African Journal of Biotechnology Vol. 8 (14), pp. 3286-3293, 20 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Evaluation of nutrient addition to diesel biodegradation in contaminated soils

C. Singh and J. Lin*

School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal (Westville), Private Bag X 54001, Durban 4000, Republic of South Africa.

Accepted 13 April, 2009

Increasing public concern towards petroleum pollution demands for new and more environmentally efficient low-cost strategies for cleaning up contaminated sites. Diesel biodegradation by microbial communities was investigated in artificially contaminated soils by supplementing commercial fertilizers under laboratory conditions. The amounts of oil degraded at each sampling day were determined by the Soxhlet extraction method, the quantities of benzene, toluene, ethyl benzene, and xylene (BTEX) compounds and hydrocarbon content in the treated and non-treated soil samples were determined by gas chromatography-mass spectroscopy. The significant enhancement of diesel degradation was observed soon after the supplementation of fertilizers in loam soil and sea sand, but not in clay soil. The fertilizer supplements stimulated higher degradations, but no significant differences in contaminated sea sand and loam soil after 60 days of incubation period. The inhibitory effect of adding fertilizers was observed in the clay soil. The breakdown of hydrocarbon $C_{>16}$ were relatively faster than the shorter chain compounds such as C_9. In conclusion, the supplementation of fertilizer stimulates the diesel degradation rate in the contaminated soil and the effects of nutrient supplementation are dependant upon soil types, existing nutrients and microbial populations.

Key words: Diesel, bioremediation, biostimulation, fertilizers.

INTRODUCTION

Over 2 billion tons of petroleum is produced annually worldwide. Environmental contamination with petroleum introduces a myriad of hydrocarbons, causing a variety of problems (Atlas and Philp, 2005). South Africa is especially vulnerable to oil spills due to the high volume of oil being transported around the coasts. Protective and preventive measures need to be taken into account to avoid spillage into the environment. Spillages of oil have become a common occurrence but not a recent one. In 1983, a major spill of 250,000 tons of oil occurred in South Africa by the tanker known as Castillo De Belluer. In June 2000, 1,300 tons of oil was spilled in the Atlantic Ocean about 30 km from Cape Town. In a separate incident, the same year, a tanker sank off the coast of Cape Town, where the spill threatened the South African penguin population and also damaged major tourist attraction sites such as Robben Island (Avian Demography Unit, 2000). In September 2002, a fire caused by the freighter Jolly Rubino spilled about 650 tons of oil 12 km south of the St. Lucia estuary (Brendan and Broderick, 2003). The cargo included fuel oil, gas and hazardous chemicals. The cargo was only being removed in February 2004, demonstrating how time-consuming a clean-up operation can be.

As an alternative to physicochemical clean-up methods resulting in negative consequences, bioremediation offers an appealing treatment technology for petroleum hydrocarbon-contaminated soils. Bioremediation processes are an effective method that stimulates the biodegradation in contaminated soils by including additives or improving the availability of materials (Swannell et al., 1996).

Biostimulation of indigenous microorganisms by the addition of inorganic nutrients such as nitrogen, phosphorous and potassium that are rapidly depleted because of 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). Bio-

*Corresponding author. E-mail: linj@ukzn.ac.za. Tel.: +27-31-2607407. Fax: +27-31-2607809.

stimulation is considered as a most appropriate remediation technique 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). Nutrient addition stimulates the degradative capabilities of the indigenous microorganisms found in the soil, as compared to the unamended samples. This allows the microorganisms to break down the organic pollutants at a faster rate (Dzantor, 1999; Ausma et al., 2002). Creating optimal environmental conditions such as pH, temperature, oxygen and bioavailability of substrates stimulates the rate of biodegradation. All microbes perform best at optimal nutritional levels of nitrogen, phosphorous, carbon and smaller quantities of other elements such as magnesium, potassium and iron. The microorganisms utilize these nutrients that stimulate the enzymes that enhance biodegradation (Margesin and Schinner, 2001). This process stimulates the numbers and activities of microbial populations, such as bacteria and fungi to effectively degrade the pollutants to harmless products (Dzantor, 1999).

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). In numerous field trials, the feasibility of adding inorganic nutrients on a periodic basis has been demonstrated as a means of sustaining elevated nutrient cones within the sediments for effective bioremediation (Lee and Levy, 1989; 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). Addition of selected nutrients in the form of organic and/or inorganic fertilizers with electron acceptors such as oxygen and with substrates such as methane, phenol and toluene stimulates pollutant degradation (Thomassin-Lacroix et al., 2002; Sarkar et al., 2005). 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).

The objective of this paper was to determine if the addition of nutrients in the form of fertilizers enhances hydrocarbon bioremediation by promoting biostimulation in different artificially contaminated soil microcosms.

MATERIALS AND METHODS

Soil collection and analyses

Three different soil types, namely clay, loam and sea sand, were used in this study. Clay and loam soil were collected from different sites on the premises at the University of KwaZulu-Natal (Westville campus) and sea sand was collected from a beach, Durban. 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 soil was analyzed for total organic carbon, nitrogen, phosphorous and potassium by the laboratory at the Umgeni waste water management centre. The pH and moisture content of the soils were obtained using standard protocols (McCauley et al., 2003).

Preliminary degradation assay

Four equal portions (150 g) of each soil sample were prepared. Each portion was artificially contaminated with 10% (v/w) diesel (obtained from a local garage and stored in the dark at ambient temperature). One was used as the control and the remaining 3 portions were treated with different commercial fertilizers namely F1, Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K); F2, Grovida-flower and fruit (8.6% N; 3.0% P; 14.4% K) and F3, Kompel-plant food (14.6% N; 4.5% P; 27.4% K), respectively. The samples were thoroughly homogenized by manual mixing to distribute the diesel oil and/or nutrients throughout the soil particles and to enhance aeration. The microcosms were incubated at 30°C and regularly watered with sterile distilled water on the different sampling days, to replace the evaporated water. The microcosms were sampled at 5 day intervals for a period of 30 days to determine the amount of diesel oil degraded.

Microcosm preparation

A scale-up study with a longer incubation period was performed thereafter. Four equal portions (2 kg) of each soil type sample were prepared. One was used as a control by autoclaving at 121°C and 15 psi for 1 h for 3 alternate days before 10% (v/w) diesel oil was added. Two portions were treated with 10% (w/w) of the 2 fertilizers, F1 and F3 respectively before addition of diesel oil. The remaining portion was contaminated with diesel oil with no additional treatment. The diesel oil contaminated soils was placed in 5 l glass beakers. The microcosms were thoroughly homogenized and incubated as described above in the preliminary assay. The microcosms were sampled at different intervals and stored at 4°C for further analysis.

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 sulphate (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 cycle's h^{-1} (Helaleh 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 % of diesel oil degradation was determined using the amount of diesel oil at day 0 as 100%.

Gas chromatography-mass spectroscopic analysis

Samples (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 sam-

Table 1. Physiochemical analyses of various soil types used in this study.

Soil type	pH	Total organic carbon (mg C/kg)	Total nitrogen (mg N/kg)	Potassium (mg K/kg)	Phosphate (mg P/kg)	Water content (%)	Soil dry mass (%)
Clay	6.57	43.5	77.2	1406	665	0.50	99.5
Loam	6.63	552	1696	1228	471	0.107	99.89
Sea sand	9.08	34.2	12	46.6	363	0.0305	99.97

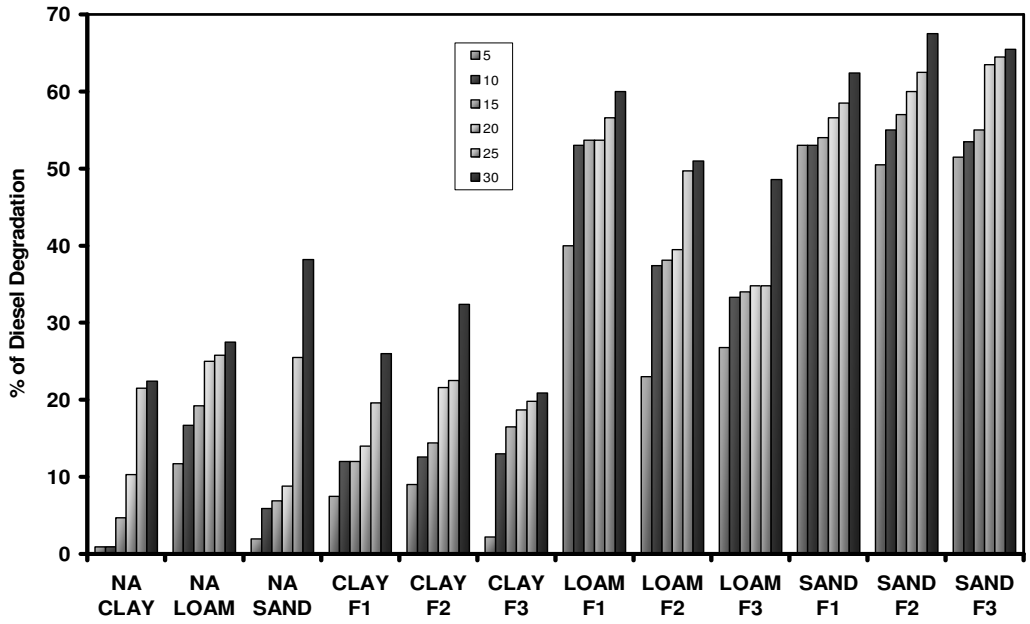

Figure 1. The % of diesel degradation in the different contaminated soils in the preliminary degradation assay. NA: non-treated; F1-F3: fertilizers. F1, Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K); F2, Grovida-flower and fruit (8.6% N; 3.0% P; 14.4% K); and F3, Kompel-plant food (14.6% N; 4.5% P; 27.4% K).

ples were carried out on a Hewlett-Packard 5890 series GC system coupled to a mass spectrophotometer VG TRIO 2000 (Eskom organic analysis laboratory, Johannesburg).

Statistical analysis

Paired t-tests (Wilkinson, 1988) 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 in Table 1 indicated that clay and loam soils were slightly acidic, while the sea sand was strongly alkaline. Clay contained higher concentrations of potassium and phosphate compared to other soil types and loam soil possessed significantly higher concentrations of total organic carbon and total nitrogen. The sea sand had low concentrations of all chemical parameters measured. These results reveal that water was not completely removed from the clay soil after drying at 105°C therefore indicating that clay has a higher water retention capacity while sea sand has the least retaining capacity.

Figure 1 shows the % of diesel degradation over a 30 day period in the different soils treated with 3 different types of local commercial fertilizers. The non-treated (NA) soils revealed lower degradation rates. The presence of fertilizer supplements did not enhance the degradation ability in the clay soil. All 3 fertilizers stimulated significantly ($P < 0.05$) higher diesel degradation rates (up to 67.5%) in sea sand and in loam soil than those in NA soil types. The presence of fertilizer F1 increased the diesel degradation rate in loam soil than other 2 fertilizers did under the same conditions.

Larger microcosms (2 kg) of different contaminated soils with or without the fertilizers supplements with a longer incubation period were set up. Figure 2 shows the effect of the fertilizer F1 on the level of diesel degradation in the different soil types over a 65 days period. In spite

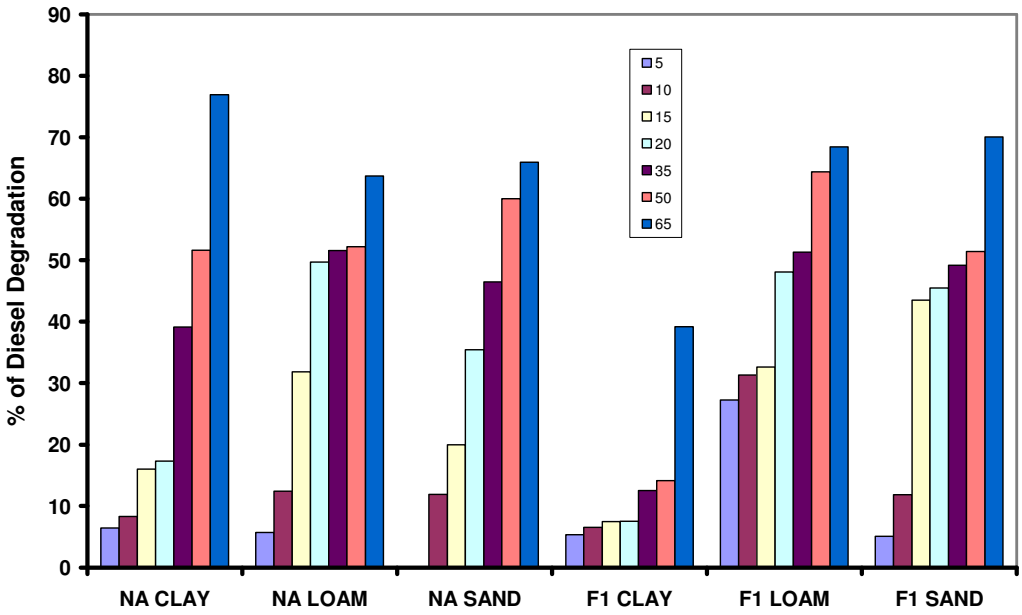

Figure 2. The % of diesel degradation in different contaminated soils supplemented with and without fertilizer F1 [Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K)] supplementation during a 65-day incubation period.

of the low degradation rate observed at the early incubation period in all non-treated soils, there were no significant differences (P > 0/05) in the degradation levels between the treated and NA soils except in clay soil after 65 days. The % of diesel degradation was observed in the range of 66 - 77% except in clay soil with F1 addition. Addition of fertilizer F1 in clay soil resulted in a lower degradation rate of 39.18%. It is also noted as observed in the preliminary assay that the presence of the fertilizers enhanced the diesel degradation rate in the early stage. A similar trend was observed while the fertilizer F3 was supplemented (data not shown). The presence of fertilizer F3 promoted diesel degradation more effectively in the sea sand (83%) compared to in the loam soil.

The different carbon lengths ($C_9 – C_{>16}$) of diesel at day o and day 65 were quantified using GC/MS. Figures 3A - 3C show degradation patterns with various carbon-length under various treatment conditions. The losses of diesel due to abiotic factors at day 65 seem negligible compared to those in day 0 (data not shown). The results obtained from the non-amended microcosms as well as the amended microcosms revealed that the indigenous microbial flora utilized various carbon length substrates as compared to ones in the autoclaved control. As the carbon length increased, the % of degradation were also increased. The degradation levels under various conditions also correspond well to those values determined using the extraction method. In loam soil, the diesel degradation was higher with the presence of fertilizer F1 while the non-treated (NA) clay soil had the highest degradation rates of the different lengths of the carbon substrates.

DISCUSSION

In this study, the chemical analyses of soils revealed that loam soil had a higher total organic carbon and nitrogen content of 552 mg C/kg and 1696 mg N/kg, respectively. Clay soil had a high 1406 mg K/kg of potassium and 665 mg P/kg of phosphate. Sand contained less amounts in all categories. Most minerals and nutrients are more soluble in acidic soils than in neutral or slightly alkaline soil (McCauley et al., 2003). Except in the contaminated clay soil, the diesel degradation rates in the contaminated soils were significantly enhanced soon after the supplementation of the fertilizer. The increases were more prominent especially in the contaminated sea sand due to the intrinsically low nitrogen and phosphorous contents.

Clay soil properties may have an impact on degradation. The small pore space in clay soil might cause low oxygen diffusion rate and limited the accessibility of the target compound for degradation by microbes (Tisdale and Nelson, 1975) especially the insolubility of diesel in water. The addition of nutrients to clay soil could not be utilized effectively or becomes toxic to soil microbiota (Ferguson et al., 2003). A study to evaluate the bioremediation of petroleum-contaminated clay soil by fixed bed experiments found that the higher the nitrogen content and the lower the organic carbon removal will be (Pala et al., 2006).

Addition of fertilizers led to faster diesel degradation rates in the initial stages in larger microcosms as observed

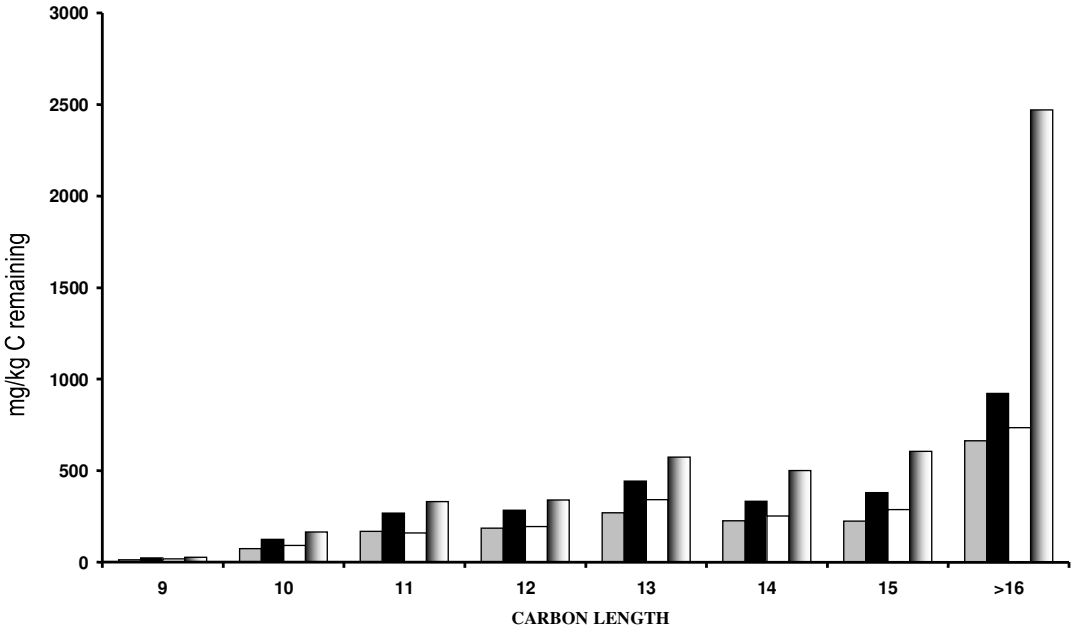

Figure 3a. Degradation pattern of total carbon substrates remaining in diesel-contaminated soil after 65 days in loam soil, Gray bar: F1 treated; Black bar: F3 treated; White bar: non-treated; Gradient bar, autoclaved soil. F1, Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K); F3, Kompel-plant food (14.6% N; 4.5% P; 27.4% K).

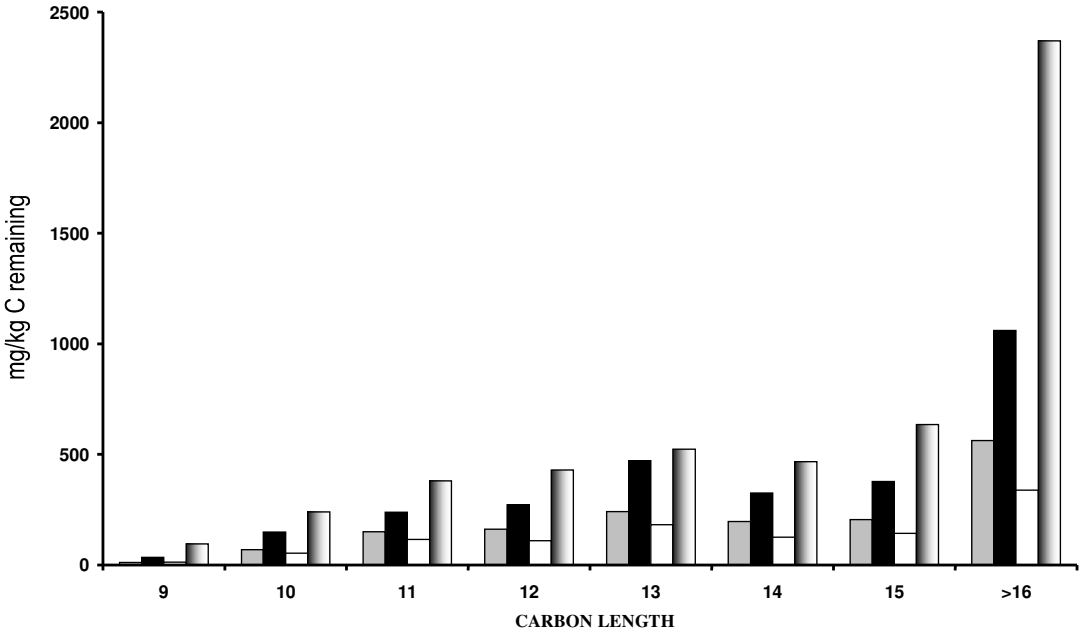

Figure 3b. Degradation pattern of total carbon substrates remaining in diesel-contaminated soil after 65 days in clay soil. Gray bar: F1 treated; Black bar: F3 treated; White bar: non-treated; Gradient bar, autoclaved soil. F1, Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K); F3, Kompel-plant food (14.6% N; 4.5% P; 27.4% K).

in the preliminary experiments. The limited or inhibitive effect of the fertilizer supplementations in clay soil was also observed. Therefore, the soil type should be an important factor in bioremediation as reported by Ghazali et al. (2004). Buddy et al. (2002) found that diesel contamination results in the development of different

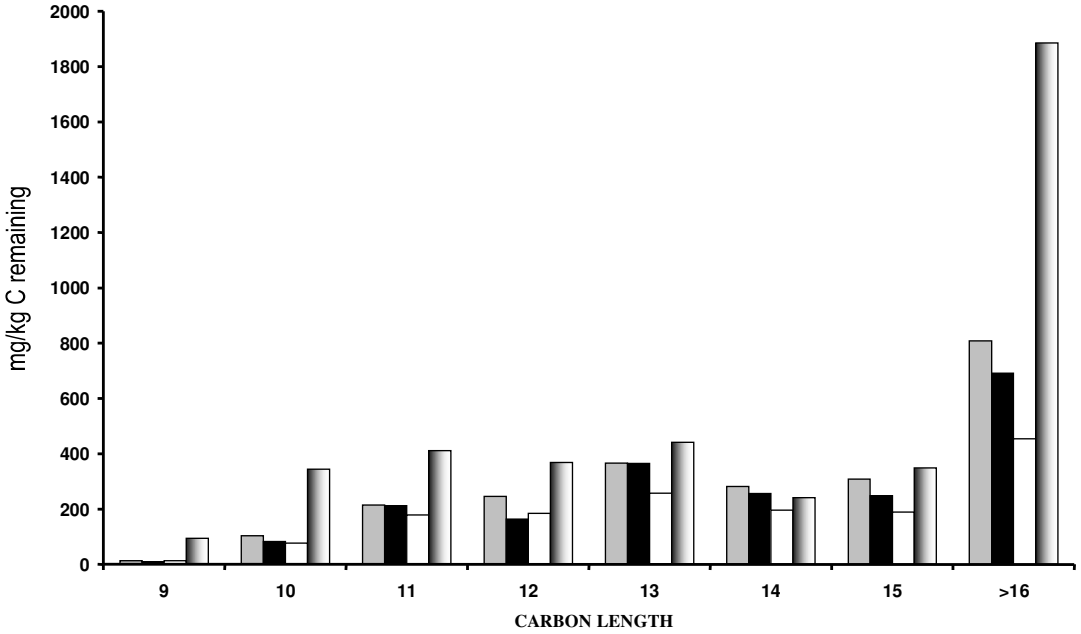

Figure 3c. Degradation pattern of total carbon substrates remaining in diesel-contaminated soil after 65 days in sea sand. Gray bar: F1 treated; Black bar: F3 treated; White bar: non-treated; Gradient bar, autoclaved soil. F1, Grovida-lawn and foliage (12.5% N; 8.3% P; 4.2% K); F3, Kompel-plant food (14.6% N; 4.5% P; 27.4% K).

community profiles in different soil types, therefore different soils have different inherent microbial potential to degrade hydrocarbons. However, in all non-treated (NA) soils, the native microbes seem to adapt to contaminated environments and were capable of utilizing the contaminant, diesel, efficiently as an energy and carbon source in a slower path. The degradation levels became less significantly different between the treated and non-treated samples after a 65 day incubation period.

The effect of the fertilizer on the diesel biodegradation process is not consistent in the literature (Alexander, 1994; Bento et al., 2005; Lee et al., 2007; Rosa and Triquis, 2007; Seklemova et al., 2001). Acceleration of the diesel biodegradation process was observed by using fertilizer to stimulate microbial growth that were responsible for decontaminating the marine environment (Rosa and Triquis, 2007) and in an alpine glacier skiing area (Margesin and Schinner, 1999; 2001). Several reports also demonstrate the positive impact on the oil biodegradation using fertilizers (Alexander, 1994; Lee et al., 2007). Marqesin et al. (2007) found that the 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 fertilization. In this study, the soils were artificially contaminated with a large quantity of diesel (10%). The supplementation of an additional nutrient source (different types of fertilizers) stimulated the degradation processes significantly. The impacts were more obvious in the sea sand sample which contained a lower nitrogen and phosphate contents than the loam soil. Walworth et al. (2006) reported that the addition of the proper amount of nitrogen nutrient can increase the biodegradation rate. However, excess nitrogen can also depress the rate of microbial activity and petroleum degradation in contaminated soils due to osmotic soil water potential depression. 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 and detailed site specific characterization studies are needed prior to deciding on the proper bioremediation approach.

The GC/MS results in our study show that the breakdown of long chain hydrocarbon ($C_{>16}$) by the microbial communities in the various soil types was relatively faster than the shorter chain compounds as reported by Whyte et al. (1998). The susceptibility to degradation was inversely 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 are more difficult to degrade than longer chains. Short chain alkanes are toxic for many microorganisms (Cookson, 1995; Mehrashi et al., 2003). *Acetinobacter* sp. isolated in our laboratory (Mandri and Lin, 2007; Singh and Lin, 2008) capable of degrading diesel and used engine oil effectively, fail to utilize the short chain hydrocarbon (less than 9) as the sole carbon source (Naidoo, 2007). The addition of fertilizer might allow a rapid degradation of long chain hydrocarbon source and results in an accumulation of the short chain hydrocarbon, and therefore slows

down the degradation process in the contaminated soils.

Without the fertilizer supplement, the microbial community degraded diesel slowly until a threshold was reached which allow an increase in the degradation process.

Conclusion

The addition of fertilizers to diesel contaminated soil (loam and sea sand) stimulated the abilities of indigenous microbial population to degradation rates especially during the initial stages of degradation. These results clearly suggest that the levels of effect in biostimulation are based on the soil type, environmental factors, the availability of nutrients and the structure of the microbial community present in the soils.

ACKNOWLEDGEMENTS

The authors express their gratitude to Dr Van Rossum from Organic Analysis Laboratory and to the staff in the Umgeni Waste Water Management Centre for analyses. The authors also thank the National Research Foundation and the University Research Committee for financial assistance.

REFERENCES

Alexander M (1994). Biodegradation and bioremediation, Academic Press, Inc. USA, pp. 1-260.

Atlas RM, Bartha R (1972). Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorous. Biotechnol. Bioeng. 14(3): 309-318.

Atlas RM, Philp J (2005). Bioremediation: applied microbial solutions for real-world environmental cleanup. ASM Press, Washington D.C., pp. 1-292.

Ausma S, Edwards GC, Fitzgerald-Hubble CR, Halfpenny-Mitchell L (2002). Volatile hydrocarbon emissions from a diesel fuel-contaminated soil bioremediation facility. Air Waste Manag. Association, 52: 769-780.

Avian Demography Unit (2000). Department of Statistical Sciences, University of Cape Town, South Africa Press Release: Rescue of seabirds in Western Cape, http://web.uct.ac.za/depts/stats/adu/oilspill/press01.htm. Retrieved on 2nd June 2008.

Bento FM, Camargo FAO, Okeke BC, Frankenberger WT (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96(9): 1049-1055

Brendan JG, Broderick AC (2003). Africa park threatened by oil spill, Marine Turtle Newsletter, 99: p. 35. http://www.seaturtle.org/mtn/PDF/MTN99.pdf, Retrieved on 28th April, 2008.

Buddy JG, Paton GI, Campbell CD (2002). Microbial communities in different soil types do not converge after diesel contamination. J. Appl. Microbiol. 92(2): 276-288.

Cookson JT (1995). Bioremediation engineering: Design and application, McGraw-Hill Inc. New York.

Del' Arco PJ, de Franca FP (2001). Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediments. Environmental Pollution, 110: 515-519.

Dzantor EK (1999). Bioremediation of contaminated soils - what it is and how to do it. University of Agriculture and Natural Resources, pp. 1007-1019.

Ferguson SH, Franzmann PD, Revill AT, Snape I, Rayner JL (2003). The effects of nitrogen and water on mineralization of hydrocarbons in diesel-contaminated terrestrial Antartic soils. Cold Regions Sci. Technol. 37: 197-212.

Ghazali FM, Rahman RNZA, Salleh AB, Basri M (2004). Biodegradation of hydrocarbons in soil by microbial consortium. Int. Biodeterioration Biodegradation, 54: 61-67.

Helaleh Murad IH, Tanaka K, Fujii SI, Korenaga T (2001). GC/MS determination of phenolic compounds in soil samples using Soxhlet extraction and derivatization techniques. Analyt. Sci. 17: 1225-1227.

Kim S, Choi DH, Sim DS, Oh Y (2004). Evaluation of bioremediation effectiveness on crude oil-contaminated sand, Chemosphere, 59(6): 845-852.

Lee K, Levy EM (1989). International Oil Spill Conference, American Petroleum Institute, Washington, DC. pp. 479-486.

Lee K, Levy EM (1991). International Oil Spill Conference, American Petroleum Institute, Washington, DC. pp. 541-547.

Lee K, Tremblay GH, Gauthier J, Cobanli SE, Griffin M (1997). International Oil Spill Conference, American Petroleum Institute, Washington, DC. pp. 697-704.

Lee K, Merlin FX (1999). Bioremediation of oil on shoreline environments: development of techniques and guidelines. Pure Appl. Chem. 71(1): 161-171.

Lee SH, Lee S, Kim DY, Kim JG (2007). Degradation characteristics of waste lubricants under different nutrient conditions. J. Hazardous Materials, 143(1-2): 65-72

Mandri T, Lin J (2007). Isolation and Characterization of Engine Oil Degrading Indigenous Microorganisms in KwaZulu-Natal, South Africa, Afr. J. Biotechnol. 6(1): 23-27.

Margesin R, Schinner F (1999). Biological decontamination of oil spills in cold environments. J. Chemical Technol. Biotechnol. 74: 381-389.

Margesin R, Schinner F (2001). Bioremediation (natural attentuation and biostimulation) of diesel-oil contaminated soil in an Alpine glacier skiing area. Appl. Environ. Microbiol. 67(7): 3127-3133.

Margesin R, Hämmerle M, Tscherko D (2007). Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: effects of hydrocarbon concentration, fertilizers, and incubation time. Microb. Ecol. 53(2): 259-269.

McCauley A, Jones C, Jacobsen J (2003). Soil pH and organic matter. Nutrient management – a self study course from the MSU extension service continuing education series., 8: pp. 1-12.

Mehrashi MR, Haghighi B, Shariat M, Naseri S, Naddafi K (2003). Biodegradation of petroleum hydrocarbons in soil. Iranian J. Public Health, 32(3): 28-32.

Molina-Barahona L, Rodriguez-Vazquez R, Hernandez-Velasco M, Vega-Jarquin C, Zapata-Perez O, Mendoza-Cantu A, Albores A (2004). Diesel removal from contaminated soils by biostimulation and supplementation with crop residues. Appl. Soil Ecol. 27: 165-175.

Naidoo E (2007). A study on the substrate specificity of oil degradaing Acetobacter isolates, Honours dissertation, University of KwaZulu-Natal, South Africa

Pala DM, de Carvalho DD, Pinto JC, Sant'Anna Jr. GL (2006). A suitable model to describe bioremediation of a petroleum-contaminated soil, Int. Biodeterioration Biodegradation, 58(3-4): 254-260.

Perfumo A, Banat IM, Marchant R, Vezzulli L (2006). Thermally enhanced approaches for bioremediation of hydrocarbon-contaminated soils. Chemosphere, 66(1): 1-6.

Plohl K, Leskovsek H (2002). Biological degradation of motor oil in water. Acta Chem. Slov. 49: 279-289.

Rosa AP, Triquis JA (2007). Bioremediation process on Brazil shoreline. Laboratory experiments. Environ. Sci. Pollut. Res. Int. 14(7): 470-476.

Sarkar D, Ferguson M, Datta R, Birnbaum S (2005). Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ. Pollut. 136: 187-195.

Seklemova E, Pavlova A, Kovacheva K (2001). Biostimulation-based bioremediation of diesel fuel: field demonstration, Biodegradation, 12(5): 311-316.

Singh C, Lin J (2008). Isolation and characterization of diesel oil-degrading indigenous microorganisms in KwaZulu-Natal, South

Africa, Afr. J. Biotechnol. 7(12): 1927-1932.
Swannell RPJ, Lee K, McDonagh M (1996). Field evaluations of marine oil spill bioremediation. Microbiological Rev. 51: 342-365.
Thomassin-Lacroix EJM, Eriksson M, Reimer KJ, Mohn WW (2002). Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Artic soil. Appl. Microbiol. Biotechnol. 59: 551-556.
Tisdale LS, Nelson WL (1975). Soil fertility and fertilizers, 3rd Edition, Macmillan Publishing Co. Inc., NY. pp. 23-45.
Venosa AD, Suidan MT, Wrenn BA, Strohmeier KL, Heines JR, Eberhart BL, King D, Holder EL (1996). Bioremediation of an experimental oil spill on the shoreline of Delaware Bay. Environ. Sci. Technol. 30(5): 1764-1775.
Walworth J, Pond A, Snape I, Rayner J, Ferguson S, Harvey P (2006). Nitrogen requirements for maximising petroleum bioremediation in a Sub-Antartic soil. Cold Regions Sci. Technol. pp. 1-8.
Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW (1998). Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp., Appl. Environ. Microbiol. 64(7): 2578-2584.
Wilkinson L (1988). Systat: The system for statistics. Evanston Systat.