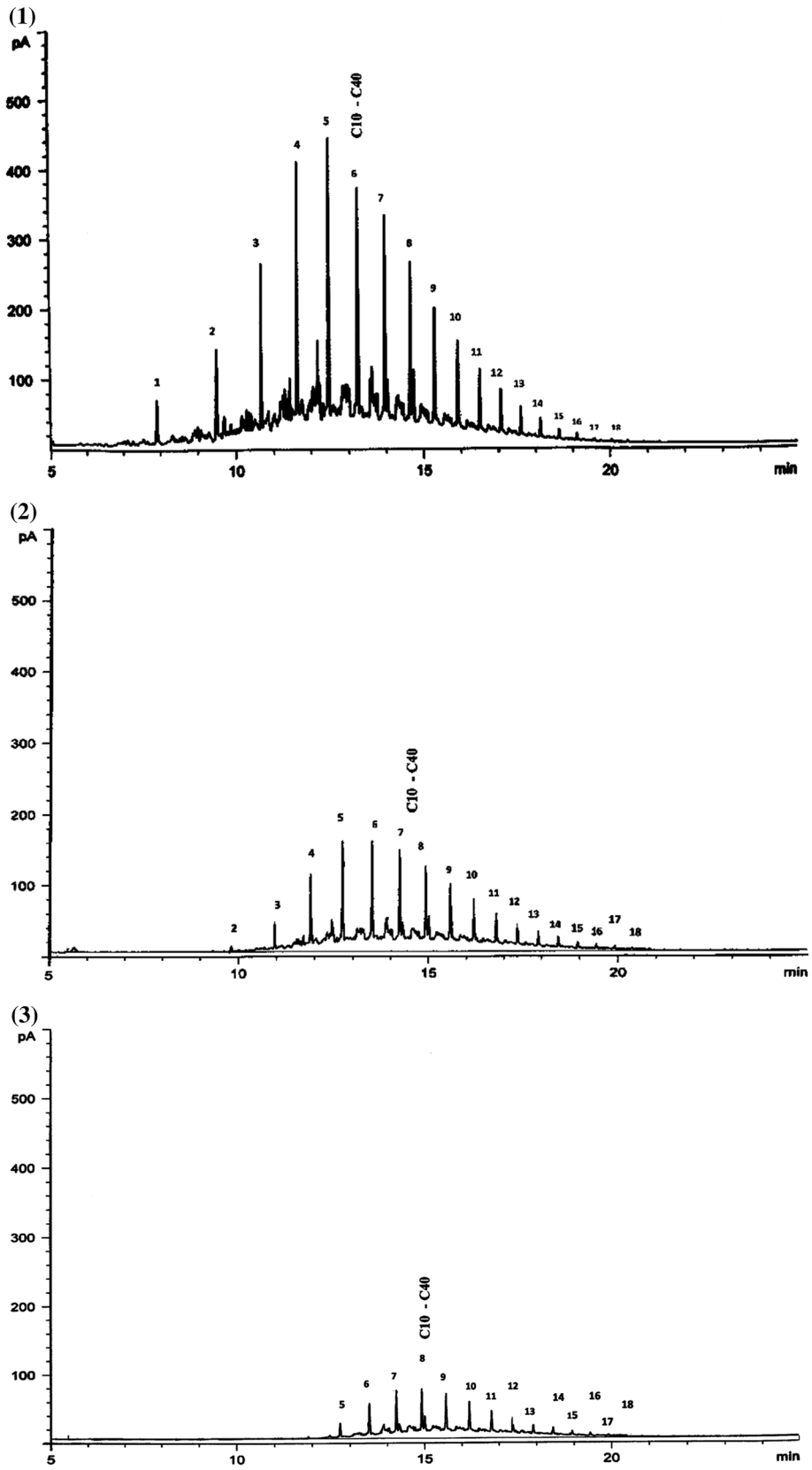

Fig. 2 Chromatographs of sample extracts (diesel dose: 9 mg TPH/g soil, 1 % bioagent dose: 60 ml/day). **1** week 0, **2** week 1, and **3** week 7

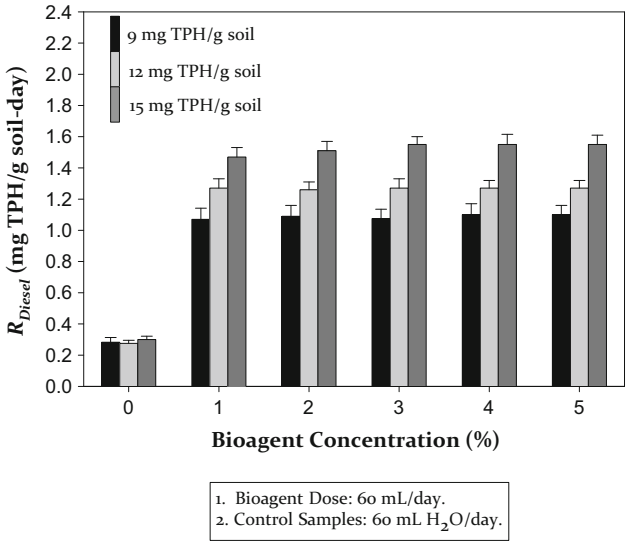

Fig. 3 Bioremediation rate of diesel fuel as a function of bioagent concentration

H_2O/kg dry air. Under such circumstances, the daily additions of 60 ml liquid (either bioagent or water) were sufficient to maintain the soil moisture content at 10–15 % by weight, which was within the recommended range for in situ bioremediation of petroleum-based hydrocarbons (Abioye 2011). Moreover, the soil pH, which ranged from 6.8 to 8.2 without control, also was close to the acceptable range of 6.0–8.0 (Abioye 2011).

Bacterial cell counts

Figure 4 illustrates the time plots of total cell count and corresponding TPH concentrations in soil that was dosed with 9 mg TPH/g soil and treated with 3 % bioagent at 60 ml/day. Also included are the copy number data of three predominant bacterial species. It is seen that a sharp increase in total cell count was coincided with active diesel degradation during the 1st week of the experiments. The total cell count started to decrease during the 2nd week and the trend persisted for the next 5 weeks. Evidently, the addition of bioagent at 60 ml/day during this period was insufficient to offset bacterial cell losses because of the unavailability of simple hydrocarbons in soil that were already degraded.

The copy number data revealed that *Sporolactobacillus* sp. were most active in the degradation of diesel in soil, followed by *Prevotella* sp. Since *Clostridium* sp. are obligate anaerobes, the species were less competitive than the other species e in the soil environment that was prepared for the experiments. The copy number of *Clostridium* sp. in test samples continued to decrease, indicating the inability of the species to grow when diesel degradation was active. The supplement of *Clostridium* sp. from the daily addition of bioagent did not reverse the trend. Therefore, it was clear that *Clostridium* sp. played an insignificant role in diesel degradation under the conditions tested. The copy number data suggested that both *Sporolactobacillus* sp. and *Prevotella* sp. grown on molasses were robust, tolerant to high diesel doses, able to utilize hydrophobic hydrocarbons, and readily adaptable to the soil environment. Since no prior acclimatization was needed to enable these properties, the finding suggested the intrinsic potential of these two species for being used as the primary agents to catalyze diesel degradation in soil.

The cell count data illustrated in Fig. 4 can be used to approximate the observed bacterial yield during the period of active diesel degradation. The increase in total bacterial cell counts was a result of two concurrent processes: the daily supplements of bioagent-contained cells to soil and the net cellular growth associated with the degradation of hydrocarbons. The average rate of increase in total bacterial cell counts during the 1st week was calculated as 1.14×10^9 CFU/day. It was estimated that 16 % of this rate (1.8×10^8 CFU/day) was attributable to the daily bioagent additions. The net cellular growth rate was therefore $0.84 \times 1.14 \times 10^9 = 0.96 \times 10^9$ CFU/day. During the same period, the diesel in soil was degraded at a rate of 1.04 mg TPH/g soil-day (or 624 mg TPH/day). The observed bacterial yield was therefore estimated to be $\frac{0.96 \times 10^9}{624} = 1.54 \times 10^6$ CFU/mg TPH.

Conclusion

The effectiveness of a bioagent to catalyze diesel degradation in soil was studied outdoors (ambient temperatures: 28–32 °C and relative humidity: 40–82 %). The bioagent was prepared via dilution from the supernatant harvested from a mesophilic (40 ± 1 °C), molasses-fed, anaerobic bioreactor without methanogenesis. *Bsporolactobacillus* sp., *Prevotella* sp., and *Clostridium* sp. were three predominant bacterial species identified in the supernatant on the basis of copy number data. The conclusions from this study are summarized as follows: Diesel degradation in soil, which was dosed with up to 15 mg TPH/g soil and treated with bioagent with concentrations up to 5 % concentration at 60 ml/day, could significantly be enhanced beyond the level that was sustained by concurrent abiotic elimination and natural attenuation. For instance, when soil was treated with 3 % bioagents, diesel degradation was completed a week after the startup of the experiments, yielding the following results at the doses of 9, 12, and 15 mg TPH/g soil (TPH: total petroleum hydrocarbons): % degradation, 83, 74, and 70; and

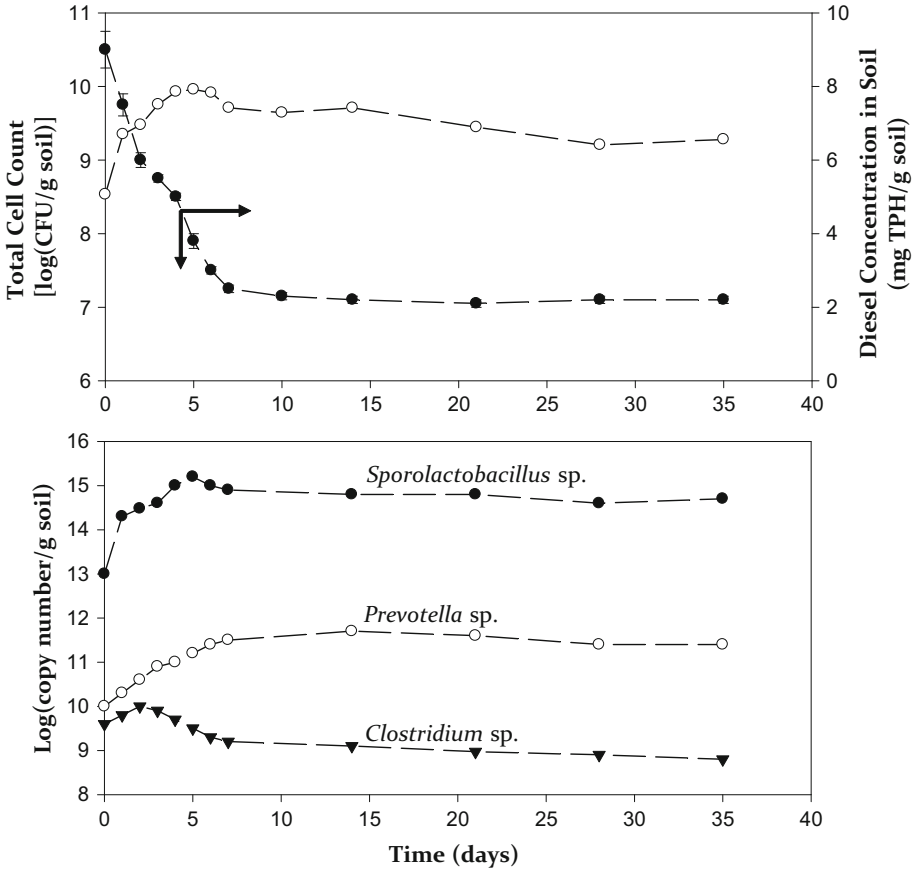

Fig. 4 Plots of total cell count, TPH concentration in soil, and copy numbers (*Sporolactobacillus* sp., *Prevotella* sp., and *Clostridium* sp.) as a function of time (TPH dose: 9 mg TPH/g soil and bioagent dose: 3 % at rlf = f)

degradation rates, 1.04, 1.35, and 1.55 mg TPH/g soil-day. By comparison, it took longer than 2 weeks to achieved 22, 17, and 15 % diesel reductions, respectively, in untreated soil when water was used in lieu of the bioagent. The associated diesel degradation rates were fairly constant at about 0.3 mg TPH/g soil-day over the range of doses tested. Under the outdoor conditions encountered, the addition of bioagents at 60 ml/day was able to maintain the soil moisture content and pH are at 10–15 % by weight and 6.8–8.2, respectively, which were within the recommended ranges for in situ bioremediation of petroleum-based hydrocarbons. Moreover, the amounts of inorganic nutrients, trace elements, and growth factor supplied by adding 3 % bioagent at 60 ml/day were satisfactory in sustaining active diesel degradation in soil at doses as high as 15 mg TPH/g soil.

Of the three predominant bacterial species identified in the bioagent, both *Sporolactobacillus* sp. and *Prevotella* sp. carried out diesel degradation. These species were robust, tolerant to high diesel doses, able to utilize hydrophobic hydrocarbons, and readily adaptable to the soil environment. More notably, prior acclimatization was unnecessary to enable these properties, despite issues related to hydrophobic and recalcitrant hydrocarbons as well as the exposure of anaerobic bacterial cultures to an unfavorable reaction environment.

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Appendix 1: PCR and RT-PCR procedures (Chang et al. 2008; Chou et al. 2011)

For PCR tests, DNA was extracted using FastDNA™ Spin Kit for Soil (MP Biomedicals, USA). The samples were processed following the procedures specified in the vendor manual. Table 3 list the primers sets used. Each reaction was performed with a mixture of 3 μl of template DNA, 2 μl (10 μM) of each primer set, 33 μl of double-distilled water and 10 μl of PCR Master Mix (BioKit, Taiwan). The thermal cycling program was: 2 min at 95 °C, 30 30-s cycles at 95 °C, 30 s at 54 °C for Prevo F/R and Sporo F/R (50 °C for the Clos L1F/R and Clos E1F/R), 30 s at 72 °C, and the program was terminated at 72 °C for 10 min. PCR results were checked with gel electrophoresis.

Table 3 Primer sets

Primer name	Sequence
Prevo F	GAGGCAGCAGTGAGGAATAT
Prevo R	GAGGCAGCAGTGAGGAATAT
Sporo F	GTGACAAACCGGAGGAAGGT
Sporo R	ATGCTGATCCGCGATTACTAG
Clos E1F	GCTGATATGACAATAATGGAAGAA
Clos E1R	GCAGCTTCCATAACTCCACCGGTTGCACC
Clos L1F	AAATCACCACAACAAATATTTGGTG
Clos L1R	ACATCCACCAGGGCAAGCCATTACTTC

The fluorescent dye, SsoFast™ EvaGreen® Supermix, was used for real-time PCR (RT-PCR) reactions using the procedure specified in the vendor's manual (Bio-Rad, Hercules, USA). Reactions were performed in a 48-well optical reaction plate and set into a 48-well MJ Mini™ Gradient Thermal-Cycler (Bio-Rad, Hercules, USA). Primers sets are listed in Table 3. Each reaction was performed in triplicate with a mixture of 2 µl of cDNA, 1 µl (3–5 µM) of each primer set, 2 µl of double-distilled water and 5 µl of enzyme Supermix. The thermal cycling program was: 60 s at 95 °C, 40 5-second cycles of at 95 °C, 5 s at 54 °C for Prevo F/R and Sporo F/R (or 50 °C for Clos L1F/R and Clos E1F/R) and followed by a dissociation stage (i.e., 15 s at 95 °C, 15 s at 65 °C, and followed by a slow temperature rise to 95 °C). Neither primer-dimer artifacts nor nonspecific PCR amplicons were observed during the melting process. The efficiency of each reaction was between 90 % and 110 %. R^2 values for all standard curves were greater than 0.99.

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