

*Full Length Research Paper*

# Bioaugmentation efficiency of diesel degradation by *Bacillus pumilus* JL<sub>B</sub> and *Acinetobacter calcoaceticus* LT<sub>1</sub> in contaminated soils

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The abilities of diesel-degrading *Bacillus pumilus* JL<sub>B</sub> and *Acinetobacter calcoaceticus* LT<sub>1</sub> were tested in contaminated soils. The effect of nutrient supplementation on bioaugmented samples was also examined. The results show that bio-augmentation and biostimulation accelerated significantly ( $p < 0.05$ ) the diesel degradation in the contaminated loam soil and sea sand. Supplementing fertilizers to the augmented loam samples did not result in a significantly higher degradation rate. Furthermore, *A. calcoaceticus* LT<sub>1</sub> alone failed to stimulate higher degradation rates in sea sand unless further supplementation of fertilizer. The results from environmental scanning electron microscopy demonstrate the population increases, then decreases in augmented samples corresponding to the level of diesel degradation. Fungi-like microorganisms became dominant in contaminated loam soil at the end of the study but not in sea sand. The study shows that it is critical not only to understand the physiology of the inoculum but also how it affects microbial community structure and function before the microorganism being introduced in the contaminated soil.

**Key words:** Diesel, bioremediation, bioattenuation, bioaugmentation, biostimulation.

## INTRODUCTION

Industrial development, population growth, urbanization and a disregard for the environmental consequences of releasing chemicals into the environment all contributed to the modern pollution situation. Over 2 billion tons of petroleum is produced annually worldwide. Spillages of oil have become a common occurrence. Depending on the site location, the level of oil contaminants in the soil may be as high as 10% (w/w) (Gogoi et al., 2003). Large quantities of hazardous substances are carelessly disposed of in the environment and are thus creating enormous pollution problems in soils and waters around the world (Atlas and Philp, 2005). Petroleum compounds are considered to be recalcitrant to microbial degradation and persist in ecosystems because of their hydrophobic nature and low volatility and thus they pose a significant

threat to the environment (Abed et al., 2002). Protective and preventive measures need to be taken into account to avoid spillage into the environment.

Since hydrocarbons are natural products, it is not surprising to find organisms that are able to degrade these energy-rich substrates (Delille et al., 2002). The ability of microbes to degrade organic contaminants into harmless constituents has been explored as a means to biologically treat contaminated environments. Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contaminants, has been established as an efficient, economic, versatile and environmentally sound treatment (Atlas and Philp, 2005; Mehrashi et al., 2003).

The extent of hydrocarbon biodegradation in contaminated soils is critically dependant upon several factors like environmental conditions and the bioavailability of the contaminants to microorganisms (El Fantroussi and Agathos, 2005; Romantschuk et al., 2000). Biostimulation is considered as a most appropriate remediation technique

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for diesel removal in soil and requires the evaluation of both intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the *in situ* process (Molina-Barahona et al., 2004). However, when few or no indigenous degradative microorganisms exist in a contaminated area or when there is no time for the natural enrichment of a suitable population, bioaugmentation can be a realistic option (Atlas and Philp, 2005; Mehrashi et al., 2003).

Identification of the key organisms that play a role in pollutant degradation processes is relevant to the development of optimal *in situ* bioremediation strategies (Abed et al., 2002; Watanabe, 2002). The microorganisms are often cultured *ex situ* before being applied to the contaminated site although they may originally have been indigenous to the site (Stallwood et al., 2005). Several bacterial strains isolated have demonstrated their abilities of utilizing diesel and used engine oil effectively as sole carbon sources (Mandri and Lin, 2007; Singh and Lin, 2008). Further studies show that the supplementation of fertilizers to diesel contaminated soils significantly enhanced diesel degradation in early stage, but there were no significant differences in the level of degradation after a longer incubation time (Singh and Lin, 2009).

The objective of this study is therefore to investigate the impact of bioaugmentation in combination with biostimulation on diesel biodegradation in diesel contaminated soils.

## MATERIALS AND METHODS

### Preparation of inoculum

Two bacterial strains JL<sub>B</sub> (*Bacillus pumilus*) and LT<sub>1</sub> (*Acinetobacter calcoaceticus*) as described earlier (Singh and Lin, 2008) were used in this study. The bacteria were grown in nutrient broth at 37°C overnight at 150 rpm. The isolates were then centrifuged at 4000 rpm and the pellet was washed three times with 0.85% (w/v) saline solution. The inoculum was standardized to a final absorbance of 1.0 at 600 nm using saline solution.

Two different soil types (loam and sea sand) were collected at the University of KwaZulu-Natal Westville campus and from a beach near Durban, South Africa, respectively. All soils were air-dried, homogenized, passed through a 7.5 mm (porous aperture) Madison Test Sieve and stored at 4°C prior to the experiment. The soils were analyzed for total organic carbon, nitrogen, phosphorous, potassium by the laboratory at the Umgeni Waste Water Management Centre as described in the previous study (Singh and Lin, 2009). The pH and moisture content of the soils were obtained using the standard protocols (McCauley et al., 2003).

### Microcosm preparation

Ten equal portions (2 kg each) of loam soil or sea sand were prepared by artificially contaminating the soils with 10% (v/w) diesel oil. Six portions were inoculated with 20 ml (1%, v/w) of individual standardized bacterial inoculum with or without the supplementation of two different commercial fertilizers (10%, w/w). The other 3 portions were inoculated with 1% consortium (10 ml each) with or without 10% (w/w) fertilizer. The remaining portion without any

supplement was used as the control. The samples were homogenized and after sampling distilled water was evenly distributed to promote aeration and moisture within the soil matrix. The contaminated soils were placed in 5 litre glass beakers and covered with the aluminum foil at 37°C. The samples were removed at a regular interval and were analyzed as described below.

## Total petroleum hydrocarbon analysis

### Diesel oil extraction

Duplicate soil samples (10 g) removed from the soil microcosms at different time intervals were mixed with an equal volume of sodium sulfate (anhydrous). The mixture was placed in a cellulose extraction thimble (Whatman). The remaining diesel oil in the contaminated soil was extracted using the Soxhlet apparatus with 200 ml of dichloromethane for 2 h at a rate of 4 cycles h<sup>-1</sup> (Murad et al., 2001). Dichloromethane of the extracted samples were evaporated using a rotary evaporator at 40°C. The remaining diesel oil was quantified by weight to determine the amount of diesel oil degraded over time. The percentage of diesel oil degradation was determined using the amount of diesel oil in the same microcosm at day 0 as 100%.

## Environmental scanning electron microscopy

Representative samples were also examined under the Environmental Scanning Electron Microscope (ESEM). The soil samples were mounted onto aluminum stubs with double sided carbon tape and viewed under low vacuum (approximately 1 torr) using the large field gaseous secondary electron detector (LFD) of the Philip XL30 ESEM at 15 kV, spot 3 - 4 (images would have the detector written as GSE on databar). High vacuum images were also obtained after being coated for 45 min with 40% gold: 60% palladium using a Palaron E5100 sputter coater (Centre for Electron Microscopy, UKZN).

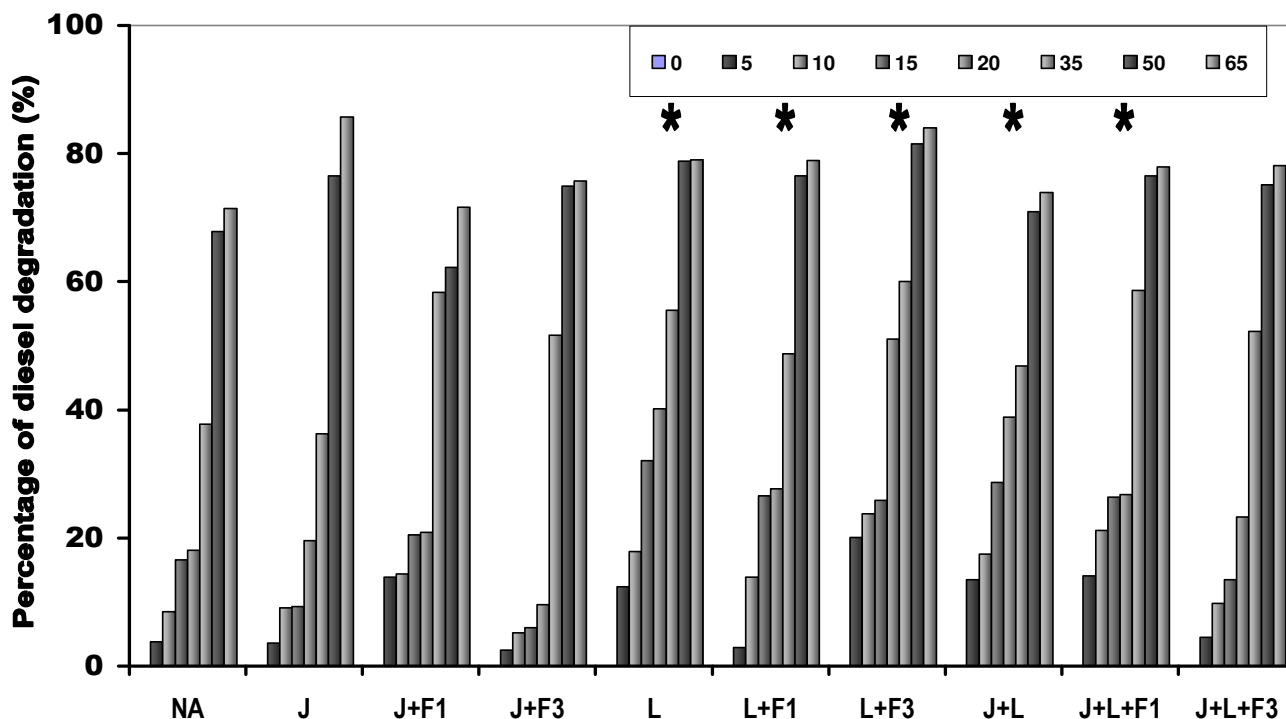
## Statistical analysis

Student t-tests were used to examine the statistical significance (SPSS version 13) between different treatments. Probability was set at 0.05.

## RESULTS

The results of the soil analyses indicated that loam soils were slightly acidic (pH 6.6) and possessed significantly higher concentrations of total organic carbon (552 mg C/kg), total nitrogen (1696 mg N/kg), potassium (1228 mg K/kg) and phosphate concentrations (471 mg P/kg), while the sea sand was alkaline (pH 9.08) and had low concentrations of all chemical parameters measured (34.2 mg C/kg; 12 mg N/kg, 46.6 mg K/kg and 363 mg P/kg, respectively).

The biodegradation rates of diesel in loam soil under various bio-remedial treatments are shown in Figure 1. A minimal of 71% and up to 85.7% of diesel degradation was achieved in contaminated loam soils after 65 days under various bio-treatment processes. The results indicate that the indigenous microbial populations are



**Figure 1.** The percentage of diesel degradation in loam soil with different amendments for 65 days. NA: non-amended; J: augmented with *Bacillus pumilus* JL<sub>B</sub>; L: augmented *Acinetobacter calcoaceticus* LT<sub>1</sub>; F1: supplemented with Fertilizer F1; F3: supplemented with Fertilizer F3; \*: significant difference,  $p < 0.05$  compared to NA.

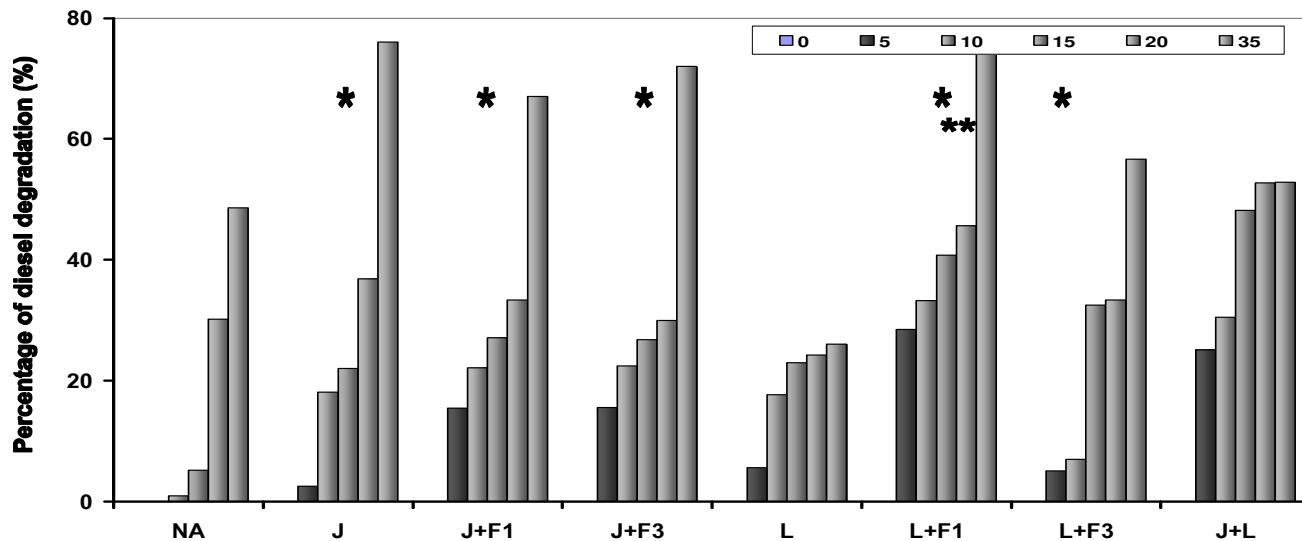
capable of degrading diesel contaminants. Addition of bacterial isolate, *B. pumilus* JL<sub>B</sub> and/or *A. calcoaceticus* LT<sub>1</sub> accelerated the diesel degradation to 85.7 and 79%, respectively, compared with 71.4% of non-augmented soil after 65 days. The combination of biostimulation and bioaugmentation also had significantly higher degradation rates ( $P < 0.05$ ) compared to the control. However, additional fertilizers to the contaminated soils which was augmented with the bacterial isolate (*B. pumilus* or *A. calcoaceticus*) did not seem to enhance a higher degradation rate than the sample augmented with the individual isolate, nor did the consortium samples. The degradation rate increased by adding *A. calcoaceticus* LT<sub>1</sub> was found to be statistically significant ( $p < 0.05$ ), but not by augmenting *B. pumilus* JL<sub>B</sub> ( $p > 0.05$ ).

The results of diesel biodegradation under various bio-remedial treatments in sea sand are shown in Figure 2. The presence of *B. pumilus* JL<sub>B</sub> isolate in contaminated sea sand with or without fertilizer resulted in a significant higher diesel degradation (76%) compared to the control (49%). The samples augmented with *A. calcoaceticus* LT<sub>1</sub> alone showed low degradation rates throughout the experiment. Further supplementation of fertilizer F1 significantly increased ( $P < 0.05$ ) the diesel degradation ability of *A. calcoaceticus* LT<sub>1</sub> in the contaminated sea sand. The fertilizer F2 supplementation increased the degradation rate, but not significantly. Although the positive impact of adding the consortium on the degradation rate

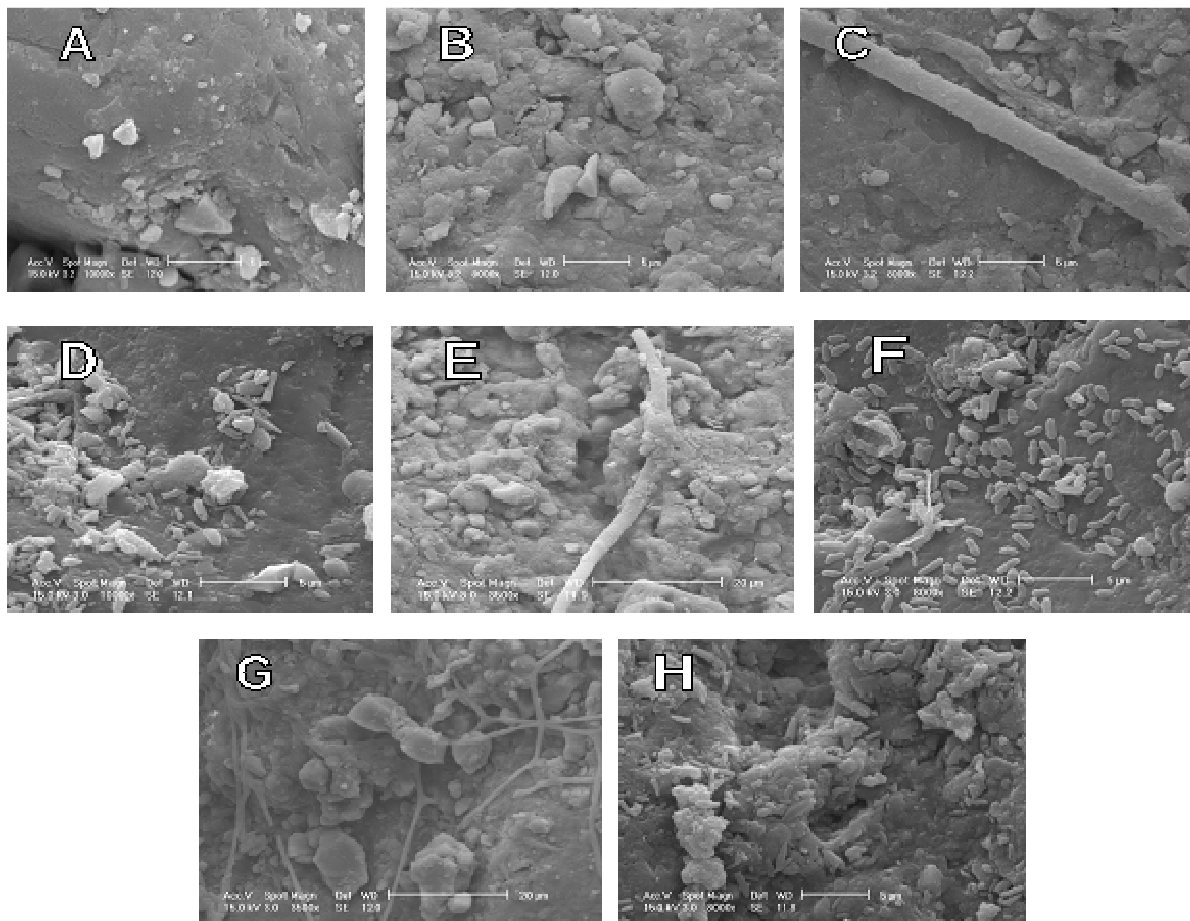
was significant, the gain might be mainly due to the degradation capability of *B. pumilus* strain JL<sub>B</sub>.

Microbial populations of soil samples during bio-remedial treatments were also examined using the environmental scanning electron microscope. The represented scanning electron micrographs of loam soil are shown in Figures 3A -H. Figure 3A represents the natural form of the loam soil before diesel contamination. Compared the micrographs of bio-attenuation (Figures 3B and 3C), with those augmented with *B. pumilus* JL<sub>B</sub> (Figures 3D and 3E) and those augmented with *B. pumilus* JL<sub>B</sub> and Fertilizer F1 (Figures 3F and 3G) at day 5 and day 65, respectively, the bacterial population in latter case was clearly higher than that in bioaugmentation alone and that in bio-attenuation was lowest at day 5. Fungi-like microorganisms seem to take over the population and become dominant thereafter as observed in 65 days (Figures 3C, 3E and 3G). The growth of fungi-like microorganisms seemed to be correspondent to the bacterial population. The higher bacterial population seems to be correlated to the higher diesel degradation in the early stage of degradation, but the higher fungi-like population at day 65 did not. Interestingly, no fungal growth was observed at 65 days in the sample treated with the consortia plus fertilizer (Figure 3H).

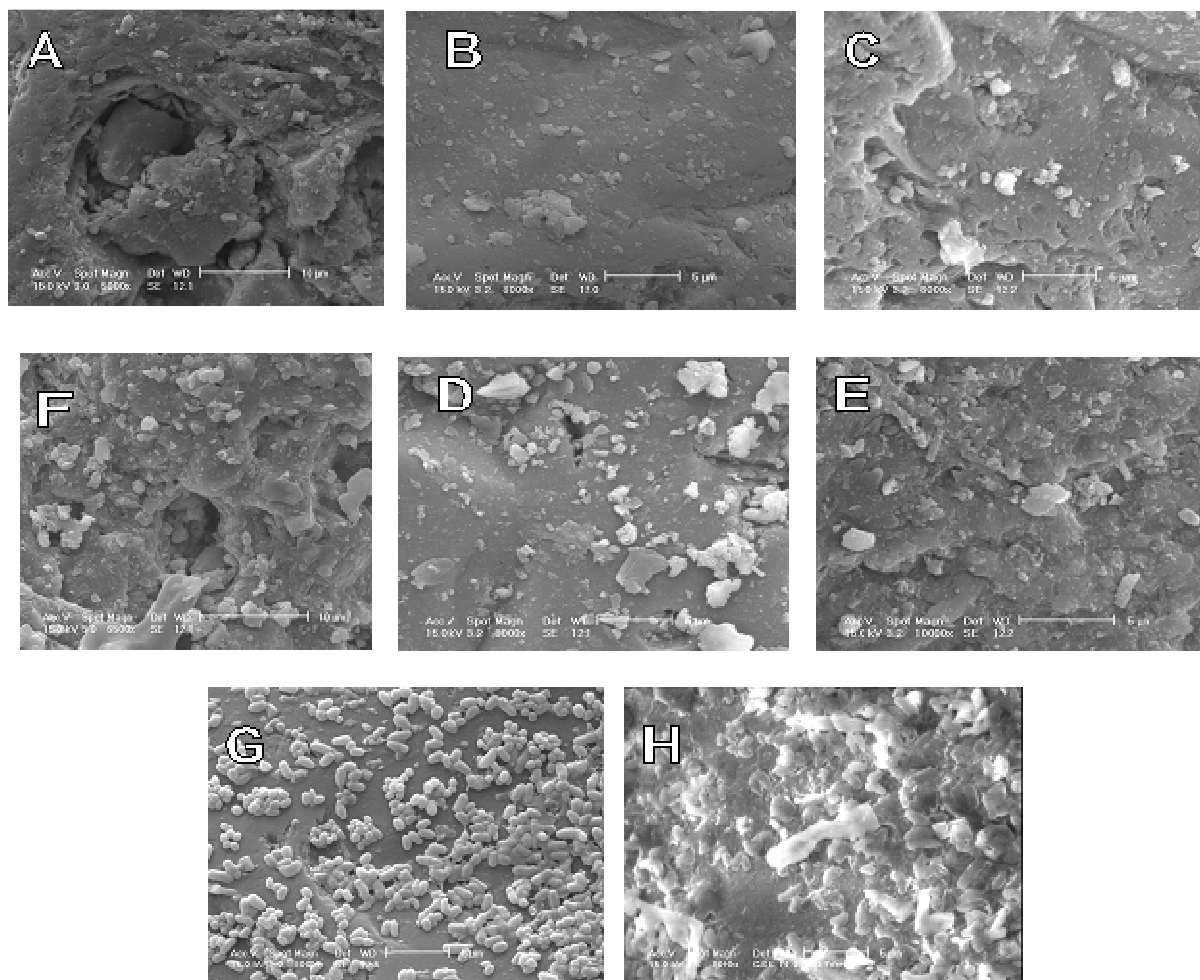
Figures 4A - H show the represented scanning electron micrographs of sea sand during diesel degradation. The image in Figure 4A shows a typical ESEM image of sea



**Figure 2.** The percentage of diesel degradation in sea sand with different amendments for 35 days. NA: non-amended; J: augmented with *Bacillus pumilus* JL<sub>B</sub>; L: augmented *Acinetobacter calcoaceticus* LT<sub>1</sub>; F1: supplemented with Fertilizer F1; F3: supplemented with Fertilizer F3; \*: significant difference,  $p < 0.05$  compared to NA; \*\*: significant difference,  $p < 0.05$  compared to L.



**Figure 3.** Environmental electron micrographs of loam soil during diesel degradation under different amendments. Loam soil at day 0 (3A), Bio-attenuation at day 5 (3B) and at day 65 (3C); Bioaugmentation with *Bacillus pumilus* JL<sub>B</sub> at day 5 (3D) and day 65 (3E); Bioaugmentation with *Bacillus pumilus* JL<sub>B</sub> plus fertilizer F1 at day 5 (3F) and day 65 (3G); Bioaugmentation with JL<sub>B</sub> and LT<sub>1</sub> plus fertilizer F1 at day 65 (3H).



**Figure 4.** Environmental Electron Micrographs of sea sand during diesel degradation under different amendments. Sea Sand at day 0 (4A), Bio-attenuation at day 5 (4B) and at day 35 (4C); Bioaugmentation with *Bacillus pumilus* JL<sub>B</sub> at day 5 (4D) and day 35 (4E); Bioaugmentation with *Bacillus pumilus* JL<sub>B</sub> plus fertilizer F1 at day 5 (4F), day 35 (4G) and day 65 (4H).

sand in its natural state without treatment or the presence of contaminants. Similar to those in the loam soil, the highest bacterial population was observed with the sample augmented with *B. pumilus* JL<sub>B</sub> and fertilizer at day 35 (Figure 4G). However, the bacterial population was decreased dramatically at day 65 (Figure 4H) and no fungi-like microorganism was observed. There was little microbial growth while augmenting the consortium with fertilizer at day 5 but increased slightly at day 35 (data not shown).

## DISCUSSION

Microorganisms play important roles in the natural environment. They contribute to the geological cycle of elements and transformation of natural chemicals. Microorganisms are extremely diverse and are capable of utilizing the contaminant as an energy and carbon source to survive in inhospitable environments (Sohal and

Srivastava, 1994; Watanabe, 2002). The sufficient autochthonous microbial population with intrinsic degradation capacities and the environmental parameters are crucial in the *in situ* bioremediation process whether bio-attenuation (Bento et al., 2005; Seklemova et al., 2001), biostimulation (Molina-Barahona et al., 2004) or bioaugmentation (Atlas and Philp, 2005; Mehrashi et al., 2003) is a realistic option.

Our other study shows that the addition of commercial fertilizers to artificially contaminated soils stimulates a rapid degradation of long chain hydrocarbon source by indigenous microorganisms (Singh and Lin, 2009). Without fertilizer supplementation, the microbial community degraded diesel slowly until a threshold was reached resulting in an increase in the degradation process. Several bacterial strains including *B. pumilus* JL<sub>B</sub> and *A. calcoaceticus* LT<sub>1</sub> have demonstrated the capacity of degrading diesel and used engine oil effectively in liquid media (Mandri and Lin, 2007; Singh and Lin, 2008). In this study, we further demonstrate that augmenting

bacterial isolate, *B. pumilus* JL<sub>B</sub> and/or *A. calcoaceticus* LT<sub>1</sub> into diesel contaminated loam soils accelerated the diesel degradation compared to non-augmented one after 65 days. The presence of isolate JL<sub>B</sub> (*B. pumilus*) in contaminated sea sand also resulted in significant higher diesel degradation rate. Although the evidence is scarce for the stimulatory effects of bioaugmentation in any contaminated environment as suggested by other researchers (Stotzky, 1997; Prince, 1998), our study and others have demonstrated its positive impact in the laboratory microcosm (Mohn and Stewart, 2000) and in the fields (Mohn et al., 2001; Ueno et al., 2006; Das and Mukherjee, 2007). Mishra et al. (2001) provided evidence that indigenous microbial population found in contaminated soils might be low if the toxic contaminants are found at high concentrations. Therefore, inoculums of hydrocarbon-degrading microorganisms can be added to the site to enhance the breakdown of the contaminant. In determining the value of inoculation in treatment systems, the benefit of time saved will have to be weighed against the expense of inoculation (Mohn et al., 2001). Recently, Alisi et al. (2009) also demonstrate the ability of a tailored microbial consortium to efficiently facilitate the bioremediation of matrices co-contaminated with diesel and heavy metals.

Several studies have reported on the roles of *Bacillus* spp. in hydrocarbon bioremediation (Annweiler et al., 2000; Ghazali et al., 2004; Ijah and Antai, 2003; Sorkhoh et al., 1993). It was postulated that *Bacillus* spp. are more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. The ability of *B. pumilus* JL<sub>B</sub> to accelerate the diesel degradation significantly in various conditions observed in this study indicating the potentials of this microorganism in clearing oil spills.

In general, the impacts of bioaugmentation and the combination of bioaugmentation and biostimulation are more significant in the contaminated sea sand (Figures 1 and 2). The sandy soils contained lower organic and mineral contents as shown in this study and might have had relatively small populations of hydrocarbon-degrading and total microorganisms (Robinson and Wookey, 1997). The pattern of diesel degradation in sea sand augmented with *A. calcoaceticus* LT<sub>1</sub> (Figure 2) might also be due to the rapid deprivation of nitrogen and mineral nutrient contents. Further supplementation of fertilizers restored the degradation process. The results also indicate that the survival of inoculated microorganisms in the environment remains a distinct challenge for bioremediation technologies that rely on bioaugmentation (El Fantroussi and Agathos, 2005).

However, the positive impacts of amending additional nutrient such as fertilizer into bioaugmented contaminated loam soils and the long-term impacts of bioaugmentation on diesel degradation are not clear. Mohn and coworkers (2001) reported that total petroleum hydrocarbon concentrations in the inoculated treatments were no longer significantly different from those in the

other amended treatments after 1 year of study. Hamdi et al. (2007) observed that bioremediation efficacy was more likely to rely on the selectivity and specialization of added microorganisms rather than on nutrient load as demonstrated in the studies of Van der Gast et al. (2004) and Alisi et al. (2009). Bento et al. (2003) also suggest that inoculating microorganisms pre-selected from their own environment might be the best approach for bioremediation of diesel oil that the microbes are more likely to survive and propagate when reintroduce to the site. Our results also show that different diesel degrading isolates such as *A. calcoaceticus* LT<sub>1</sub> demonstrate different diesel degrading capacity while inoculating them into different soil environments.

The results of ESEM (Figure 3) in this study show that the augmented microorganisms (MOs) were dominant in the contaminated soil, but indigenous fungi-like rapidly replaced their position. It may be possible that other indigenous microorganisms (fungi-like MOs in our study) utilized the metabolic products generated by the diesel degrading bacteria and at the same time inhibited their growth. It is also possible that lower diesel concentration and accumulation of the metabolic products limited the bacterial growth and then allow the fungi-like MOs to utilize the remaining nutrients for replication. It has been shown that prolonged incubation of *P. putida* GPO1 on n-alkane-containing medium resulted in the loss of n-alkane-oxidizing activity probably due to the down-regulation of the *alkBFGHJKL* operon (Chen et al., 1996). Yuste et al. (1998) and Canosa et al. (1999; 2000) demonstrated the involvement of a catabolite repression or positive feedback mechanisms in regulation of the n-alkane degradation depending on the carbon source available to the microorganisms. Such mechanisms allow both rapid induction of the n-alkane utilization pathway and a fast down regulation thereof when the n-alkanes are consumed. Ratajczak and coworkers (1998) also observed the induced expression of *alkM* on the presence of n-alkanes with chain lengths above C<sub>6</sub>. Moreover, an inhibitory effect of oxidized n-alkane derivatives on the expression of *alkM* in *Acinetobacter* ADP1 has been noticed. The phenomenon can potentially cause a problem for a long-term biotechnological process based on n-alkane utilization because it would rely on an enzyme system required for good productivity while at the same time being deleterious to the host if it is being over-expressed.

At present, the effectiveness for bioaugmentation of diesel contaminated soil remain debatable. Therefore, it is critical to understand not only the physiology of the inoculum but also how it affects microbial community structure and function in the soil environment to which it is being introduced. In principle, persistence and growth of an inoculated strain in any soil will depend on its ability to utilize local resources and also may require displacement of a component of the indigenous community (Cunliffe and Kertesz, 2006). Under a tailor-made micro-

bial formula, bioaugmentation could emerge as one of only a few environmental friendly techniques for pollution management (El Fantroussi and Agathos, 2005).

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