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Full Length Research Paper

The effect of fertilizer amendment on diesel biodegradation in contaminated soils

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Diesel biodegradation and the effect of application frequency were investigated in artificially contaminated soils (loam soil and sea sand) amended with commercial fertilizers and the effect of application frequency. Two sets of four equal portions (2 kg each) of diesel-contaminated soils were amended with 10% (w/w) of 2 different commercial fertilizers at 4 different but regular intervals for up to four times during a 7-week study period. The microcosms were then incubated at 30°C and sampled weekly. One sterilized soil microcosm contaminated with 10% (v/w) diesel and supplemented with 10% (w/w) fertilizer and one naturally attenuated microcosm served as controls. The amount of diesel remaining in each sample was determined using the Soxhlet extraction method and compared to the amount of diesel present in the same sample at day 0. The quantities of hydrocarbon contents in the supplemented samples and the controls were determined by GC-MS. The total heterotrophic population of each sample was also monitored. The population increase was found to correspond to the higher diesel degradation percentage in the study on sea sand. A significant enhancement ($p<0.05$) of diesel degradation was observed after the supplementation of fertilizers especially in sea sand compared with those in both controls. There were no significant differences in diesel degradation with the fertilizer application frequency. In conclusion, supplementation of diesel contaminated soils with commercial fertilizer results in a significant increase on diesel biodegradation. Additional supplementation did not further stimulate the degradation under the same conditions.

Key words: Diesel, bioremediation, fertilizers, application frequency.

INTRODUCTION

Every year, 1.3 million tonnes of oil are discharged into the environment (http://www.nature.com/nrmicro/journal/v8/n7/pdf/nrmicro2404.pdf) due to leakage from underground and aboveground storage tanks, as well as other accidental releases (Gallego et al., 2001; Juteau et al., 2003). Prolonged exposure to high concentration of diesel may result in the development of liver and kidney disease, and potential damage to the bone marrow (Propst et al., 1999; Lloyd and Cackette, 2001; Mishra et al., 2001; Atlas and Philp, 2005). South Africa is especially vulnerable to oil spills due to the high volume of oil being transported around its coastline where oil spillages have become a common occurrence (Avian Demography Unit, 2000; Brendan and Broderick, 2003). Recent Gulf oil spillage, as much as 1 million gallons of oil per day were leaking into the water, threatening wildlife along the Louisiana coast, is the largest oil spill in U.S. history. Researchers also found methane in the contaminated site at concentrations 100 to 10,000 times higher than usual (http://news.sciencemag.org/scienceinsider/2010/06/huge-oil-plumes-confirmed-but-ef.html). The magnitude of the environmental and economic catastrophe in the Gulf of Mexico will take years to calculate (http://news.sciencemag.org/scienceinsider/2010/06/new-estimate-bumps-oil-flow-to-a.html). The fact that beaches

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are not perpetually covered with tar balls because of oil spillages is due to the activity of microorganisms that can degrade the released petroleum.

Numerous studies have shown that bioremediation is an efficient and reliable method for the treatment of hydrocarbon-contaminated sites (Huesemann and Moore, 1993; Li et al., 1995; Zhou and Crawford, 1995; Liebeg and Cutright, 1999; Gogoi et al., 2003; Nano et al., 2003). Biostimulation is a type of natural remediation in which conditions are manipulated and optimized in order to improve degradation of pollutants by indigenous microorganisms (Margesin et al., 2000) and is currently considered as the most appropriate remediation technique for diesel removal in soil. Although the potential capability of the indigenous microflora to degrade oil is a function of the physical and chemical properties of the soil and oil, the environmental conditions, and the biota themselves, it is generally accepted that nutrient availability is the most common limiting factor (Atlas and Bartha, 1972; Kim et al., 2004). During the process of biostimulation, additional nutrients in the form of organic and/or inorganic fertilizer is introduced into the contaminated site, thus resulting in increased populations of indigenous microflora (Swannell et al., 1996; Dzantor, 1999; Seklemova et al., 2001; Molina-Barahona et al., 2004) that produce the catabolic enzymes that enhance biodegradation (Margesin and Schinner, 2001; Molina et al., 2004; Bento et al., 2005).

In all these studies, however, nutrients were only supplemented into the contaminated site during the initial stage of the bioremediation treatment. Therefore degradation was enhanced for only a limited period of time after which nutrients became depleted and degradation slowed down. This could have occurred before the complete removal of the contaminant was achieved since nutrient supplemental period was short. Therefore, the purpose of the study is to investigate whether nutrient addition to a contaminated site at periodic intervals can further stimulate the process on diesel degradation.

MATERIALS AND METHODS

Soil collection and analyses

Two different types of soil, namely loam and sea sand, were used in this study. Loam soil was collected from different sites on the premises of the University of KwaZulu-Natal (Westville campus), and sea sand was collected from the Blue Lagoon Beach, Durban, South Africa. All soils were air-dried, homogenized, passed through a 7.5 mm (porous aperture) Madison Test Sieve and stored at 4°C prior to further analysis. The pH and moisture content of the soils were obtained using the protocols of McCauley et al. (2003). Total volatile solids (%), total Kjeldahl nitrogen (mg N/kg) and total phosphate (mg P/kg) contents of the soil were determined according to the standard methods for the examination of water and wastewater (Clustery et al., 1998). The nitric acid extractable potassium was determined by measuring the emission signal using ICP-OES (ICP Varian 30). All the analyses were undertaken in the laboratory at the umgeni waste water management Centre, Pietermarizburg, South Africa.

Microcosm preparation

Ten equal portions (2 kg) of each soil type were prepared in 5 L glass beakers. One portion served as a background control by autoclaving at 121°C and 15 psi for 1 h for three alternative days before 10% (v/w) diesel was added (autoclaved soil + diesel). A naturally attenuated control was set up by contaminating one portion with 10% (v/w) diesel without any nutrient supplementation throughout the experiment (Natural soil + diesel). Four portions of each soil type were supplemented with 10% (w/w) commercial fertilizer F1, Grovida lawn and foliage (12.5% N; 8.3% P; 4.2% K), then contaminated with 10% (v/w) diesel (Natural soil + fertilizer+ diesel). During the study period, additional fertilizers were added to 3 contaminated soil samples after 10 days (Natural soil + 4 times application of fertilizer+ diesel), 15 days (Natural soil + 3 times application of fertilizer+ diesel) and 21 days (Natural soil + 2 times application of fertilizer+ diesel) intervals, respectively. A similar experiment was set up with the other four portions using a different fertilizer (F2) (Kompel plant food; 14.6% N; 4.5% P; 27.4% K).

The samples were thoroughly homogenized with a stainless steel spatula for five minutes, to distribute the diesel and/or nutrients through the soil particles and to enhance aeration after each nutrient supplementation. The diesel-contaminated soil samples were covered with aluminum foil and incubated at 3°C. The microcosms were watered weekly with sterile distilled water to replace evaporated water. The microcosms were sampled weekly for seven weeks to determine the amount of diesel degraded and the bacterial population. At the end of the experiment, 100 g of each sample was sent for GC/MS analysis to determine the diesel content.

Total petroleum hydrocarbon analysis

10 g of each soil sample were removed in duplicate from the soil microcosms and mixed with an equal mass of anhydrous sodium sulfate. The mixture was placed in a Whatman cellulose extraction thimble. The diesel remaining in this sample was extracted with 200 ml of dichloromethane (DCM) for 2 h at a rate of 4 cycles h^{-1} using the Soxhlet apparatus (Helaleh et al., 2001). The DCM fraction was collected in a pre-weighed 250 ml round bottomed flask and the DCM evaporated using a rotary evaporator at 40°C. The remaining diesel was quantified by weight to determine the amount of diesel degraded over time. The percentage of diesel oil degradation was determined using the amount of diesel in 10 g contaminated soil sample after the same extraction process at day 0 as 100%.

Gas chromatography-mass spectroscopic analysis

Samples of soil (100 g each) removed at the initial and final stages of the experiment were analyzed by GC/MS to determine the quantity and composition of the total hydrocarbons. GC/MS analyses of all samples were carried out in the Escom organic |analysis laboratory, Johannesburg. A Hewlett-Packard 5890 series GC system coupled to a mass spectrophotometer VG TRIO 2000 was used for the analysis. The GC/ MS was equipped with a SPB-1701 capillary column (30 m × 0.25 mm i.d × 0.25 µm film thickness) for separation, and helium carrier gas flow was 0.9 µl min^{-1} (set at 100°C). The injection port temperature was maintained at 250°C. The headspace was set at 60. kPa. The column oven was

Table 1. Physical and chemical characteristics of loam soil and sea sand in this study.

Characteristics	Loam soil	Sea sand
Total volatile soil (%)	3.29	0.43
Total nitrogen (mg n/ kg)	304	393
Phosphate (mg p/ kg)	242	618
Potassium (mg k/ kg)	685	120
pH	7.88	7.86
Gravimetric H_20 content (%)	4.67	0.078
Soil dry mass (%)	95.33	99.92

initially held at 100°C for 2 min, increase to 200°C at a rate of 10°C min^{-1}, then to 250°C at 20°C min^{-1} (held for 5 min). Data was acquired in the full scan detection mode from 45 to 350 a.m.u at the rate of one scan per second. The concentration of each carbon length was determined by comparing to a known concentration of the standard.

Enumeration of the heterotrophic count (HPC)

A soil suspension was prepared by mixing 1 g of composite soil with 9 ml of saline solution (pH 7.0). The soil suspension was serially diluted to 10^{-4} and 0.1 ml of each dilution was spread onto nutrient agar (Merck) and incubated for 24 to 48 h at 30°C (Okerentugba and Ezeronye, 2003). Two plates were inoculated for each dilution.

Statistical analysis

ANOVA was used to determine the statistical significance (SPSS version 13) between different treatments. Probability was set at 0.05.

RESULTS

The results of the soil characterization are represented in Table 1. The pH values of loam soil and of sea sand were almost the same (pH 7.88 and 7.86, respectively). Loam soil contained higher concentrations of total organic carbon (552 mg C/kg) and potassium (685 mg K/kg) whereas sea sand contained greater concentrations of nitrogen (393 mg N/kg) and phosphate (618 mg P/kg). The moisture content of loam soil was 4.67%, with 95.33% of soil dry mass. Sea sand had lower moisture content (0.078 %) with 99.92% of soil dry mass. These results reveal that loam soil has a higher water retention capacity compared to sea sand. The results in Figure 1 reveal that the biodegradation of diesel occurred in non-supplemented loam soil microcosms and those supplemented with fertilizer F1. The supplemented microcosms demonstrated significant differences compared to two controls at week 7 ($p<0.05$) with the highest percentage of diesel degradation (62.4 %) in the four-time application microcosm compared to that observed in the naturally attenuated (25.9%) and sterilized (5.3%) ones.

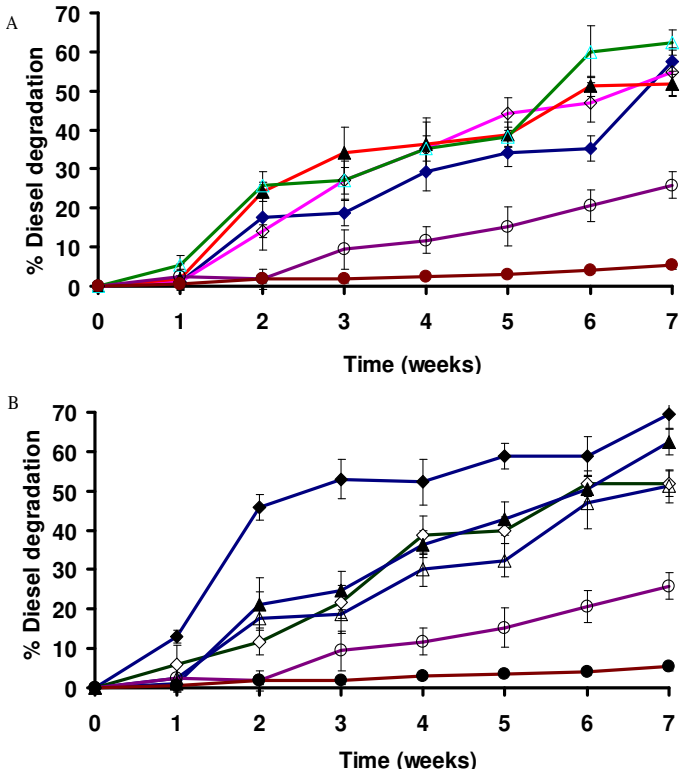


Figure 1. Percentage of diesel degradation in contaminated loam soil supplemented with (A) F1 and (B) F2 at different nutrient supplementations after 7 weeks of incubation (●: sterilized control; ○: non-supplemented control; supplemented one time; ◇: two times; ▲: three times; △: four times).

A significant increase in diesel degradation was also observed in the loam soil microcosms which were supplemented with fertilizer F2 compared with the two controls (Figure 1B). However, no trend between the percentage of degradation and the supplementation frequency was observed as the highest percentage of degradation was achieved in the microcosm supplemented once (1×) at the beginning of the experiment. There was no statistical difference observed in diesel degradation between various supplemented samples. A significant increase in diesel degradation ($p<0.05$) was also observed in the sea sand microcosms supplemented with F1 or with F2 compared with both controls (Figures 2A and B). The percentage of diesel degradation in supplemented and naturally attenuated sea sand microcosms with the exception of the sterilized control were higher than those found with the respective loam soil ones. The highest percentage (80.6 and 84.7%) of diesel degradation after 7 weeks occurred in the sea sand samples supplemented with fertilizer F1 and F2 respectively compared to supplemented loam soil samples (62.4 and 69.4%) under the same conditions. Diesel degradations in the naturally attenuated controls were 50.6 and 25.6% respectively

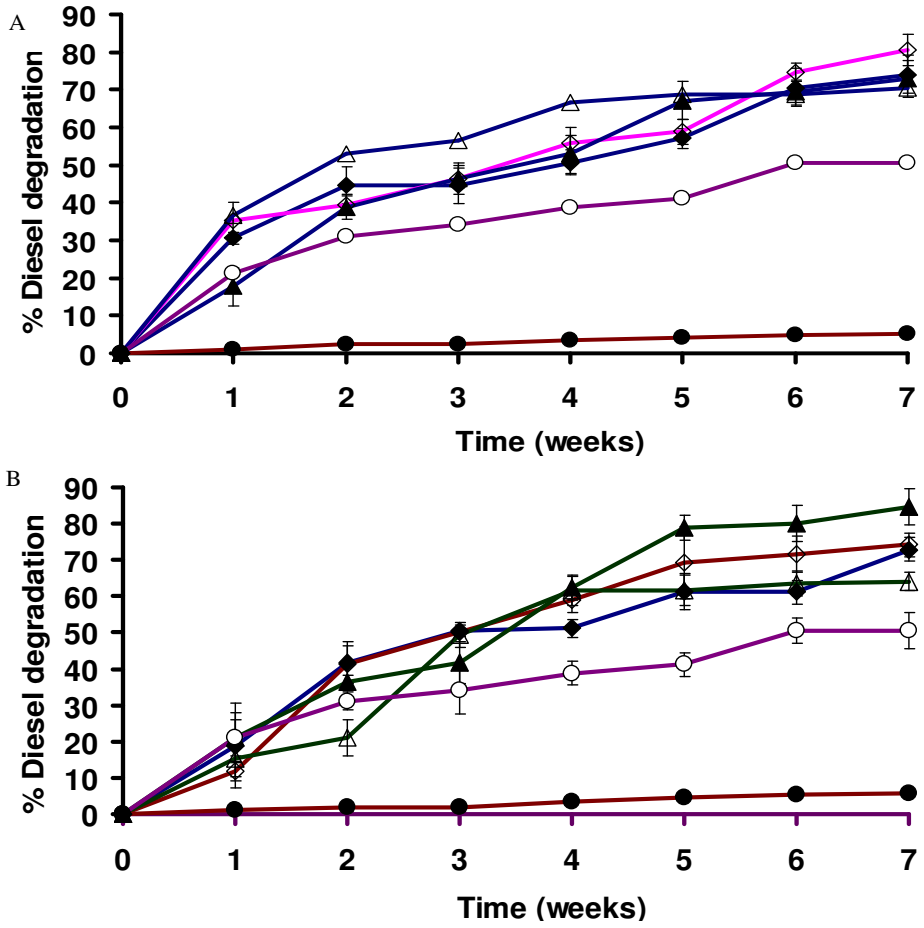


Figure 2. Percentage of diesel degradation in contaminated sea sand supplemented with (A) F1 and (B) F2 at various intervals after 7 weeks of incubation. (●: sterilized control; ○: non-supplemented control; ◆: supplemented one time; ◇: two times; ▲: three times; △: four times).

for sea sand and loam soil samples respectively. The hydrocarbon compositions ($C_9 – C_{27}$) of diesel in the day-0 and week-7 samples were analyzed by GC/MS.

The selected gas chromatograms of F1-supplemented and naturally attenuated loam soil samples and of F2-supplemented and naturally attenuated sea sand samples are shown in Figures 3 and 4, respectively. Decreases in the intensities of hydrocarbon ($C_9 – C_{27}$) in all supplemented and naturally attenuated loam soil and sea sand microcosms were observed compared with those in the sterilized samples at day 0. A slight decrease in the hydrocarbon intensity in sterilized week-7 sample was observed which might be due to evaporation. Furthermore, the distribution pattern of the hydrocarbon content in the biostimulated samples shifted to lower carbon lengths compared to those in the other microcosms indicating an accumulation of hydrocarbon with lower carbon length. However, an increase in intensity of C_{12} at day 0 from 758 mg/kg loam soil to 999.8 mg/kg loam soil at week-7 was also observed indicating that some level of diesel degradation might have taken place in the sterilized sample. The decrease in the intensities of hydrocarbon of both soil samples confirmed the degradation of diesel by the indigenous populations in the diesel-contaminated soil. However, it was not as effective in the soil samples supplemented with fertilizer. Degradation of alkanes was more pronounced in sea sand.

The growth patterns of heterotrophic microorganisms present in diesel-contaminated loam soil supplemented with F1 are revealed in Figure 5A. There was no growth detected in all sterilized samples during the study period. It was observed that there were increases in bacterial population from 2.0 x 10^6 to 3.2 x 10^6 cfu/ml during the 1st week in all supplemented microcosms and the number of microorganisms decreased (5.0 x 10^5 cfu/ml in week 6) thereafter. However, in the naturally attenatuated soil, the number of heterotrophic microorganisms continued to increase until week five (5.0 x10^7 cfu/ml). The growth patterns of heterotrophic microorganisms in diesel-contaminated loam soil supplemented with F2 are

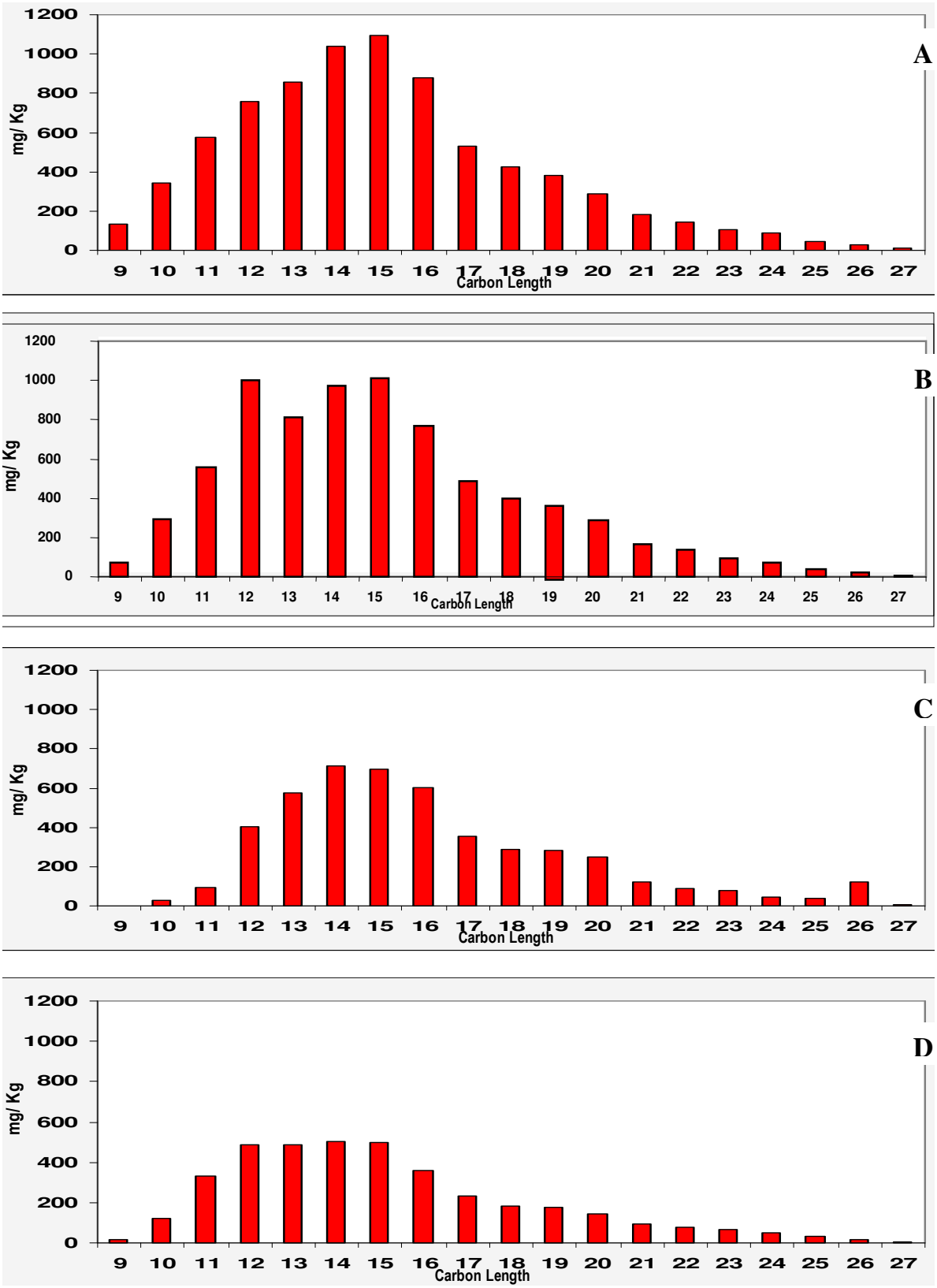


Figure 3. GC/MS analysis of diesel contents of representative diesel-contaminated loam soil samples. A: sterilized loam soil at day 0; B: sterilized loam soil at week 7; C: Untreated loam soil at week 7; D: F1-L-4x at week 7 (contaminated loam soil supplemented every 15 days with F1)

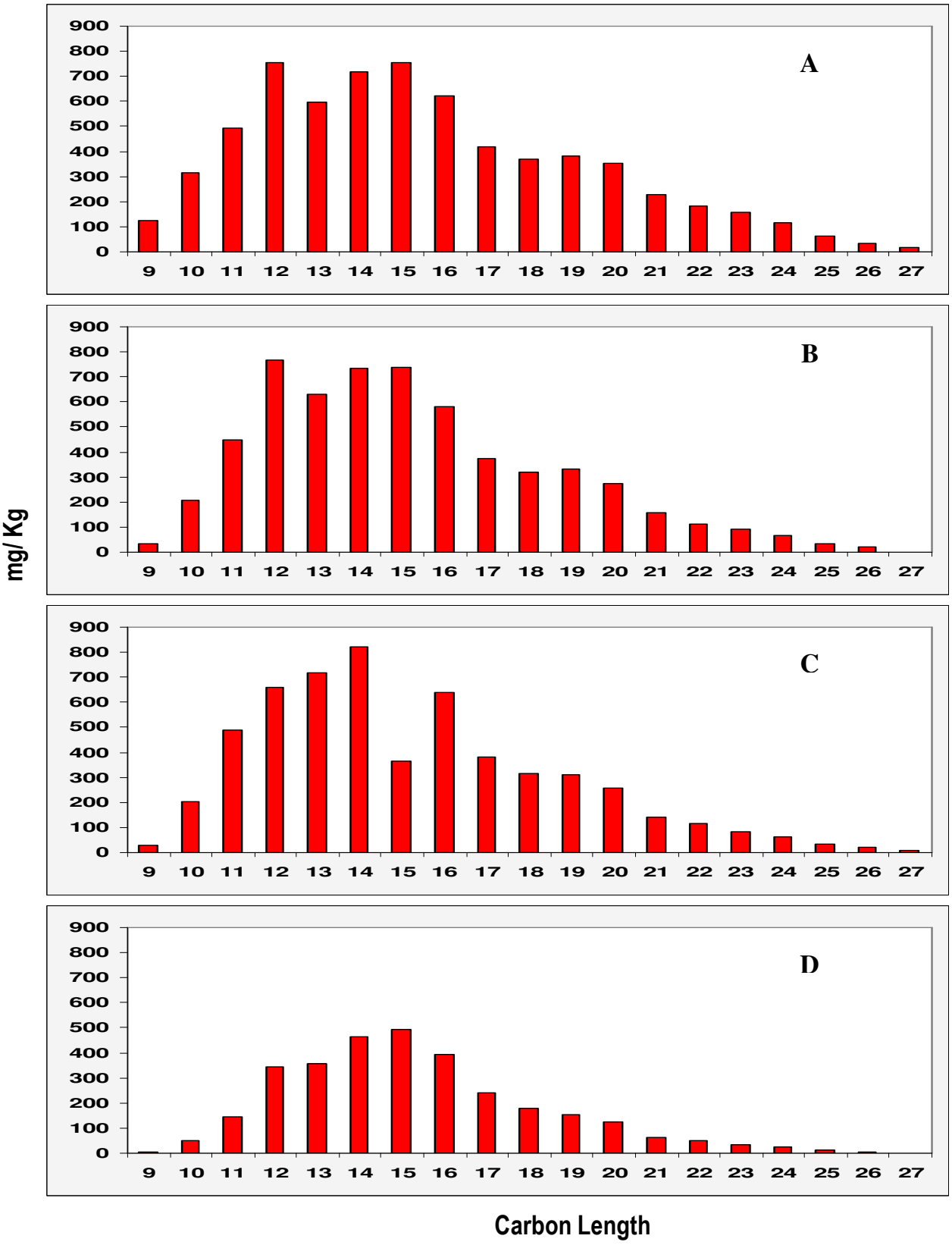
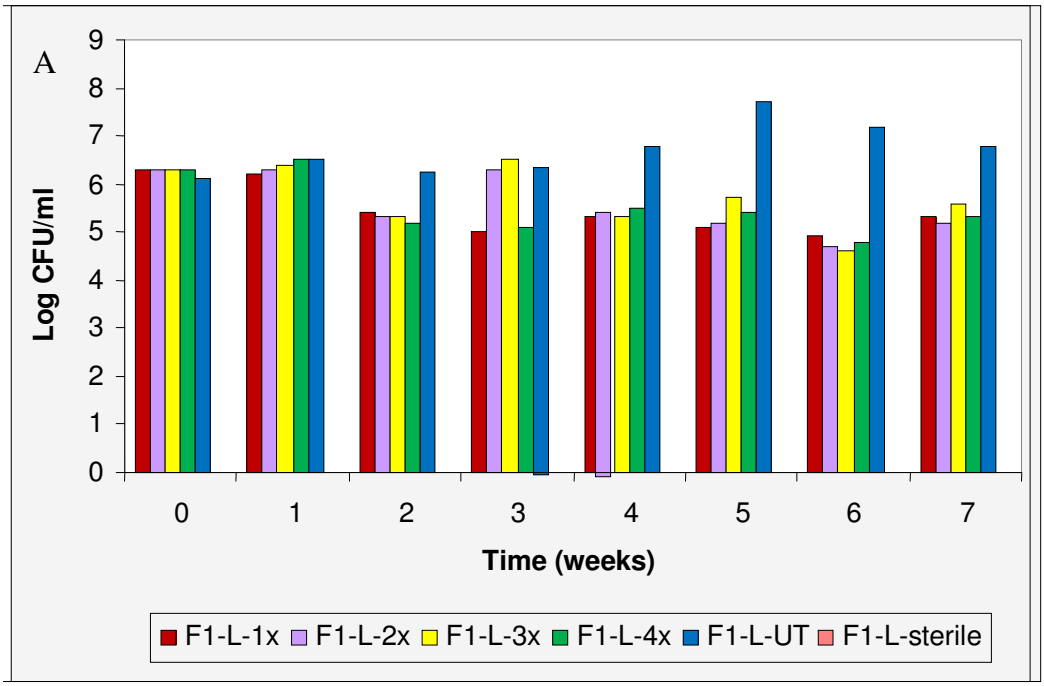


Figure 4. *GC*/MS analysis of diesel contents of representative diesel-contaminated sea sand samples. A: sterilized sea sand at day 0; B: sterilized sea sand at week 7; C: Untreated sea sand at week 7; D: F2-S-3x at week 7 (sea sand supplemented every 20 days with F2).



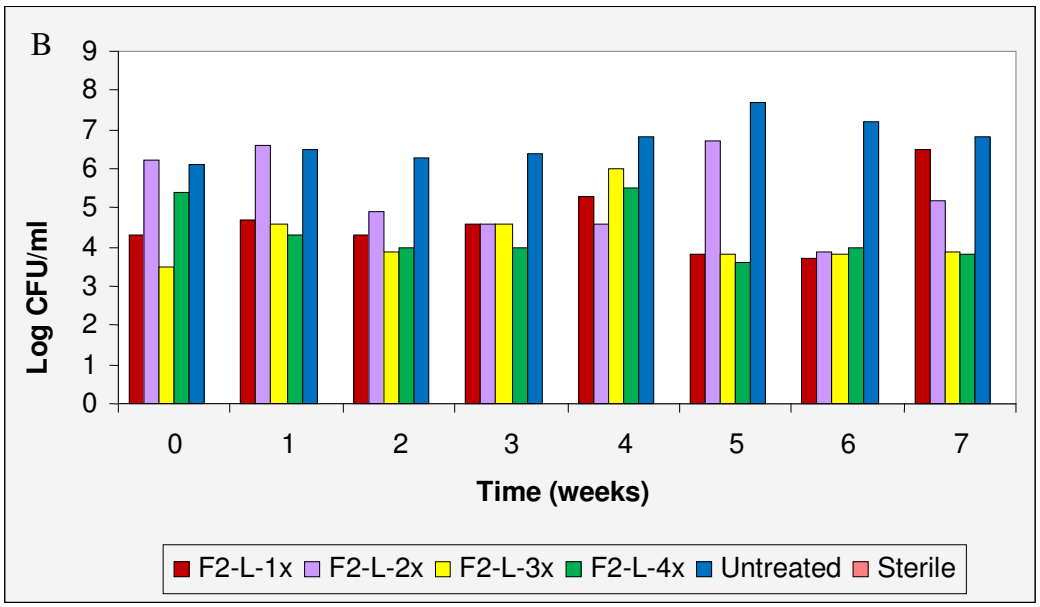


Figure 5. Enumeration of bacteria in diesel-contaminated loam soil supplemented with F1 (A) or with F2 (B) during 7 weeks of incubation

revealed in Figure 5B**.** The supplementation of F2 onto the contaminated soil inhibited the growth of microbial population compared to F1 and naturally attenuated microcosm. Fertilizer supplementation had an inhibitory effect on the microbial population in the contaminated sea sand. However, the population increase was found to be higher (Figures 6A and B) during the diesel degradation, which seems to be corresponding to the higher diesel degradation percentage in the study.

DISCUSSION

Our study demonstrates the stimulating effect in amending fertilizer to diesel-contaminating sites. A significant increase in diesel degradation ($p<0.05$) was also observed in the loam soil microcosms (Figure 1) and in the sea sand microcosms (Figure 2) supplemented with F1 or with F2 compared with both controls. Biostimulation of indigenous microorganisms by the addition of

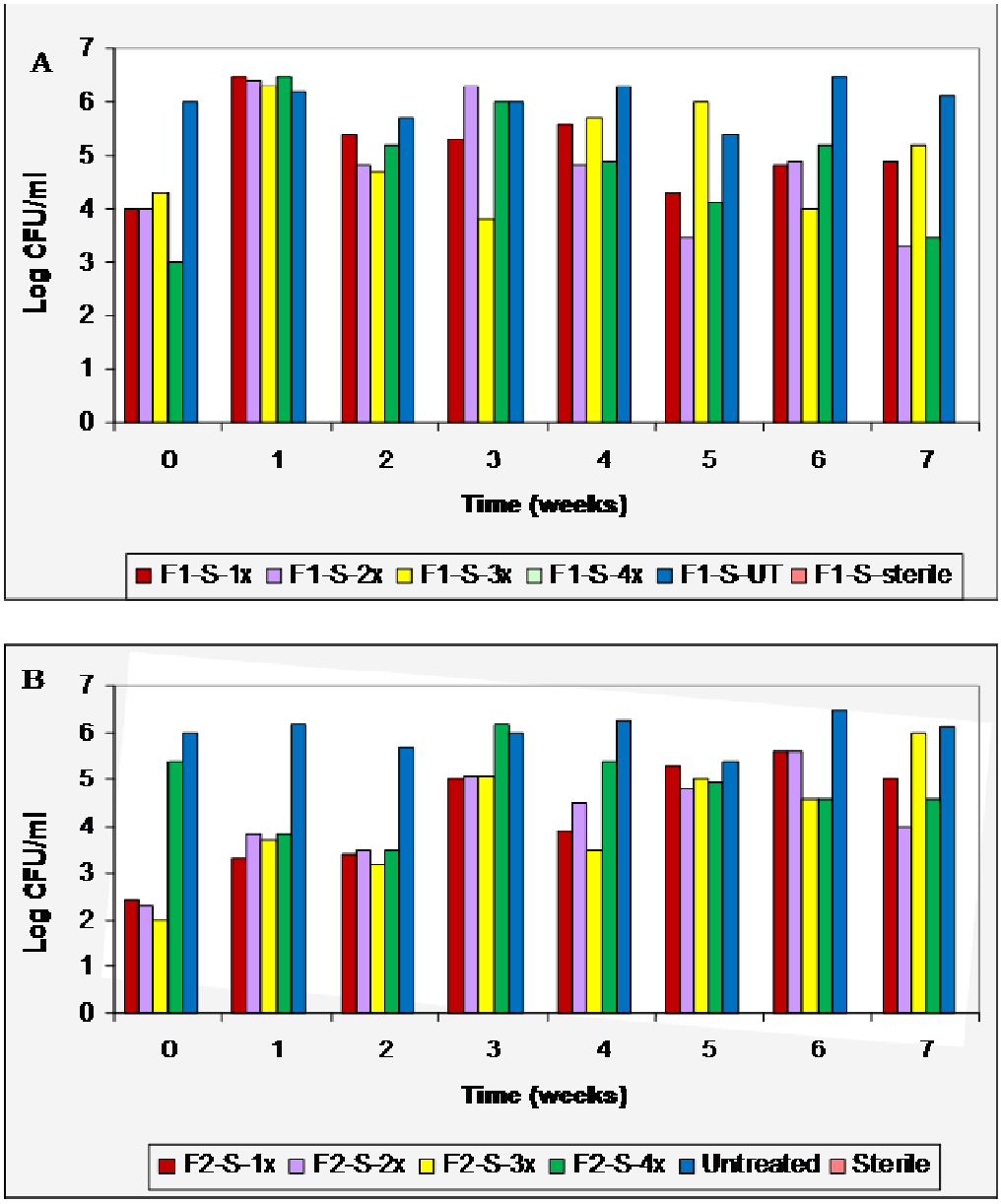


Figure 6. Enumeration of bacteria in diesel-contaminated sea sand supplemented with F1 (A) and F2 (B) during 7 weeks of incubation

inorganic nutrients such as nitrogen, phosphorous and potassium to balance the high carbon content due to hydrocarbon contamination has been widely used in diesel contaminated soils (Molina-Barahona et al., 2004; Perfumo et al., 2006). In numerous field trials, the feasibility of adding inorganic nutrients has been demonstrated as a means of sustaining elevated nutrient cones within the sediments for effective bioremediation (Lee and Levy, 1991; Venosa et al., 1996). Controlled studies suggest that optimum rates of degradation could be sustained by retaining high but nontoxic, levels of nutrients (Venosa et al., 1996; Lee et al., 1997). A range of additional nutrient sources have been used including inorganic fertilizers, urea, sawdust, compost, manure, and biosolids (Rosenberg et al., 1992; Walworth and Reynolds, 1995; Cho et al., 1997; Williams et al., 1999; Namkoong et al., 2002) as well as in this study. One of the most widely used products for natural environment amendment is oleophilic fertilizers. This source of nutrients has been widely applied in marine oil spills (Bragg et al., 1994; Diez et al., 2005) since it adheres to hydrocarbons and supplies nutrients at the oil-water interface. Other advantages of inorganic agricultural fertilizers as bioremediation agents include low cost,

availability and ease of application. Furthermore, these organic nutrient formulations may also provide trace elements and other growth factors required by bacteria (Lee and Merlin, 1999). Current developments have anticipated the effectiveness of these nutrients in the treatment of hydrocarbon-polluted groundwater and soil (Gallego et al., 2001).

Despite its wide use, reports on the effect of fertilizer on the diesel biodegradation process are not consistent in the literature. Several reports demonstrate a positive impact of fertilizers on oil biodegradation (Alexander, 1994; Margesin and Schinner, 1999, 2001; Lee et al., 2007; Rosa and Triquis, 2007). Bento et al. (2005) found that natural attenuation was more effective than biostimulation in Hong Kong soil. Another field study found that adding nutrients had no effect on decontamination of polluted soils (Seklemova et al., 2001). Walworth et al. (2006) reported that the addition of the proper amount of nitrogen nutrient could increase the biodegradation rate. However, excess nitrogen can also depress microbial activity and petroleum degradation in contaminated soils due to the depression of osmotic soil water potential. This study also shows that nutrient addition stimulates the diesel-degrading capabilities of the indigenous microorganisms significantly in the contaminated soils (Figures 1 and 2), as compared to the naturally attenuated (without nutrient supplementation) ones. The effect of fertilizer supplements on diesel degradation was also evidenced by the results of GC/MS (Figures 3 and 4).

Significant reduction in diesel content (C9-C27) was observed in the biostimulation samples compared to the natural attenuation and the sterilized controls. Margesin et al. (2007) found that hydrocarbon concentration and incubation time are important factors during bioremediation of diesel-contaminated soil. The higher the initial contamination, the more marked was the effect of fertilizer supplements. Accidental release of large amounts of hydrocarbons into ecosystems produces extremely high C/N and/or C/P ratios, which are not favourable for microbial growth (Leahy and Colwell, 1990). In this study, 170 g of diesel (10%, density of 0.85 g/ml) was introduced onto 2 kg of microcosm resulting in a high C/N ratio of 28 (calculated based on total nitrogen content in loam soil in Table 1). Laboratory and field studies have demonstrated that the optimal C:N:P ratio for hydrocarbon biodegradation is 100:15:3 (33:5:1) (Zitrides, 1983; Rosenberg and Gutnick, 1986; Venosa et al., 1996; Riser-Roberts, 1998; Boufadel et al., 1999; Wrenn et al., 2006).

Therefore, sufficient quantities of nitrogen and phosphate are required to stimulate the growth of indigenous microorganisms present in contaminated soils. The addition of 25.2 g of nitrogen source from the supplementation of 10% fertilizer increased the C:N ratio of the soil closer to the bacterial C/N requirements. This allowed the microorganisms to break down the organic pollutants at a faster rate as shown in our study and others (Dzantor, 1999; Ausma et al., 2002). The results were supported by Margesin and Schinner (1997) and Molina-Barahona et al. (2004) that showed a significant increase in diesel degradation by biostimulation using C:N ration of 10:1. Bento et al. (2005) demonstrated that the natural attenuation process was more efficient in degrading diesel than the biostimulation process. However, the soil samples used in the above study contained a high content of nitrogen source resulting in high C:N ratio (~ 5:1) before the addition of further nutrient sources. Subsequent supplementation of nutrients did not stimulate the degradation process. Molina et al. (2004) further demonstrate that a higher C:N ratio (100:30) slows down the degradation rate.

This study also shows that the biostimulation and the natural attenuation processes in diesel-contaminated sea sands were more effective than in contaminated loam soil. The percentage of diesel degradation was up to 80.6, 84.7 and 50.6% in sea sand compared with 62.3, 69.4 and 25.6% in loam soil supplemented with F1, F2 and no supplementation (naturally attenuation), respectively, under the same conditions. This correlates with studies conducted by Bento et al. (2005) in which beach sand demonstrated the most degradation of total petroleum hydrocarbons. Therefore, the soil type should be an important factor in bioremediation (Ghazali et al., 2004). The small pore space in loam soil might cause low oxygen diffusion rate and limits the accessibility of the target compound for degradation by microbes as suggested by Tisdale and Nelson (1975). Buddy et al. (2002) found that diesel contamination results in the development of different community profiles in different soil types, therefore different soils have different inherent microbial potential to degrade hydrocarbons.

In this study, the native microbes especially in the sea sand seem capable of utilizing diesel efficiently as energy and carbon source without fertilizer supplements. The results in this study as well as others suggest that the soil types and the indigenous microbial population should be taken into account in impact and risk assessment. Therefore, detailed site specific characterization studies are needed prior to deciding on the proper bioremediation approach. Despite the enhancement of diesel degradation capacities of indigenous microorganisms by supplementing fertilizer onto the contaminated soil, there is no significant difference in the percentage of diesel degradation at the end of the study period by increasing the frequency of fertilizer supplementation. Adding fertilizer onto the contaminated sea sand actually suppressed the initial microbial population as shown in Figures 6. The reduction in population however did not affect the diesel degradation capacity in the remaining microbial population.

The remaining microbial population increased steadily up to week 4 and the diesel concentration decreased.

The higher population observed in the naturally attenuated samples both in loam soil and in sea sand did not translate into a higher degradation rate. There was probably more competition in the existing microbial population that utilized nutrients (such as nitrogen source) other than the carbon source, diesel. The microbial population in the biostimulation samples slowly decreased after the initial increases. In the literature, alkane degradation ability by the microorganisms depends on the structure and length of hydrocarbon source. Longer carbon chain compounds are easier to degrade than those shorter than C_9 with the exception of methane (Cookson, 1995; Whyte et al., 1998). A wide variety of microorganisms can readily degrade longer-chain aliphatic hydrocarbons. Different patterns can be proposed for liquid *n*-alkanes, C_{12} -C_{16}, low solid *n*-alkanes C_{17} -$C_{28,}$ and high solid alkanes above C_{28} (Setti et al., 1993). The susceptibility to degradation is proportional to increasing carbon numbers (Del' Arco and de Franca, 2001; Plohl and Leskovsek, 2002). It was found that the chain compounds shorter than C_9 were more difficult to degrade than longer chains. *Acetinobacter* sp isolated in our laboratory (Mandri and Lin, 2007; Singh and Lin, 2008) capable of degrading diesel and used engine oil effectively, failed to utilize the short chain hydrocarbon (less than 9) as the sole carbon source (Nadoo, 2007). The addition of fertilizer might allow a rapid degradation of long chain hydrocarbon source as shown in Figures 3 and 4 but results in an accumulation of the short chain hydrocarbon. Short chain alkanes are reported to be toxic for many microorganisms (Cookson, 1995; Mehrashi et al., 2003) resulting in a decrease of microbial population.

Conclusion

Bioremediation can be a viable and effective response to soil contamination by petroleum hydrocarbons. Micro-organisms present in all soil samples showed the ability to degrade diesel. This study demonstrates that addition of fertilizers to diesel-contaminated soil (loam and sea sand), stimulated the microbial population and showed increased degradation rates especially during the initial stages of degradation. There is no significant effect in the level of diesel degradation, however, by increasing the supplementation frequency. These results clearly suggest that the effects of biostimulation are based on several factors such as soil type, environmental factors, nutrients and the structure of the microbial community present in the soils. Detailed site-specific characterization studies are needed prior to deciding on the proper bioremediation approach.

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