

Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation

Fatima M. Bento^a, Flávio A.O. Camargo^a, Benedict C. Okeke^b,
William T. Frankenberger^{b,*}

^a Department of Soil Science, Federal University of Rio Grande do Sul, Porto Alegre RS 91540-000, Brazil

^b Department of Environmental Sciences-084, University of California, 2217 Geology Building, Riverside, CA 92521-0424, USA

Received in revised form 14 September 2004; accepted 14 September 2004

Available online 21 November 2004

Abstract

Bioremediation of diesel oil in soil can occur by natural attenuation, or treated by biostimulation or bioaugmentation. In this study we evaluated all three technologies on the degradation of total petroleum hydrocarbons (TPH) in soil. In addition, the number of diesel-degrading microorganisms present and microbial activity as indexed by the dehydrogenase assay were monitored. Soils contaminated with diesel oil in the field were collected from Long Beach, California, USA and Hong Kong, China. After 12 weeks of incubation, all three treatments showed differing effects on the degradation of light (C₁₂–C₂₃) and heavy (C₂₃–C₄₀) fractions of TPH in the soil samples. Bioaugmentation of the Long Beach soil showed the greatest degradation in the light (72.7%) and heavy (75.2%) fractions of TPH. Natural attenuation was more effective than biostimulation (addition of nutrients), most notably in the Hong Kong soil. The greatest microbial activity (dehydrogenase activity) was observed with bioaugmentation of the Long Beach soil (3.3-fold) and upon natural attenuation of the Hong Kong sample (4.0-fold). The number of diesel-degrading microorganisms and heterotrophic population was not influenced by the bioremediation treatments. Soil properties and the indigenous soil microbial population affect the degree of biodegradation; hence detailed site specific characterization studies are needed prior to deciding on the proper bioremediation method.

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Keywords: Diesel oil contamination; Soil; Bioremediation; Natural attenuation; Biostimulation; Bioaugmentation; Petroleum hydrocarbons

1. Introduction

Among hydrocarbon pollutants, diesel oil is a complex mixture of alkanes and aromatic compounds that frequently are reported as soil contaminants leaking from storage tanks and pipelines or released in accidental spills (Gallego et al., 2001). One of the best approaches to restoring contaminated soil is to make use

of microorganisms able to degrade those toxic compounds in a bioremediation process.

Bioremediation is an attractive approach of cleaning up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant (Frankenberger, 1992). To warrant a practical application, any bioremediation process should demonstrate that removal of contaminants is the primary effect of biodegradation, and that the degradation rate is greater than the natural rate of decontamination. One of the difficulties of developing bioremediation strategies lies in achieving as good or better results in the field as in the laboratory (Juhasz et al., 2000).

* Corresponding author. Tel.: +1 909 787 3405; fax: +1 909 787 2954.

E-mail address: william.frankenberger@ucr.edu (W.T. Frankenberger).

Strategies for inexpensive and natural bioremediation include natural attenuation, biostimulation, bioventing, bioaugmentation, landfarming, composting, and phytoremediation (Skipper, 1999). Diesel oil bioremediation in soil can be promoted by stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil (biostimulation) (Seklemova et al., 2001) or through inoculation of an enriched microbial consortium into soil (bioaugmentation) (Richard and Vogel, 1999; Barathi and Vasudevan, 2001).

For optimum biodegradation conditions, it is important to know the characteristics of the contaminated site before beginning treatments. Basic information such as residual oil concentration, population density of the oil-degrading microorganisms and the biodegradation potential, are key factors to be considered for bioremediation of oil-polluted sites. In most field studies, enhancing biodegradation of petroleum hydrocarbons depends on the specific microbial population present. The composition of the microbial population is affected by the environmental conditions and the composition of the hydrocarbons (Admon et al., 2001). In this study, we performed a comparative evaluation of natural attenuation, biostimulation and bioaugmentation for bioremediation of diesel oil contaminated soils.

2. Methods

2.1. Soil samples and microorganisms

Two diesel oil contaminated soils were collected from Long Beach, California (USA) and Hong Kong (China). The soil properties of both samples are provided in Table 1. The diesel oil-degrading microbial population was 4.96 and 4.38 log bacteria ml⁻¹ in the Long Beach and Hong Kong soils, respectively. The hydrocarbon-degrading bacterial consortium used in bioaugmentation were those previously isolated from the Long Beach soil, identified by 16S-rRNA gene sequence as *Bacillus cereus*, *Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus pumilus*, *Acinetobacter junii* and *Pseudomonas* sp. (Bento et al., submitted for publication).

2.2. Bioremediation treatments

We carried out three treatments to evaluate the efficiency of diesel oil degradation in the contaminated

soils, using sterilized soils as controls. The treatments were: (a) *natural attenuation* (soil's natural ability to degrade the contaminant); (b) *biostimulation* (adding nutrients to improve the natural biodegradation rate); and (c) *bioaugmentation* (addition of a microbial consortium from selected species isolated from a contaminated soil plus nutrients).

2.3. Microcosm description

Contaminated soils (450 g samples) were placed in a set of aluminum pans with a surface area of 441 cm² and a volume of 1764 cm³. We mixed the soils weekly to provide sufficient air and oxygen. The microcosms were covered with aluminum foil and kept at room temperature (27 °C). The soil was moistened by the addition of 20 ml of sterile distilled water every week until the end of the experiment. The control was sterilized three times by autoclaving at 121 °C for 30 min. The soils were biostimulated by adding 250 mg kg⁻¹ of (NH₄)₂SO₄ and 100 mg kg⁻¹ of K₂HPO₄. The same conditions provided in the biostimulation treatment were used in the bioaugmentation treatment plus the addition of 40 ml of 2.6 × 10⁸ cells ml⁻¹ of a bacterial consortium previously isolated from the Long Beach soil.

2.4. Soil analyses

The contaminated soils were sampled at 0, 1, 2, 6, 8, and 12 weeks for chemical and microbiological analyses. Composite samples were obtained by mixing 5 g of soil collected from five different areas of the microcosm. A portion of the composite soil (1 g) was placed in sterile bottles for microbiological analyses, and the remainder was analyzed chemically. Total petroleum hydrocarbons (TPH) were determined using a modified EPA 8015 technique. Briefly, the soil sample was extracted with methylene chloride and an aliquot of the extract was injected into a gas chromatograph (HP 5890, Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID). The column used was a DB-1 (30 m by 0.25 mm) with helium carrier gas at a flow rate of 2 ml min⁻¹, hydrogen gas at a flow of 30 ml min⁻¹ and air at a flow of 300 ml min⁻¹. The temperature program used was 50 °C 2 min⁻¹, 35 °C min⁻¹, 270 °C 5 min⁻¹. The extractable petroleum hydrocarbons were identified and quantified by comparison of a sample chromatogram with calibration chromato-

Table 1
Physical and chemical characteristics of soils

Soil sample	pH (1:1)	CEC (cmol _c kg ⁻¹)	Organic carbon (g kg ⁻¹)	Total N (g kg ⁻¹)	Sand (g kg ⁻¹)	Silt (g kg ⁻¹)	Clay (g kg ⁻¹)	TPH (C ₁₂ -C ₂₃) (mg kg ⁻¹)	TPH (C ₂₃ -C ₄₀) (mg kg ⁻¹)
Long Beach	6.3	6.2	15.1	2.38	610	320	70	2800	9450
Hong Kong	7.7	10.8	38.1	1.85	780	150	70	3300	7450

grams of diesel oil. Percentage of degradation was calculated by the following expression: Percentage of degradation = $[(\text{TPH control} - \text{TPH treatment}) / \text{TPH control}] \times 100$.

Microbial cell numbers were estimated using the most probable number (MPN) method (Braddock and Catterall, 1999). Briefly, 1 g soil samples were added to 10 ml of distilled water and vortexed for 30 min and serially diluted to 10^{-10} . Sterile Tryptic Soy Broth was dispensed (250 μl) into 96-well microtitre plates and the wells were incubated (five replicates) with 10 μl of the respective dilutions of soil samples for total heterotrophic microorganisms. Bacterial growth was determined by turbidity. For diesel fuel-degrading microorganisms, we used a Bushnell–Hass medium and a tetrazolium chloride (TTC) solution as the indicator. After inoculation with dilutions of the soil samples, microtitre plates were inoculated with 10 μl of diesel oil (sterilized through 0.2 μm membrane). The static cultures were incubated without agitation at room temperature (27 °C) for 10–14 days. At the end of this period, each plate was scored visually by violet color development (indicating reduction of the tetrazolium dye via respiration) for the diesel oil-degraders. Microbial population were then determined using statistical tables found in Standard Methods of Soil Analysis (Lorch et al., 1995).

Soil microbial activity was estimated by the dehydrogenase assay. Dehydrogenase activity was determined by monitoring the rates of reduction of 2,3,5-triphenyltetrazolium chloride to triphenylformazan as described by Alef (1995), calculated as μg of formazan g^{-1} of soil after 24 h, and expressed as relative activity (%) in relation to the control activity (100%).

2.5. Statistical analysis of data

Experiments were conducted using two independent replicates. Data were subjected to analysis of variance and the averages were compared by Tukey multiple range test at $p \leq 0.001$.

3. Results

3.1. Bioremediation treatments and diesel oil degradation

Chromatographic analysis allowed us to estimate the degradation of the diesel oil in the light ($\text{C}_{12}\text{--}\text{C}_{23}$) and the heavy fractions ($\text{C}_{23}\text{--}\text{C}_{40}$). The effect of the bioremediation treatment on the degradation of the light fraction of TPH in the two soils is shown in Table 2. Microbial degradation of TPH was higher in the soil sample from Long Beach than in the Hong Kong soil. In the Hong Kong soil, degradation was dependent on incubation time, and reached 50% only upon natural attenuation after 2 weeks (Table 2). The Long Beach soil showed the highest percentage of degradation in the first week of incubation, but part of the loss may be attributed to volatilization as well.

Among the bioremediation treatments, the highest degradation rate (63–84%) of the light fraction of TPH was observed in the Long Beach soil when a consortium of pre-selected bacteria (bioaugmentation) was added. The effect of nutrient addition (biostimulation) to this soil promoted significant degradation (72%) of the light fraction only after six weeks of incubation. In the Hong Kong soil, natural attenuation resulted in the highest degradation (47%) of the light fraction of TPH.

The bioremediation treatments affected the degradation of the heavy fraction of TPH in both soils, but more so in the Long Beach soil (Table 3). The pattern of degradation in this soil was similar to the light fraction. Only after 12 weeks, did the degradation of the heavy fraction in the Long Beach soil decrease significantly, upon natural attenuation (Table 3).

The bioremediation treatments did not affect the degradation of the heavy TPH fraction in the Hong Kong soil at the beginning of incubation (Table 3). In the sixth week, 19% degradation was observed upon natural attenuation. Further incubation did not achieve a substantial increase in degradation. In the Hong Kong soil, approximately 28–31% degradation of the heavy TPH

Table 2
Percentage of degradation of the light fraction ($\text{C}_{12}\text{--}\text{C}_{23}$) of total petroleum hydrocarbons present in soils contaminated with diesel oil

Weeks	Long Beach soil			Hong Kong soil		
	Attenuation	Biostimulation	Bioaugmentation	Attenuation	Biostimulation	Bioaugmentation
<i>Percentage of degradation^a</i>						
01	41.9 \pm 0.24Cd	45.8 \pm 0.13Bc	83.8 \pm 0.45Aa	0.0 \pm 0.40Cd	1.2 \pm 0.02Bd	5.0 \pm 0.09Ac
02	55.6 \pm 0.12Bb	16.3 \pm 0.34Cd	68.4 \pm 0.27Ac	10.1 \pm 0.11Ac	5.1 \pm 0.08Bc	7.6 \pm 0.08Ac
06	60.0 \pm 0.33Ba	71.5 \pm 0.29Aa	74.0 \pm 0.31Ab	49.0 \pm 0.22Aa	27.7 \pm 0.21Bb	25.5 \pm 0.21Bb
08	49.2 \pm 0.41Bc	69.2 \pm 0.39Aa	71.3 \pm 0.17Abc	31.0 \pm 0.08Ab	27.3 \pm 0.32Bb	23.6 \pm 0.32Cb
12	36.2 \pm 0.17Ce	59.4 \pm 0.24Bb	68.1 \pm 0.33Ac	47.1 \pm 0.21Aa	35.3 \pm 0.24Ba	43.8 \pm 0.18Aa

^a Percentage of degradation was calculated by the following expression: % of degradation = $[(\text{TPH control} - \text{TPH treatment}) / \text{TPH control}] \times 100$. Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.001$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.001$). \pm Standard error ($n = 2$).

Table 3

Percentage of degradation of the heavy fraction (C₂₃–C₄₀) of total petroleum hydrocarbons present in soils contaminated with diesel oil

Weeks	Long Beach soil			Hong Kong soil		
	Attenuation	Biostimulation	Bioaugmentation	Attenuation	Biostimulation	Bioaugmentation
<i>Percentage of degradation</i> ^a						
01	29.2 ± 0.30Cc	33.3 ± 0.16Bb	86.1 ± 0.25Aa	0.0 ± 0.02Bd	0.0 ± 0.06Bd	5.0 ± 0.12Abc
02	55.9 ± 0.22Ba	12.2 ± 0.09Cc	68.3 ± 0.21Ac	8.2 ± 0.07Ab	3.3 ± 0.13Cc	6.0 ± 0.12Bb
06	57.8 ± 0.44Ca	67.1 ± 0.29Ba	73.1 ± 0.34Ab	19.0 ± 0.19Aa	2.0 ± 0.07Ccd	4.2 ± 0.11Bc
08	50.8 ± 0.27Cb	68.1 ± 0.33Ba	71.8 ± 0.20Abc	3.1 ± 0.10Bc	7.1 ± 0.09Ab	2.1 ± 0.09Bd
12	27.8 ± 0.18Bc	35.6 ± 0.33Ab	37.3 ± 0.31Ad	9.3 ± 0.21Bb	28.2 ± 0.23Aa	30.9 ± 0.22Aa

^a Percentage of degradation was calculated by the following expression: % of degradation = [(TPH control – TPH treatment)/TPH control] × 100. Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.001$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.001$). ± Standard error ($n = 2$).

fraction was achieved upon biostimulation and bioaugmentation treatments after 12 weeks (Table 3).

The effects of the bioremediation treatments on the cumulative percentage of degradation and weekly rate of degradation of TPH in both soils contaminated with diesel oil are summarized in Table 4. After 12 weeks of incubation, the greatest percentage of degradation of the light (75%) and heavy fractions of TPH (73%) was observed in the Long Beach soil when pre-selected bacteria (bioaugmentation) were added to the microcosm. This pattern was also observed in the weekly rate of degradation, where bioaugmentation was responsible for the highest degradation rates in both fractions of TPH of the Long Beach soil. Addition of nutrients (biostimulation) had the least effect on the degradation of both fractions of TPH in both soils.

In comparing the two soils, the Long Beach sample showed the most degradation in both TPH fractions. In the Hong Kong soil, a higher percentage of degradation was found upon natural attenuation of the light fraction. In the heavy fraction, the percentage of degradation of TPH by biostimulation in the Hong Kong soil

was very close to that of the bioaugmentation treatment. However, the weekly rate of degradation in the Hong Kong soil was two times greater under natural attenuation than bioaugmentation with a consortium of bacteria isolated from the Long Beach soil.

3.2. Microbial activity

Dehydrogenase activity in soil has been used to monitor microbial activity as an index for the total oxidative activity (Alef, 1995). We relied on diesel oil as a carbon source in estimating the activity of the diesel-degrading microbial population (Frankenberger et al., 1989). Dehydrogenase activity varied, depending on the incubation time, bioremediation treatments and soils (Table 5). Increasing the incubation time increased the relative activity of dehydrogenase in both soils, with the highest activity detected upon natural attenuation of the Hong Kong soil (Table 5).

Among the bioremediation treatments in the Long Beach soil, the highest microbial activity occurred upon bioaugmentation (Table 5). The addition of pre-selected

Table 4

Cumulative percentage of degradation and weekly rate constant (k) of degradation of the light and heavy fractions of total petroleum hydrocarbons present in two soils contaminated with diesel oil

Treatments	TPH fractions			
	Light fraction (C ₁₂ –C ₂₃)		Heavy fraction (C ₂₃ –C ₄₀)	
	Long Beach soil	Hong Kong soil	Long Beach soil	Hong Kong soil
<i>Percentage of degradation</i> ^a				
Attenuation	48.7 ± 0.33Ab	23.3 ± 0.18Ba	45.7 ± 0.41Ab	7.5 ± 0.08Ba
Biostimulation	45.8 ± 0.25Ab	16.0 ± 0.28Bb	45.2 ± 0.39Ab	6.2 ± 0.11Ba
Bioaugmentation	75.2 ± 0.17Aa	17.8 ± 0.31Bb	72.7 ± 0.37Aa	7.3 ± 0.11Ba
<i>Weekly degradation rate</i> ^b				
Attenuation	0.451	0.000021	0.205	0.105
Biostimulation	0.324	0.000008	0.106	0.00012
Bioaugmentation	0.577	0.000011	0.296	0.0405

Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.001$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.001$). ± Standard error ($n = 2$).

^a Percentage of degradation accumulated in 12 weeks.

^b Estimated using cumulative degradation (mg TPH kg⁻¹ soil) and the exponential equation: Degradation = Degradation_{max} (1 – e^{-kx}) was used to calculate the degradation rate constant (k).

hydrocarbon degraders increased the microbial activity by 2.45-fold in the beginning, and by 3.30-fold at the end of the incubation period (12 weeks). The addition of nutrients such as nitrogen and phosphorus stimulated the activity of the indigenous microbial population up to the sixth week, but after that, we observed a small decrease in dehydrogenase-related activity. Natural attenuation in the Long Beach soil showed the least microbial activity compared with the other bioremediation treatments. Upon natural attenuation, we found a continual increase in dehydrogenase activity, reaching almost 2.00-fold at the end of the incubation period.

In the Hong Kong soil, we observed the least dehydrogenase-related activity (0.75-fold) in the first week of natural attenuation. However, microbial activity increased substantially in subsequent weeks with this treatment, reaching the highest level (4.05-fold) observed in the 12th week. Biostimulation showed an initial increase in dehydrogenase activity in the first week and after the eighth week of incubation. Decreases in dehydrogenase activity were observed between the second and sixth weeks. In the bioaugmentation treatment, microbial activity was not affected by incubation time and was the lowest at the end of the experiment (Table 5).

3.3. Microbial enumeration

The number of diesel oil-degrading microorganisms was highest in the Long Beach soil in the first week

(Table 6). In this soil, the addition of pre-selected bacteria (bioaugmentation) or nutrients (biostimulation) resulted in the greatest number (7.50 and 7.30 log bacteria ml⁻¹, respectively) at the beginning of incubation, compared to natural attenuation (4.96 log bacteria ml⁻¹). After the first week, the number of diesel-oil degrading microorganisms decreased and was not affected by the bioremediation treatments until the end of the experiment. In general, the diesel oil-degrading microorganisms were not affected by the bioremediation treatments in the Hong Kong soil.

The number of heterotrophic microorganisms present in the contaminated soils upon the bioremediation treatments is shown in Table 7. The ratio of diesel oil-degrading microorganisms/heterotrophic microorganisms indicates that there are more heterotrophic microorganisms and that this ratio was the smallest in the Long Beach soil, mainly in the first two weeks. Thereafter, the ratios remained similar in both soils and was not affected by the bioremediation treatments (Table 7).

The population density of the microorganisms influenced by the bioremediation treatments was estimated to determine if a possible relationship existed to microbial activity. An increase in microbial activity after the second week of incubation was not followed by an increase in the number of diesel-degrading microorganisms and heterotrophic population in both soils. This negative relationship could indicate that specialized microorganisms were adjusting to the changing

Table 5
Microbial activity estimated by dehydrogenase-related activity (relative to the control) of soils contaminated with diesel oil

Weeks	Long Beach soil			Hong Kong soil		
	Attenuation	Biostimulation	Bioaugmentation	Attenuation	Biostimulation	Bioaugmentation
<i>Fold of control</i>						
01	0.83 ± 0.07Bc	1.22 ± 0.25Bb	2.45 ± 0.25Ac	0.75 ± 0.08Bc	2.20 ± 0.15Bb	1.96 ± 0.20Ac
02	1.37 ± 0.19Bbc	1.59 ± 0.31Bb	2.75 ± 0.13Abc	2.05 ± 0.12Bbc	1.62 ± 0.16Bb	2.35 ± 0.23Abc
06	1.87 ± 0.21Bab	2.93 ± 0.24Aa	2.71 ± 0.23Abc	1.68 ± 0.10Bab	1.17 ± 0.22Aa	2.92 ± 0.17Abc
08	2.06 ± 0.13Ba	3.00 ± 0.41Aa	3.40 ± 0.41Aab	3.11 ± 0.22Ba	1.77 ± 0.30Aa	2.77 ± 0.31Aa
12	2.00 ± 0.33Bab	2.40 ± 0.11Ba	3.30 ± 0.29Aa	4.00 ± 0.36Bab	3.57 ± 0.28Ba	3.00 ± 0.33Aab

Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.05$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.05$). ± Standard error ($n = 2$).

Table 6
Number of diesel oil-degrading microorganisms in soils contaminated with diesel oil

Weeks	Long Beach soil			Hong Kong soil		
	Attenuation (log bacteria ml ⁻¹)	Biostimulation (log bacteria ml ⁻¹)	Bioaugmentation (log bacteria ml ⁻¹)	Attenuation (log bacteria ml ⁻¹)	Biostimulation (log bacteria ml ⁻¹)	Bioaugmentation (log bacteria ml ⁻¹)
01	4.96 ± 0.22Ca	7.30 ± 0.32Ba	7.50 ± 0.25Aa	3.84 ± 0.16Cc	5.51 ± 0.33Aa	4.79 ± 0.23Bb
02	3.04 ± 0.34Cc	3.36 ± 0.27Bc	3.90 ± 0.19Ac	4.40 ± 0.39Bb	4.50 ± 0.28Bb	5.00 ± 0.25Ab
06	3.34 ± 0.18Bc	3.54 ± 0.14Ac	3.60 ± 0.20Ac	4.89 ± 0.27Aa	4.69 ± 0.19Ab	4.89 ± 0.17Ab
08	4.23 ± 0.41Bb	4.34 ± 0.44Bb	4.54 ± 0.42Ab	4.73 ± 0.26Bab	4.69 ± 0.18Bb	5.69 ± 0.45Aa
12	3.34 ± 0.27Cc	3.54 ± 0.23Bc	3.96 ± 0.24Ac	4.66 ± 0.12Ab	3.80 ± 0.22Bc	4.20 ± 0.20Ac

Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.05$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.05$). ± Standard error ($n = 2$).

Table 7
Number of total heterotrophic microorganisms present in soils contaminated with diesel oil

Weeks	Long Beach soil			Hong Kong soil		
	Attenuation (log bacteria ml ⁻¹)	Biostimulation (log bacteria ml ⁻¹)	Bioaugmentation (log bacteria ml ⁻¹)	Attenuation (log bacteria ml ⁻¹)	Biostimulation (log bacteria ml ⁻¹)	Bioaugmentation (log bacteria ml ⁻¹)
01	7.35 ± 0.78Ba (0.67) ^a	9.15 ± 0.36Aa (0.79)	8.61 ± 0.65Aa (0.87)	5.59 ± 0.43Bb (0.68)	5.27 ± 0.23Cd (1.04)	6.10 ± 0.57Abc (0.78)
02	7.90 ± 0.66Ca (0.38)	8.50 ± 0.69ABa (0.39)	8.90 ± 0.82Aa (0.43)	5.20 ± 0.52Ac (0.84)	5.90 ± 0.88Bb (0.76)	6.40 ± 0.77Abc (0.72)
06	5.89 ± 0.51Abc (0.56)	6.38 ± 0.45Abc (0.55)	6.11 ± 0.74Ab (0.59)	5.69 ± 0.42Bb (0.85)	5.84 ± 0.94ABbc (0.80)	6.07 ± 0.48Ac (0.81)
08	5.38 ± 0.49Cc (0.78)	5.69 ± 0.80ABc (0.46)	6.38 ± 0.56Ab (0.71)	5.69 ± 0.55ABb (0.83)	5.54 ± 0.30Bcd (0.89)	5.84 ± 0.22Ac (0.97)
12	7.96 ± 0.91Aab (0.42)	7.32 ± 0.53Ab (0.48)	6.89 ± 0.79Ab (0.57)	7.96 ± 0.78Ba (0.58)	7.38 ± 0.44Ca (0.51)	8.38 ± 0.34Aa (0.50)

Same capital letters are not statistically different among treatments by the Tukey test ($p < 0.05$) and same lower caps letter are not statistically different among weeks by the Tukey test ($p < 0.05$). ± Standard error ($n = 2$).

^a Ratio of diesel oil-degrading microorganisms/heterotrophic microorganisms.

substrate conditions, increasing their metabolic activity in a stressed environment, thus limiting the growth of the microbial population (Devinny and Chang, 2000).

4. Discussion

The highest rate of degradation of the light fraction of TPH (C₁₂–C₂₃) was observed in the first two weeks of incubation in the Long Beach soil. During that period, more than 50% of the TPH was degraded, with a small and continual decrease in degradation until the end of the experiment (12 weeks). In the beginning, diesel oil-degrading microorganisms were stimulated by labile hydrocarbon sources (probably linear and open-chain hydrocarbons) that induced a high percentage of degradation. As those forms decreased, microbial populations had to use the more recalcitrant hydrocarbons (probably aromatic hydrocarbons with higher molecular weight) less efficiently. With the decrease in labile carbon sources, nutrients were most likely limited in supporting microbial growth. It is also possible that degradation of higher molecular weight hydrocarbons may produce toxic intermediates that can inhibit diesel-degrading microorganisms (Frankenberger, 1992).

Independent of the fraction of TPH (light or heavy) and the bioremediation treatment, the soils themselves had varying effects on degradation of diesel oil and on microbial populations as well as activity. The soil collected from Long Beach, California showed the highest TPH degradation, reaching more than 70% degradation in 12 weeks of incubation. Upon bioaugmentation, this soil had a weekly rate of degradation of 58%. The Hong Kong soil showed a weak response in biodegradation of its TPH components. We observed that TPH degradation in the latter soil and microbial activity were highest

upon natural attenuation, which is an indication that the activity of the indigenous microorganisms in the Hong Kong soil limited the activity of the introduced bacteria. The success of bioremediation is dependent of the level of metabolic and genetic adaptation of the microbial populations to their environment (Devinny and Chang, 2000).

Biostimulation of soil microorganisms upon the addition of N and P resulted in the least degradation of diesel oil among the bioremediation treatments evaluated. In a field demonstration of bioremediation based on biostimulation, Seklemova et al. (2001) found that the addition of nutrients had no effect on the decontamination of a forest soil contaminated with diesel oil. On the other hand, Gallego et al. (2001) evaluated in situ bioremediation techniques and demonstrated that in laboratory conditions it is possible to degrade up to 90% of diesel oil, using inorganic N and P. Addition of nutrients including N and P is standard practice for increasing hydrocarbon degradation (Atlas and Bartha, 1998). By adding these nutrients, the C/N and C/P ratios of the soil is closer to the bacterial C/N and C/P requirements. In our work, the less effect of biostimulation on diesel oil degradation might be due to nutrient limitation in terms of bioavailability. Mills (1994) evaluated the effect of phosphorus sources and concentration (100–1000 mg kg⁻¹) on diesel fuel degradation, and reported that degradation depended on phosphorus availability. Some sources might supply enough phosphorus to restore the microbial C/P relationship, but become unavailable because of their low solubility. Knowledge of bioavailability of nutrients is necessary in planning of an efficient bioremediation strategy.

Monitoring the indigenous microorganisms within a contaminated site is the simplest approach for cleanup of hydrocarbons (Devinny and Chang, 2000). In the Hong Kong soil, natural attenuation resulted in the

highest degradation of the light fraction of TPH. The indigenous microbial population present in the Hong Kong soil degraded diesel oil more efficiently than the microbial consortium introduced from the Long Beach soil. This efficiency could be explained by the autochthonous adaptation that allows microorganisms to be physiologically compatible with their habitat, compared with transient allochthonous organisms that do not occupy a functional niche (Atlas and Bartha, 1998).

The best performance in diesel oil degradation was with bioaugmentation of the Long Beach soil. In the field, bioaugmentation of contaminated soil with commercial preparations has been less promising. When commercial products are used, microbial species that may be much different than those of the local environment are often applied. Most tests indicate that the benefits of these products do not justify their cost (Frankenberger, 1992). New bioaugmentation approaches have been described in the literature, and selection and culturing for organisms directly from the local site have shown to give the best results (Devinny and Chang, 2000). Capelli et al. (2001) reported a decrease in TPH of almost 70% in laboratory studies inoculated with pre-selected bacteria. In our study, the addition of a pre-selected microbial consortium degraded 73–75% of the light (C_{12} – C_{23}) and heavy (C_{23} – C_{40}) fractions of the TPH present in the Long Beach soil contaminated with diesel oil, but had no effect on the Hong Kong soil. Thus, the best bioaugmentation performance can be approached by the use of microorganisms that are already present in the soil and increasing their abundance. With the increase of a specific microbial community and nutrient addition, this approach reduces cleanup time substantially. Indigenous microorganisms are well adjusted to their own environment. An immediate increase in the population density of these microbes could ensure rapid degradation of the pollutant.

Acknowledgements

We wish to thank Fred Sims with Weston Environmental Consultants in providing the soil sample from Hong Kong. F.A.O. Camargo and F.M. Bento are grateful to the Brazilian National Research Council (CNPq) for a scholarship concession, especially to SEBIE staff Katiane Lima and to the Department of Environmental Science (UCR) for the opportunity to participate as visiting scientists.

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