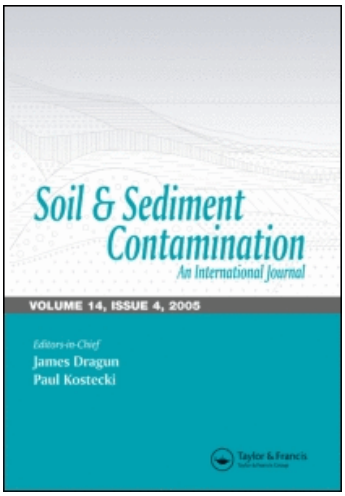

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Influence of Bulking Agents, Fertilizers and Bacteria on the Removal of Diesel from a Newfoundland Soil

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Laboratory experiments in culture flasks, containing diesel-contaminated Newfoundland soil samples, were undertaken to compare the influence of fertilizers, microorganisms and bulking agents on bioremediation. In Phase I experiments only one fertilizer (cow manure or poultry manure), one bulking agent (sand or hay), or one inoculum (cold-tolerant indigenous bacteria or exogenous commercial bacteria) was added to a soil sample. In Phase II experiments, Design-Expert® Version 6 design of experiment software determined the combinations of fertilizers, bulking agents and inocula to be mixed with the soil samples to study the interactions among the amendments. The maximum diesel removal at 90 days occurred in the sample with sand (Phase I) and in the sample with cow manure, an inoculum of cold-tolerant indigenous bacteria, and sand (Phase II). Diesel removal at 45 days for the same two samples was 85.4% (Phase I) and 91.9% (Phase II), suggesting the cow manure and/or cold-tolerant bacteria inoclum accelerated the process. The poultry manure, commercial bacteria and hay were less effective than their counterparts. The commercial bacteria were more sensitive to diesel concentration than the indigenous cold-tolerant bacteria. The addition of sand, cow manure, and poultry manure improved diesel removal.

Keywords Aerobic biodegradation, cold-tolerant bacteria, cow manure, poultry manure, sand, total petroleum hydrocarbons

Introduction

Widespread leaking of underground storage tanks has drawn attention to hydrocarbon pollution of soil and groundwater, and in Newfoundland (NL) remediation of contaminated sites has dealt primarily with the removal of petroleum hydrocarbons. Remediation of soils with gasoline, diesel, jet fuel, or heating oils has made use of the enzymes contained in microbial cells and controlled conditions with respect to nutrients, oxygen, moisture, and temperature. Diesel or the middle distillates of crude oil in the C_9 to C_{20} hydrocarbon range consist of 30% alkanes, 45% cyclic alkanes, 24% aromatics (Frankenberger et al., 1989), and 4% polyaromatics (Heath et al., 1993) and are second only to benzene in terms of the most frequently treated type of contamination (Zytner et al., 2001).

Biostimulation with inorganic salts, horse manure, poultry litter, or domestic sewage sludge can aid soil bioremediation by as much as doubling the hydrocarbon removal rate (Williams et al., 1999; Cunningham and Philip, 2000; Gallego et al., 2001). A 14-week

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diesel biodegradation field study in Petawawa sand revealed that biostimulation with a commercial fertilizer was more important than tillage or bioaugmentation (Demque et al., 1997).

Wood chips, sawdust, leaves, hay, wheat bran, chopped wheat, straw, shredded tires, pine bark, peat, loam, or vermiculite have been used as bulking agents to improve removal of petroleum hydrocarbons or 3, 4-dichloroanaline and benzo(a)pyrene from contaminated soils (Vasudevan and Rajaram, 2001; Rhykerd et al., 1999; Morgan et al., 1993). Low density bulking agents lower the bulk density of soil and may help form water stable aggregates, increase soil porosity and oxygen diffusion and thereby stimulate microbial activity (Rhykerd et al., 1999).

A highly populated, diverse consortia of indigenous microorganisms is ideal for bioremediation of petroleum-contaminated soils (Bhattacharya et al., 2003); however, bioaugumentation has been recommended for petroleum-contaminated soils having low indigenous hydrocarbon-degrading microbial populations (Mishra et al., 2001).

Many previous studies have demonstrated that bioremediation is effective for petroleum-contaminated soils (Rahman et al., 2002), though research with cold-tolerant microorganisms has been less extensive. The objective of this research was to investigate and compare the influence of cold-tolerant microorganisms, commercial microorganisms, fertilizers and bulking agents on the aerobic bioremediation of diesel-contaminated soil (anaerobic bioremediation was not considered).

In St. John's, NL, the daily average temperature is below 12°C ten months of the year and is 15.4°C and 15.5°C in July and August (Environment Canada, 2002), and soil indigenous bacteria will be primarily psychrophiles and psychrotrophs. Psychrophiles can grow at 0°C, grow optimally at 16°C, and cannot grow above 20°C, whereas psychrotrophs can grow at 0°C but exhibit optimal growth from 20°C to 25°C (Margesin and Schinner, 1994).

Experiments were conducted in two phases. In the Phase I experiments, one inoculum (indigenous mostly psychrophilic and psychrotrophic bacteria or exogenous commercial bacteria), one bulking agent (clean Ottawa sand or hay), or one fertilizer (poultry manure or liquid cow manure) was added at a time. In the Phase II experiments, a 2^3 factorial design generated by Design-Expert® Version 6 design of experiment (DOE) software, by Stat-Ease, Inc., Minnesota, MN, USA, was used to select combinations of inocula, bulking agents, and fertilizers to be added to the contaminated soil to study their interactions and their main effects.

Materials and Methods

All experiments were conducted in duplicate, and all media and mineral solutions were sterilized in an autoclave at 121°C and 103 kPa for 30 minutes and Perotti et al. (2001) used the same duration to sterilize cow manure. Thirty minutes was selected to preserve the nutritional value of the manures, and was considered sufficient for sterilization given that the manures originated in an anaerobic environment (i.e. in the guts of cattle and poultry) and were to be tested under aerobic conditions.

All of the test methods were conducted at room temperature or higher, although cold-tolerant bacteria in addition to commercial bacteria were studied. Higher temperatures were used to accelerate the procedures because with cooler temperatures diesel bioremediation could have been prolonged. However, under field conditions and with a very large pile of diesel-contaminated soil, elevated temperatures and bioremediation will

occur in the interior of the pile even if the ambient outdoor temperatures are below freezing (Mr. Terry Dollard, personal communication, Universal Environmental Services Inc., 2004).

Sources of Diesel-Degrading Bacteria and Analytical Procedures

A clean, native sandy soil from Memorial University of Newfoundland botanical gardens provided an uncontaminated sample and a diesel-contaminated sandy soil near the university printing plant diesel generator storage tank was used as a source of diesel-degrading bacteria. The soils were air-dried and sieved with a #10 US (2 mm) sieve. To grow the diesel-degrading bacteria, 140 g of diesel-contaminated soil and 6.5 kg of clean soil were combined in an empty fish tank in the lab, and kept moist for four weeks by regularly adding deionized water. Only this mixed soil, hereafter referred to as the soil, was used in the experiments.

The soil pH, water content and (Walkey-Blackey) organic carbon were determined as described in the "Analytical Methods Manual" of the Research Branch, Agriculture Canada (Sheldrick, 1984), and the particle size was obtained with ASTM method D422-63 (ASTM, 2002). The Kjeldahl method, used to estimate the soil nitrogen content, employed a Kjeltec, 1002 steam distillation unit and 2020 Block Digester, according to application note AN 300 in the Tecator Co. (1996) operation manual. The soil phosphorus content was estimated using the vanadomolybdophosphoric acid colorimetric method of Olsen and Sommers (1982). The potassium, magnesium, calcium, and iron contents of the soil were estimated by EPA methods 7610, 7450, 7140, and 7380, respectively (USEPA, 1986).

Clean Ottawa sand was purchased from Fisher Scientific, and hay was obtained from Rushmere Farms, Whitbourne, NL. Rushmere Farms and Ocean View Farm, Bay Bulls, NL, supplied the poultry manure and cow manure, respectively**.** The organic carbon, nitrogen and phosphorus contents of the manures were determined using the same methods as were used for the soil.

Diesel was purchased from an Ultramar filling station in St. John's, and patented, powdered, commercial bacteria were purchased from Universal Environmental Services Inc., St. John's.

Procedures with the Cold-Tolerant and Commercial Bacteria

Four procedures were followed with the indigenous and commercial bacteria: one to enumerate the heterotrophic and diesel-degrading bacteria, two procedures (plate inoculation and shake flask) to assess the diesel-degrading capability of the bacteria, and one to produce inocula for the Phase I and II soil microcosm experiments.

To isolate the heterotrophic and diesel-degrading microorganisms for enumeration, 1 g of soil was suspended in 9 mL of 0.85% (v/v) NaCl (sterile saline) solution, and 1 g of commercial bacterial powder was suspended in 45 mL of sterile saline solution. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared with the bacterial suspensions. Two replicates of 0.1-mL portions of each dilution were spread plated on trypticase soy agar (TSA, Difco Laboratories, Detroit, Michigan, USA) plates and on synthetic medium with Bacto agar (a gelling agent) plates. Sugars in the TSA provided the carbon source for the heterotrophic bacteria. Diesel provided the only carbon source for the diesel-degrading bacteria in the synthetic medium, which was composed of 0.13% HN_4NO_3, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.5% KH_2PO_4, 0.5% K_2HPO_4, 1.5%

agar, and 0.2% diesel, following the procedure of Gallego et al. (2001). The TSA agar and synthetic medium agar inoculated plates were incubated at 37°C for 24 and 72 hours, respectively. Colonies appearing on the plates were estimated using a Quebec colony counter.

The plate inoculating technique to assess the diesel-degrading capability of the bacteria employed TSA and the synthetic medium of Gallego et al. (2001), except that 1% (v/v) Bacto agar was used. Two replicates of 0.1-mL portions of (the above) bacterial suspensions were spread plated on the synthetic agar plates and incubated at 30°C for 72 hours, and then plated onto the TSA plates, exposed to diesel vapors (provided by 0.5 mL of diesel in a capillary tube plugged with cotton), and incubated at 30°C for 24 hours.

The minimal mineral salt (MMS) medium, used in the shake flask experiments to determine the diesel-degrading capability of the bacteria, and in the inocula production, in 1 L consisted (in g) of: NH_4NO_3 (2.5), $CuSO_4{\cdot}5H_2O$ (10), H_3BO_3 (10), $FeCl_3$ (0.01), $ZnSO_4{\cdot}7H_2O$ (70), MoO_3 (10), $MgSO_4{\cdot}7H_2O$ (0.5), $MnSO_4{\cdot}5H_2O$ (10), KH_2PO_4 (0.56), and K_2HPO_4 (4.74). After the MMS medium was autoclaved, diesel was added where necessary.

The shake flask experiments (in 125 mL culture flasks) to evaluate the diesel-degrading capability of the bacteria employed a liquid medium consisting of 50 mL MMS medium, 1 mL of the 1:10 dilution of bacteria in saline solution, and 1% diesel (v/v) providing the only carbon source (MMS, bacteria and diesel). A control (MMS and bacteria), a blank (MMS and diesel), and a control blank (MMS) were also evaluated and the two blanks (without bacteria) were used to zero the UV spectrophotometer. The inoculated culture flasks were incubated at 30°C in a Gyratory Water Bath Shaker (Model G76D, New Brunswick Scientific Co. Inc., Edison, N.J., USA) at 170 rpm for two weeks. Increased turbidity indicated the growth of diesel-degrading bacteria and optical density (OD) measurements were made using a UV Spectrophotometer at 600 nm.

The inocula for the liquid cultures were prepared as follows: 1 mL of the soil suspension and the commercial bacteria suspension were inoculated separately into 50 mL MMS solution in 125 mL culture flasks. Diesel (1%, v/v) and succinate (a co-substrate to accelerate microbial growth) (1%, w/v) were added to the flasks to induce the diesel-degrading bacteria. The microbial consortia were collected by centrifugation at 10,000 rpm and 8,720 × g for 15 minutes and the cell pellets were washed in sterile saline solution three times to remove dissolved nutrients and residual diesel. The obtained cold-tolerant bacteria and commercial bacteria cell masses were suspended separately in 75 mL saline solutions and stored at 0–4°C until used.

Phase I and Phase II Biodegradation Experiments

A total of 32 soil microcosm experiments, including duplicates, were conducted in 250 mL culture flasks, containing 100 g of the mixed soil (adjusted to pH 7.0 with 0.5 N NaOH and allowed to dry to a water content of 30% to 35%) and closed with plastic foam stoppers to permit oxygen entry. The neutral pH of the soil created conditions that are normally considered favorable for bacterial activity. The soil was spiked with diesel to give a concentration of 10,000 mg/kg or 1%, which is typical of the concentration that could occur at a diesel contaminated site. The water content was maintained at approximately 30% (without innocula) or 35% (with innocula) by weighing the flasks every two days and adding sterile deionized water to replace any loses. The tests were conducted in a laboratory at room temperature.

The 100 g of mixed soil contained 10 g of cow or poultry manure, 50 g of sand, 5 g of hay or 5 mL of an inoculum in the Phase I experiments and combinations of these quantities in the Phase II experiments.

Only in the Phase I abiotic controls were the soil samples autoclaved, and in all of the other experiments the samples to which the commercial bacteria inocula were added also contained the indigenous cold-tolerant bacteria. The inocula, bulking agents, and manures were examined separately in the Phase I experiments and in combination in the Phase II experiments. The combinations were determined using the Design-Expert® Version 6 software for Design of Experiments, by Stat-Ease, Inc., Minneapolis, MN, USA.

At 45 and 90 days, the flasks were sampled to estimate the total petroleum hydrocarbon (TPH) concentrations. All ex-situ techniques experience losses through volatilization (Heitzer and Sayler, 1993) and the TPH reduction in the Phase I and Phase II experiments would have been due to both degradation and volatilization. No measurement at 0 days was taken to show the initial volatilization, but the abiotic control (Phase I) was included to provide an indication of the volatilization occurring throughout the experiment. TPHs were extracted according to EPA method 3541 (USEPA, 1986), employing a Soxtec HT-2 extraction system with a temperature-controlled oil bath (Tecator Co., 1996). The soil extracts were cleaned according to EPA method 3600C (USEPA, 1986). Activated silica gel (1 g, 60–120 mesh) was packed into a Pasteur pipette, and the soil extract (1 mL; final volume) was added drop-wise and then washed into the pipette column. The nitrogen blowdown (or evaporation by blowing with nitrogen), EPA method 3540C, was used to collect a final concentration from the 5 mL of the eluate. The cleaned extracts were transferred into 5 mL vials and 2 mL of each extract were pipetted into 2 mL vials and stored at 4°C until analysis with a gas chromatograph (GC).

TPH measurements were performed at Maxxam Analytics Inc., St. John's, according to EPA method 8015B (USEPA, 1986). An Agilent 6890 Series model G1530A GC with an Agilent Flame Ionization Detector (FID) and Agilent GC ChemStation software (Rev.A.08.03, 847) were used. The system detects a TPH concentration as low as 15 mg/kg or approximately 0.15% of the initial soil diesel concentration of 10,000 mg/kg. Two μL of cleaned soil extract were injected into the GC/FID using an Agilent Auto Sampler, 7683 series injector, model G2613A and programmed according to the Atlantic Risk-based Corrective Action (RBCA or "Rebecca") method (1999). An Rtx-5 sil MS Dual (7.5 m $\times$ 32 mm $\times$ 1.0 μm film) column with helium carrier gas at a flow rate of 1.5 mL/min was used. The oven temperature programming was 55°C (no hold); 50°C/min to 95°C (no hold); 20°C/min to 150°C (no hold) and 60°C/min to 290°C (hold 7.5 min). The injector split/splitless at 250°C was splitless for 0.5 min with the FID set at 300°C.

Results and Discussion

Characteristics of Soil and Manures

The geotechnical and geochemical properties of the mixed soil and manures are presented in Table 1. The soil analysis revealed a sandy loam with an absence of clay.

Microbial Enumeration Results

The soil and the commercial microbial sample contained 1.54×10^5 and 7.25×10^7 heterotrophic microbes per gram, respectively, and 5.5×10^5 and 3×10^4 diesel-degrading

Table 1
Geotechnical and geochemical properties of mixed soil and manures

Parameters	Materials		
	Mixed Soil	Poultry Manure	Cow Manure
pH	4.1		
Water content (%)	48.3		
Organic carbon (%)	2.14	14	2.8
N (%)	0.125	2.7	0.4
P (%)	0.0375	0.2	0.14
K (%)	0.1		
Mg (%)	0.2		
Ca (%)	0.1		
Fe (%)	2.4		
Particle size distribution			
Sand (%)	85.8		
Silt (%)	14.2		

microbes per gram, respectively. The commercial sample had a greater number of heterotrophic microbes whereas the soil had more diesel-degrading microbes.

Diesel-Degrading Capability of the Bacteria

In the plate inoculating technique, when the diesel-degraders (from the soil and commercial samples) that had been induced on synthetic agar plates, and then grown on TSA plates in the presence of diesel vapors, were viewed under a microscope, they were seen to have become numerous and it was inferred that they had utilized the diesel vapors and possessed the ability to degrade diesel.

The shake flask experiment results are presented in Table 2. The optical density (OD) of the two blanks was always zero since they contained no microorganisms. The average increase in OD of the cold-tolerant bacteria inoclum, MMS, and diesel was 0.84; their initially colorless solutions turned brown, and the average reduction in OD of the controls was 0.034. These results suggest growth of the cold-tolerant bacteria on the diesel hydrocarbons and death of some cold-tolerant bacteria in the absence of any carbon source. The average decrease in OD of the commercial bacteria inoclum, MMS, and diesel was 1.14, giving final negative values, whereas the controls increased negligibly (by 0.008). These results suggest that the commercial bacteria were killed in the presence of the (1%, v/v) diesel and were more sensitive than the cold-tolerant bacteria to the diesel concentration.

Phase I Experiments

Duplicate and average values of diesel removal in the Phase I experiments after 45 and 90 days are shown in Table 3. The percentage TPH removal is based on the initial diesel concentration of 10,000 mg/kg. Immediately after spiking the soil with diesel, a preliminary pilot test determined that approximately 7.5% or 750 mg/kg of the spiked diesel was not recovered by the Soxtec extraction system.

Table 2
Determination of bioremediation potential of microorganisms from the laboratory shake flask experiments with inocula

Indigenous Cold-tolerant Bacteria Population					
With Diesel as a Carbon Source			Without a Carbon Source		
Design	Initial OD[a]	Final OD	Design	Initial OD	Final OD
MSS[b] + D[c]	0.00	0.00	MSS	0.00	0.00
MSS + D + PB I[d]	1.081	1.924	MSS + PB I	1.070	1.036
MSS + D + PB II[d]	1.076	1.916	MSS + PB II	1.070	1.040
Exogenous Commercial Bacteria Population					
With Diesel as a Carbon Source			Without a Carbon Source		
Design	Initial OD	Final OD	Design	Initial OD	Final OD
MSS + D	0.00	0.00	MSS	0.00	0.00
MSS + D + CB I[e]	0.448	−0.699	MSS + CB I	0.133	0.145
MSS + D + CB II[e]	0.617	−0.524	MSS + CB II	0.135	0.140

[a]OD = Optical Density.
[b]MSS = Mineral Salt Solution.
[c]D = diesel.
[d]PB I, PB II = samples 1 and 2 with Indigenous Cold-tolerant Bacteria.
[e]CB I, CB II = samples 1 and 2 with Exogenous Commercial Bacteria.

Table 3
Percent diesel removal for duplicate samples and their average values in Phase I experiments after 45 and 90 days

		After 45 days			After 90 days		
Treatment and Sample Nos.		Degradation[a] (%)	Average (%)	Range (%)	Degradation[a] (%)	Average (%)	Range (%)
S[b]	1,2	85.4, 85.3	85.4	0.1	96.1, 97.1	96.6	0.5
H[c]	3,4	82.0, 70.5	76.3	7.5	78.7, 79.4	79.1	0.4
CB[d]	5,6	85.2, 70.5	77.9	9.5	86.7, 89.2	88.0	1.4
PB[e]	7,8	71.4, 77.6	74.5	4.2	85.7, 83.8	84.8	1.1
PM[f]	9,10	82.7, 70.8	76.8	7.8	83.8, 87.0	85.4	1.9
CM[g]	11,12	81.0, 77.7	79.4	2.1	89.1, 85.7	87.4	1.9
AC[h]	13,14	89.7, 74.2	82.0	9.5	64.8, 74.3	69.6	6.8
NA[i]	15,16	65.2, 71.2	68.2	4.4	75.7, 86.3	81.0	6.5

[a]Diesel removal values are for duplicate samples.
[b]S = sand.
[c]H = hay.
[d]CB = commercial bacteria.
[e]PB = cold-tolerant bacteria.
[f]PM = poultry manure.
[g]CM = cow manure.
[h]AC = abiotic control and only autoclaved sample.
[i]NA = natural attenuation control without added nutrients, bulking agents or inocula.

For the biostimulation experiments, both manures enhanced diesel removal, with the cow manure being more effective than the poultry manure, and with the cow and poultry manures the resulting C:N:P ratios were 100:1.2:0.75 and 100:7.9:0.64, respectively, indicating that the resulting nitrogen content with the cow manure was lower. C:N:P ratios of 100:1.7:0.1 (Dibble and Bartha, 1979) and 100:0.4:0.04 (Huddleston, 1979) are in good agreement with the findings here. A soil nitrogen content that is too high can have a harmful effect on the microorganisms (Walworth et al., 1997).

In the commercial bacteria bioaugmentation experiments two sets of consortia were present in the non-sterilized soil samples and this may have contributed to the higher 90-day diesel removal observed with commercial bacteria than with cold-tolerant bacteria. The results of mixing the two types of bacteria may confirm that more diverse microbial populations are better.

Table 3 shows that the clean Ottawa sand performed better than the hay as a bulking agent, and the addition of sand resulted in the highest rate of diesel removal among the Phase I treatments. Sand is inert and sandy soils are known to drain well, unlike clayey or organic soils, which retain fluids. The sand may have enhanced oxygen diffusion and facilitated the diesel removal. During the experiments, one visual observation was that microbial growth appeared on the hay, though the growth had not been present before the experiments. This microbial growth on the hay may have indicated that microbes used the hay as well as the diesel as a carbon source, especially since ultimately (at 90 days) diesel removal was better by natural attenuation than by adding hay, although Morgan et al. (1993) reported that hay enhanced remediation of 3, 4-dichloroanaline and benzo(a) pyrene.

Similar behavior is seen in the TPH gas chromatogram of biodegradation at 90 days for the soil with sand (sample No. 2) (Figure 1a) and with hay (sample No. 4) (Figure 1b). The n-alkanes (i.e., large singular peaks) are easily identifiable in a diesel GC profile and are biodegraded rapidly compared to other compounds (De Jonge et al., 1997). Standards provided by Maxxam Analytics, Inc. (courtesy of Mr. Robert Whelan) indicated that n-hexadecane (C16) and n-Heneicosane (C21) occurred at 7.797 min and 5.886 min, respectively. From the total ion chromatogram of diesel (Šepeč et al., 1996), other n-alkanes in Figure 1 (and Figure 2) were inferred. The internal standard used for the GC analysis, 1,2-diphenylbenzene (o-terphenyl) is located between C19 and C20. In Figure 1a the n-alkanes have been well degraded whereas in Figure 1b the n-alkane peaks are still prominent.

Table 3 also shows that less diesel removal at 90 days occurred in the natural attenuation controls than when either inoclum, fertilizer or sand were added. Soil bioaugmentation and biostimulation increase biodegradation more than for soils undergoing natural attenuation (Trindade et al., 2005).

Two of the 16 Phase I experiments (sample Nos. 3 and 13) showed increasing soil diesel concentrations with time, which is impossible, with the greater error occurring for the abiotic control. The sample No. 13 error is probably associated with the 45-day measurement, and due to hydrocarbon losses during extraction or nitrogen drying. Its duplicate showed similar diesel removal at 45 and 90 days (74.2% and 74.3%, respectively), which is reasonable since only volatilization and photodegradation would be expected in the abiotic control. Lighter diesel compounds such as the C10 to C14 n-alkanes are more volatile than higher carbon chain n-alkanes (Namkoong et al., 2002), whereas the C16 to C20 n-alkanes are considered non-volatile (De Jonge et al., 1997) but are biodegraded readily. With increasing carbon number, biodegradation of n-alkanes decreases and the trend is more noticeable for C12 to C16 and than for C16 to C25 n-alkanes (Mohanty and Mukherji, 2008).

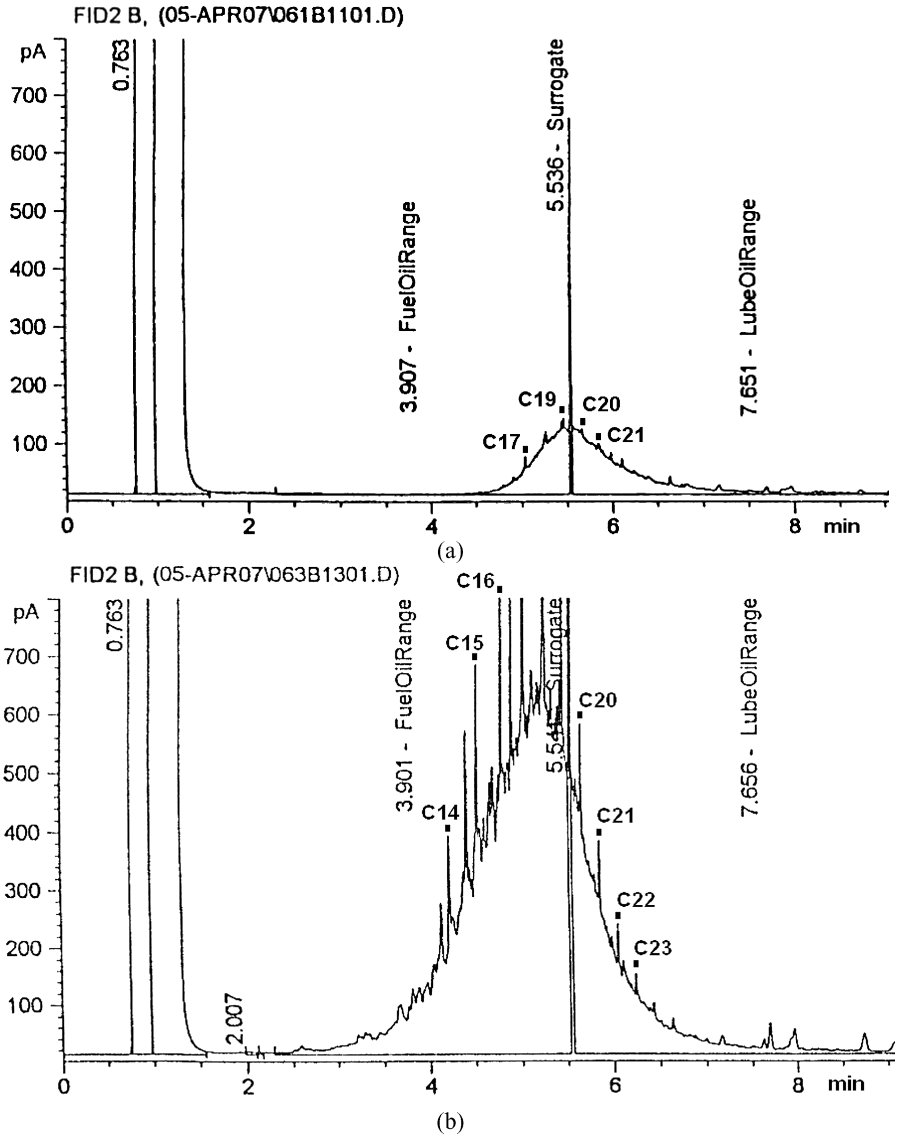

Figure 1. The TPH chromatograms of residual hydrocarbons after 90 days with bulking agents a) sand and b) hay; internal standard at 5.6 min.

Also seen at 90 days (in Table 3) is that greater diesel removal occurred during natural attenuation with microorganisms present than for the abiotic control. The gas chromatograms at 90 days for the abiotic control (sample No. 14) (Figure 2a) and the natural attenuation control (sample No. 16) (Figure 2b) also show more degradation of the C21 and C22 n-alkanes for the natural attenuation sample where biodegradation could occur in addition to photodegradation.

Comparing the C14, C15, C20, C21, C22 and C23 n-alkanes in the gas chromatograms for hay (Figure 1b) and natural attenuation (Figure 2b) it appears that the hay interfered with the diesel removal. TPHs may easily adsorb onto or be absorbed by organic materials

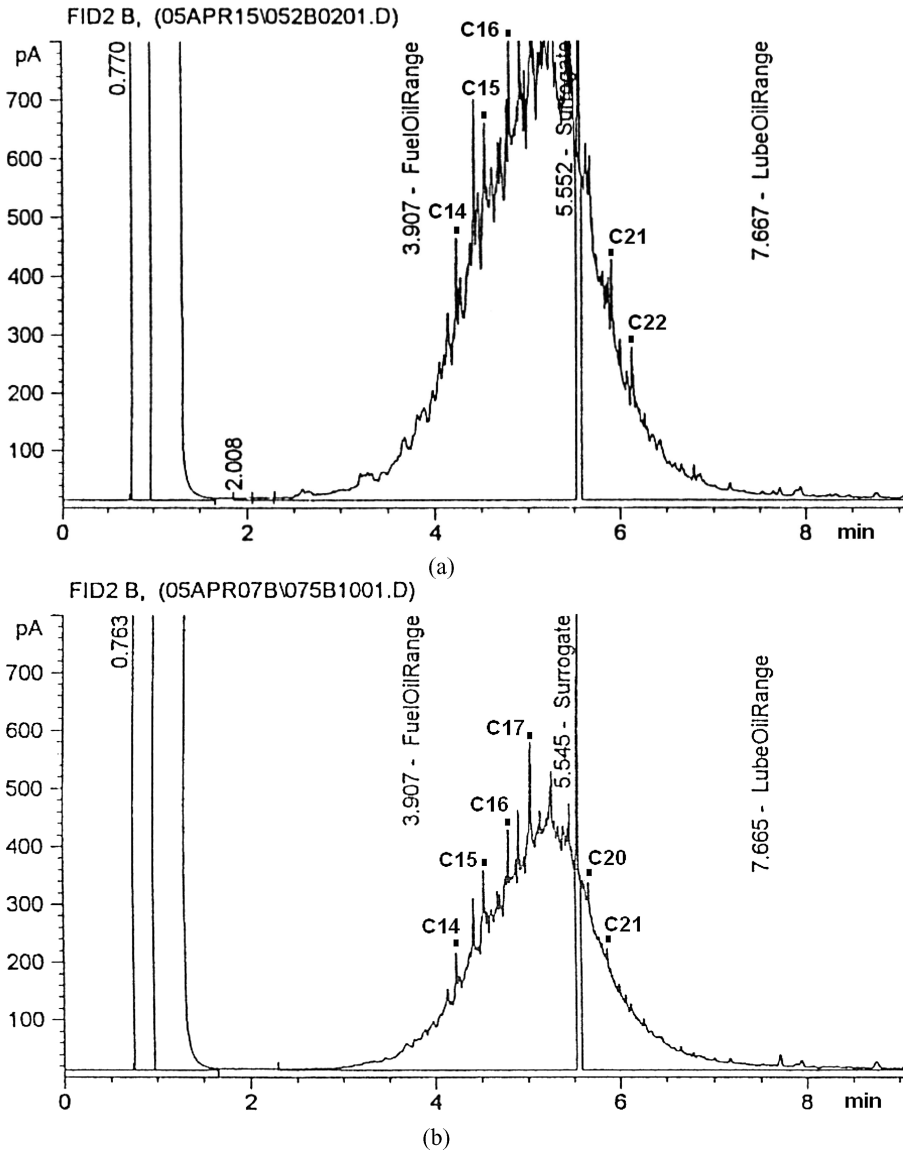

Figure 2. The TPH chromatograms of residual hydrocarbons after 90 days for the a) abiotic control and b) natural attenuation control; internal standard at 5.6 min.

and this sorption can either increase or decrease TPH bioavailability. Bonding between the hay and some of the diesel may have caused a lesser quantity of diesel to be biodegraded and it may also have prevented some physicochemical degradation and volatilization of the diesel. The microorganisms may also have been interested in the hay as a food source, causing them to consume less of the diesel.

In Table 3 the average diesel remaining at 45 days and 90 days for the 16 samples was 2248 mg/kg and 1604 mg/kg, respectively, and the corresponding average measurement errors are ± 0.67% and ± 0.94%, respectively.

Table 4
Percent diesel removal for duplicate samples and their average values in Phase II experiments after 45 and 90 days

Treatment and Sample Nos.		After 45 days			After 90 days		
		Degradation[a] (%)	Average (%)	Range (%)	Degradation[a] (%)	Average (%)	Range (%)
PM[b] + PB[c] + H[d]	17,18	83.4, 77.5	80.5	3.7	88.5, 88.3	88.4	0.1
CM[e] + PB + H	19,20	75.3, 74.6	75.0	0.5	93.0, 89.0	91.0	2.2
CM + CB[f] + H	21,22	74.5, 72.9	73.9	1.1	89.0, 90.3	89.7	0.7
CM + CB + S[g]	23,24	85.1, 85.8	85.5	0.4	95.2, 94.3	94.8	0.5
CM + PB + S	25,26	93.1, 90.7	91.9	1.3	98.5, 93.9	96.2	2.4
PM + PB + S	27,28	84.6, 86.1	84.7	0.6	91.6, 88.7	90.2	1.6
PM + CB + S	29,30	85.0, 84.3	84.7	0.4	91.4, 91.8	91.6	0.2
PM + CB + H	31,32	79.8, 58.3	69.1	15.6	85.6, 87.0	86.3	0.8

[a] Diesel removal values are for duplicate samples.
[b] PM = poultry manure.
[c] PB = cold-tolerant bacteria.
[d] H = hay.
[e] CM = cow manure.
[f] CB = commercial bacteria.
[g] S = sand.

Phase II Experiments

Diesel removal in the Phase II experiments after 45 and 90 days is shown in Table 4 and the percentage TPH removed is based on the initial diesel concentration of 10,000 mg/kg. All of the 16 Phase II experiments exhibited a decrease in diesel concentration with time. At 90 days the highest removal of 96.2% occurred in the treatment with cow manure, cold-tolerant bacteria and sand, and the second highest TPH reduction of 94.8% occurred in the treatment with cow manure, commercial bacteria and sand.

It is thought that the commercial bacteria appeared to perform better than the cold-tolerant bacteria in the Phase I experiments because there were two sets of consortia in the unsterilized samples. However, in the Phase II experiments the treatment with cow manure, cold-tolerant bacteria and sand ultimately (at 90 days) outperformed the cow manure, commercial bacteria and sand combination, in spite of two sets of consortia still being present where the commercial bacteria were introduced. Comparing the two microbial consortia, the commercial samples contained fewer diesel-degrading microorganisms that were less tolerant to diesel and possibly they were also less tolerant of the cow manure in the Phase II experiments. (With the cow manure and hay combination, the cold-tolerant bacteria also did better than the commercial bacteria.)

At 90 days the best Phase II treatment with cow manure, cold-tolerant bacteria and sand with an average diesel removal of 96.2%, was comparable to the 96.6% removal obtained in the best Phase I treatment with sand. At 45 days the average removal for the same two treatments were 91.9% and 85.4%, respectively, and it appears the cow manure may have accelerated the diesel biodegradation in the Phase II experiments. The results suggest that the sand was the better bulking agent and the cow manure was the better fertilizer. Clean Ottawa sand is abundant and inexpensive and would not introduce an organic co-substrate, competitive organisms or toxic metabolites. Sand might be economical compared with

mechanical tillage for increasing porosity and soil aeration, and comparing the costs of adding sand or tilling the soil could be an area for future research.

Sample 32 at 45 days shows only 58.3% diesel removal; it is the lowest value in Tables 3 and 4, and the gas chromatograms showing the peaks for sample 32 at 45 days are significantly more pronounced than for any of the other 32 samples. One possible explanation is that there could have been something with the poultry manure, commercial bacteria and hay combination that slowed the acclimatization of the bacteria so that diesel removal was initially hindered, but then caught up since the 90 days results are similar to those of the duplicate sample.

In Table 4 the average diesel present at 45 and 90 days for the 16 samples was 1931 mg/kg and 900 mg/kg, respectively, giving average measurement errors of $\pm$ 0.78% and $\pm$ 1.67%, respectively. Sample 25 at 90 days measured 150 mg/kg of diesel and its measurement error was $\pm$ 10%.

Conclusions

Fertilizer addition resulted in a higher diesel removal in the Phase I experiments. In the Phase I and Phase II experiments the addition of cow manure resulted in an overall higher TPH removal rate than the addition of poultry manure. The resulting C:N:P ratios with cow manure and poultry manure were 100:1.2:0.75 and 100:7.9:0.64, respectively.

This study supports the use of sand as a bulking agent since in the Phase I experiments the highest TPH removal rate (96%) was achieved using clean Ottawa sand, and in the Phase II experiments the highest diesel removal was achieved with the treatment containing cow manure, cold-tolerant bacteria and sand. The hay as a high organic substrate retarded the diesel removal, possibly by partially replacing the diesel as a carbon source and adsorbing the microorganisms.

The microbial inoculation of cultured cold-tolerant bacteria and commercial bacteria in the Phase I experiments improved TPH degradation compared to the natural attenuation controls. The microbial inoculation of cultured commercial bacteria may have helped more in the Phase I experiments only because it increased the microbial diversity since in the shake flask experiments and the Phase II experiments the cold-tolerant bacteria appeared to perform better than the commercial bacteria. The diesel-degrading microbes in the soil outnumbered those in the commercial microbial product, were less sensitive to diesel concentration and appeared to be more effective at enhancing biodegradation. The natural attenuation control performed better than the abiotic control (as expected) and better than the hay (Phase I) since the hay appeared to inhibit diesel removal.

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