Remediation Techniques Applied in Residual Clayey Soil (Oxisol) Contaminated by Diesel and Biodiesel

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Abstract. . Biodegradation in residual basaltic soil contaminated by diesel fuel and biodiesel was assessed by three different techniques comprising natural attenuation, bioaugmentation, and bioventing. The soil was described in terms of its physicochemical and microbiological characteristics, showing favorable bioremediation conditions. Natural attenuation was monitored throughout the experiment. Bioaugmentation was performed using bacteria pre-selected from the soil at the concentration of ~3.0 x10^6 CFU mL^{-1}. In the bioventing process, the soil was aerated during a period of 4 h a day at a pressure of 280 kPa. Bioremediation was assessed by counting the colony forming units (CFU g^{-1} of soil) and by degradation of contaminants using gas chromatography for 120 days. The highest microbial density was observed at 30 days in natural attenuation and at 60 days in bioaugmentation and bioventing. Biodiesel-contaminated soils had the largest microbial activity and highest degradation of carbon chains. At 120 days, bioaugmentation was the most efficient bioremediation technique, with the largest biodegradation rate for the light fraction of diesel (78.5%) and for biodiesel esters (98.6%). Bioventing was more efficient than natural attenuation for both contaminants, indicating the presence of aerobic bacteria at the analyzed soil depth.

Keywords: bioremediation, microorganisms, contaminants, aeration, residual soil.

1. Introduction

The contamination of soils and groundwater by petroleum hydrocarbons has grown in recent decades, especially because of the high frequency of contamination episodes and of the impacts of these episodes on the environment. Even though large accidental oil spills are often reported in the media, it is estimated that the major source of contamination by diesel fuel is related to small fuel leaks in containers, to mechanical or human error during unloading operations, or even to accidents during the transportation of this chemical.

According to Wolicka *et al.* (2009), the risk posed to the environment depends on the characteristics of the contaminated area (surface and/or depth) and on the chemical composition of petroleum hydrocarbons. In the case of fuel leaks into the soil, a wide range of physicochemical and biological processes are indicated for remediation of these hydrocarbons. Physicochemical processes such as air injection, soil vapor extraction, vapor removal and bioremediation are some of the remediation processes used in current practice. (Sharma & Reddy, 2004).

Bioremediation processes have become recognized as alternatives for the treatment of soils contaminated by organic substances and have been preferred as they are based on natural and relatively simple methods that are less aggressive and more appropriate for the maintenance of ecological balance, aside from their low cost compared to other remediation processes (Bento *et al.*, 2005, Mathew *et al.*, 2006).

The bioremediation of organic pollutants by biodegradation is based on the capacity of microbial populations to modify or decompose pollutants. Biodegradation occurs by the action of soil microorganisms (natural attenuation) or inoculation of contaminated soil with bacteria (bioaugmentation), under natural process or stimulated by nutrients such as nitrogen and phosphorus (biostimulation) or by oxygen (bioventing) (Troquest *et al.*, 2003).

The soil natural microbiological activity or the inoculated microorganisms are used in bioremediation processes to reduce the concentration and/or toxicity of different pollutants, including petroleum hydrocarbons (Alexander, 1994; Rajashekara Murthy *et al.*, 2010; Castro-Gutiérrez *et al.*, 2012; Lalevic *et al.*, 2012). The major microorganisms that potentially degrade hydrocarbons in the soil include the genera *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium*, *Bacillus*, *Aspergillus*, *Mucor*, *Fusarium*, *Penicillium*,

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Submitted on April 8, 2013; Final Acceptance on August 2, 2013; Discussion open until April 30, 2014.

Rhodotorula, *Candida* and *Sporobolomyces*, as reported by Leahy & Colwell, 1990; Bento *et al.*, 2003; Alvarez & Illman, 2006; Sørensen *et al.*, 2011.

Several studies have been recently carried out on bioremediation of soils contaminated by biodiesel and by diesel (Trindade, 2002; Bento *et al.*, 2005; Byun *et al.*, 2005; Hwang *et al.*, 2006; Mariano, 2006; Wolicka *et al.*, 2009; Bücker *et al.*, 2011; Sørensen *et al.*, 2011; Moliterni *et al.*, 2012). However, little is known about the biodegradation potential of these contaminants in residual soil (Oxisol). This kind of soil is very common in different parts of the world and it is located mainly on developing countries.

The aim of the present study was to assess the biodegradation potential of a residual clayey soil (Oxisol) contaminated by diesel and biodiesel oil using three bioremediation processes: natural attenuation, bioaugmentation, and bioventing.

2. Materials and Methods

The experiments were undertaken *ex situ* using a residual clayey soil (oxisol) for addition of contaminants. Undisturbed soil samples were obtained at a depth of 1.2 m, from B horizon. The physicochemical characteristics of the soil are shown in Table 1. With respect to particle sizes, the residual soil contains 70% clay, 6% silt and 24% fine sand (ASTM D422). Although the soil presents high clay amount in its composition, it can be observed in Table 1 that the soil has high hydraulic conductivity. The oxisol has well-structured aggregates and are composed of mixture of kaolinite and Fe oxide (hematite). This makes the soil composed with stable aggregates and they are responsible for high infiltration and, as consequence, high hydraulic conductivity.

For all tests undisturbed soil samples were molded into cylindrical shape, 100 mm high and 75 mm in diameter and were placed in bioreactors made of screw-capped polyvinyl chloride (PVC) tubes with 100 mm in diameter and 200 mm in length. The samples were inserted inside the PVC tube and the lateral space was filled with commercial gypsum slurry, thus furthering a state of containment after its cure. This was necessary to avoid preferential flow between soil and the tube wall, since the cured gypsum has hydraulic conductivity a hundred times lower than soil used in this work.

Natural soil samples were contaminated with diesel fuel (of type C, sold at gas stations in Brazil) and with animal derived biodiesel (B100). The contaminants were autoclaved to clean all microorganisms that could be present in oil and only allow soil microorganism in the biodegradation process. The contaminant was poured on the samples in an amount equal to the void volume of the sample. The percentage of absorbed contaminant was about 4% in relation to the dry soil mass for all samples. The contaminants physicochemical characteristics are specified in Table 2.

The density of soil microbial communities was evaluated using culture media in plates by spread plate technique. Although this method has limitations about the growth of microorganisms in culture medium (as it is estimated that 1% of the microorganisms are cultivable), this form of indirect evaluation is most adopted as a method for evaluation of the occurrence, density and diversity of microorganism in soil.

First, the soil sample was homogenized for 2 min with sterile distilled water using 1:9 ratio. Later, dilutions up to 10^{-5} were carried out and 100 μL of each dilution was added to the surface of Petri dishes containing Plate Count Agar (PCA). The dishes were incubated at 30 °C for 48 h and microbial growth was determined by counting the colony forming units (CFU), and the results were expressed as CFU g^{-1} of soil.

Thereafter, the different colonies were transferred to culture media for purification and isolated growth and later identification by physiological and biochemical tests (Cappuccino & Sherman, 1996; Quinn *et al.*, 2002; Tortora, *et al.*, 2008).

The resistance of the isolated bacteria to hydrocarbons was tested using the Bushnell-Hass Broth (BH), con-

Table 1 - Physicochemical characteristics of natural soil used in bioremediation.

Parameters/Soil	Values	Parameters/Soil	Values
Natural moisture (%)	34	H_2O pH	5.4
Specific Gravity – G	2.7	P (mg/dm^3)	4
Liquid Limit (ω_L) (%)	53	K (mg/dm^3)	28
Plastic Limit (ω_P) (%)	42	H+Al ($cmol_c/dm^3$)	6.2
Plasticity Index (PI) (%)	11	CEC ($cmol_c/dm^3$)	8.6
Saturation rate (%)	74.2	Saturation – Bases (%)	28
Porosity (%)	55	Saturation – Al (%)	50
Void ratio	1.24	Saturation – K (%)	0.8
Organic Matter (%)	< 0.8	Hydraulic Conductivity (m/s)	$1.4x10^{-5}$

Table 2 - Physicochemical characteristics of conventional diesel oil and chemical characteristics of animal-derived biodiesel.

Characteristics *	Diesel (%)	Characteristics**	Biodiesel (%)
Sulfur (%)	0.3	Myristic acid (14:0)	5.03
Carbon (%)	86	Palmitic acid (16:0)	29.09
Hydrogen (%)	13.4	Palmitoleic acid (16:1)	3.22
Oxygen (%)	0	Stearic acid (18:0)	25.33
Aromatics (% v/v)	31.5	Oleic acid (18:1)	35.85
Viscosity at 40 °C (cSt)	2.5 to 5.5	Linoleic acid (18:2)	1.48
Cetane number (CN)	42	Saturated	59.45
Density at 15 °C (kg/m^3)	0.849	Unsaturated	40.55
Cloud point °C	1	Cetane number (CN)	69
Calorific value (MJ/kg)	42.30	Viscosity at 40 °C (cSt)	5.14
Moisture (ppm)	58	Cloud point °C	8-10
		Calorific value (MJ/kg)	39.33
		Moisture (ppm)	1390

Source: * NPA (2010), ** Schuller (2007).

taining 1% of diesel and tetrazolium chloride (TTC) as indicator according to the method proposed by Braddock & Catterall (1999) referenced by Bento *et al.* (2005) and Cerqueira & Costa (2009).

The inocula were incubated at 30 °C and 150 rpm for 14 days. Microbial growth was qualitatively determined by violet color development (indicating reduction of the indicator via respiration).

The inoculum used for bioaugmentation was prepared with the isolated microorganisms that were able to degrade diesel fuel. An aliquot of 1,000 μL of each bacterial suspension was used in 50 mL of liquid BH medium, kept under orbital agitation at 120 rpm and 28 °C. After 24 h of incubation, successive dilutions in peptone water at 0.1% were performed and Petri dishes containing PCA were inoculated for the count of microorganisms in the inoculum. The bioaugmentation inoculum had the concentration of approximately 3.0 x 10^6 CFU mL^{-1} of the microbial consortium. Afterwards, 1,000 μL of the inoculum from each isolate was slowly added (poured) on the 500-gram soil samples in each bioaugmentation treatment.

An aeration device was developed for the bioventing process (Fig. 1). It is composed by an air compressor and a water filter (this was used to avoid the air contamination by the compressor oil). This equipment allowed air to flow upwards in samples. This air was used as a source of biostimulation for microbial growth. It was used a daily continuum ventilation for four hours at pressure of 280 kPa.

Bioremediation trials were carried out in triplicates, and soil samples were assessed at predefined times, namely at 30, 60, 90 and 120 days, as outlined in Table 3.

Moisture and pH were measured accordingly. Moisture losses as high as 8.75% were observed during bioventing treatments (compared to moisture at baseline). With regard to pH, slight acidification was observed in all experiments, but the largest variation occurred in treatment bioventing + biodiesel of the bioventing procedure, in which pH dropped from 5.4 to 4.4.

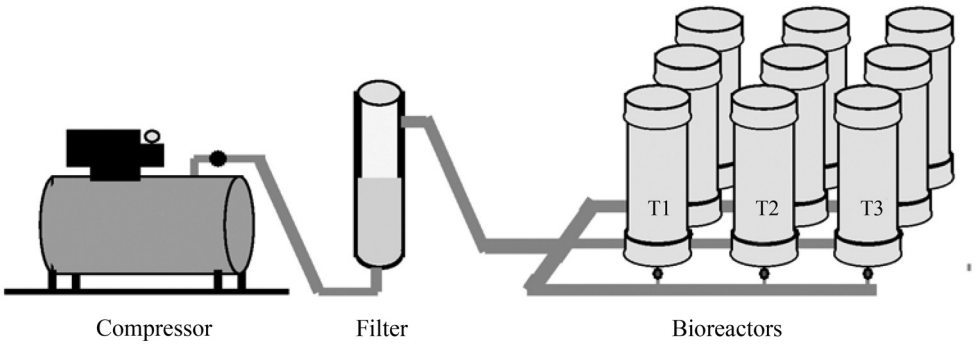

Figure 1 - Aeration device layout.

Table 3 - Treatments used in the bioremediation experiment.

Treatments	Contaminants	Bioremediation procedures
Natural Attenuation (T1)	No contaminant	Control
Natural Attenuation (T2)	Diesel	Natural Attenuation
Natural Attenuation (T3)	Biodiesel	Natural Attenuation
Bioaugmentation (T4)	No contaminant	Control*
Bioaugmentation (T5)	Diesel	Bioaugmentation *
Bioaugmentation (T6)	Biodiesel	Bioaugmentation *
Bioventing (T7)	No contaminant	Control**
Bioventing (T8)	Diesel	Bioventing**
Bioventing (T9)	Biodiesel	Bioventing**

*Bacterial inoculum (~3.0 x 10^6 CFU mL^{-1}). **Period: four daily hours at a pressure of 280 kPa.

The Soxhlet method (USEPA, 1996) was used for the extraction of soil contaminants. The contaminants were quantified by a Varian Gas Chromatograph (model 3400) equipped with a flame ionization detector. Hydrogen was used as carrier gas at a flow rate of 2 mL. min^{-1}. Injector and detector temperatures were 250 °C and 300 °C, respectively. A PE = WAX capillary column (30 m x 0.25 mm x 0.25 μm) was used for determination of hydrocarbons, with an initial oven temperature of 35 °C, increasing from 5 °C min^{-1} to 245 °C, and remaining at the latter temperature for 20 min. The CP Sil 88 capillary column (50 m x 0.25 m x 20μm) was used for determination of fatty acids, with an initial temperature of 140 °C, increasing 1 °C min^{-1} until reaching 185 °C. Hydrocarbons and fatty acids were identified by comparing retention times with specific standards quantified by normalization of the areas.

The experiments were conducted independently in triplicates. The count of microorganisms in CFU g^{-1} of soil was normalized by logarithmic transformation and assessed by analysis of variance (ANOVA) and Tukey's test at 5% for comparison of the means.

3. Results and Discussion

The microorganisms isolated from the natural soil (used in bioaugmentation) were identified as bacteria of the species *Bacillus circulans* and *Pseudomonas aeroginosa.* These bacteria are referenced in the literature as organic pollutant degraders (Bento & Gaylard, 2001; Alvarez & Illman, 2006; Labbé *et al.*, 2007).

The population density of microorganisms is influenced by bioremediation treatments and should be estimated in order to determine the relationship between microbial activity and the degradation rate. The Table 4 shows the means for the total number of microorganisms (CFU/g soil) and significant differences with a 95% confidence interval (Tukey's test) for the bioremediation of natural soils, contaminated by diesel and biodiesel, through natural attenuation, bioaugmentation and bioventing. Figure 2 shows the results for microbial count (Log CFU g^{-1}) of soils, comparing the same contaminant under effect of the three different treatments.

It can be observed in Fig. 2a that microbial count in the control experiment decreased over time, being statistically significant compared to the initial time after 60 days ($p = 0.037$). On the other hand, soils contaminated by diesel and biodiesel and submitted to natural attenuation maintained the concentration of microorganisms when compared to initial time ($p > 0.05$) all through the bioremediation process for treatment T2 (diesel used as contaminant) and up to 90 days for treatment T3 (biodiesel used as

Table 4 - Microbial count in different treatments and the statistical differences concerning the interaction between time and treatment.

Time (d)	Total plate count (Log CFU g^{-1} of soil)								
	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	3.28^{Ab}	3.28^{Ab}	3.28^{Ab}	3.89^{Aa}	3.89^{Aa}	3.89^{Aa}	3.28^{Ab}	3.28^{Ab}	3.28^{Ab}
30	2.73^{ABb}	2.94^{Ab}	3.30^{Ab}	3.67^{Ba}	2.97^{Bb}	2.74^{Cb}	2.84^{Bb}	2.93^{Ab}	2.89^{Bb}
60	2.55^{Bcd}	2.93^{Abcd}	3.07^{Aabd}	3.55^{BCa}	3.19^{Bab}	3.61^{Ba}	3.64^{Aa}	2.34^{Bc}	3.23^{Aab}
90	2.21^{Bc}	2.82^{Aab}	2.88^{ABab}	3.23^{Ca}	3.07^{Bab}	2.79^{Cab}	2.58^{Cbc}	2.19^{Bc}	2.57^{Cbc}
120	2.39^{Ba}	2.78^{Aa}	2.41^{Ba}	2.55^{Da}	2.79^{Ba}	2.38^{Da}	2.49^{Ca}	2.13^{Ba}	2.58^{BCa}

Values with the same lowercase letter superscript, in each row, are not significantly different at the 95% confidence level, based on Tukey's range test. Values with the same uppercase letter superscript, in each column, are not significantly different at the 95% confidence level, based on Tukey's range test.

contaminant). Although treatment of natural attenuation did not differs significantly over time, the presence of contaminant contributed to maintain the concentration of autochthonous microorganisms in the soil during the study period, to be sources of carbon and energy to the metabolism, determinant factor for the bioremediation.

Bioaugmentation in treatments T4, T5 and T6 (Fig. 2b) revealed that the microbial count decreased at 30 days of bioremediation compared to the initial time (comparison by the Tukey's test denoted by capital letters in Table 4, $p < 0.05$). Nonetheless, comparing the microbial count at 30 days of treatment for the three experiments indicates that the addition of contaminant (T5 and T6) caused microbial concentration to fall more sharply than that of T4 (3.67 significantly higher than 2.97 and 2.74 at a significance level lower than 0.05, compared to treatments T4, T5 and T6 at 30 days, small letters on Tukey's test, Table 4). The inoculated bacteria, although they were in the growth stage, needed time to adapt to the contaminated medium.

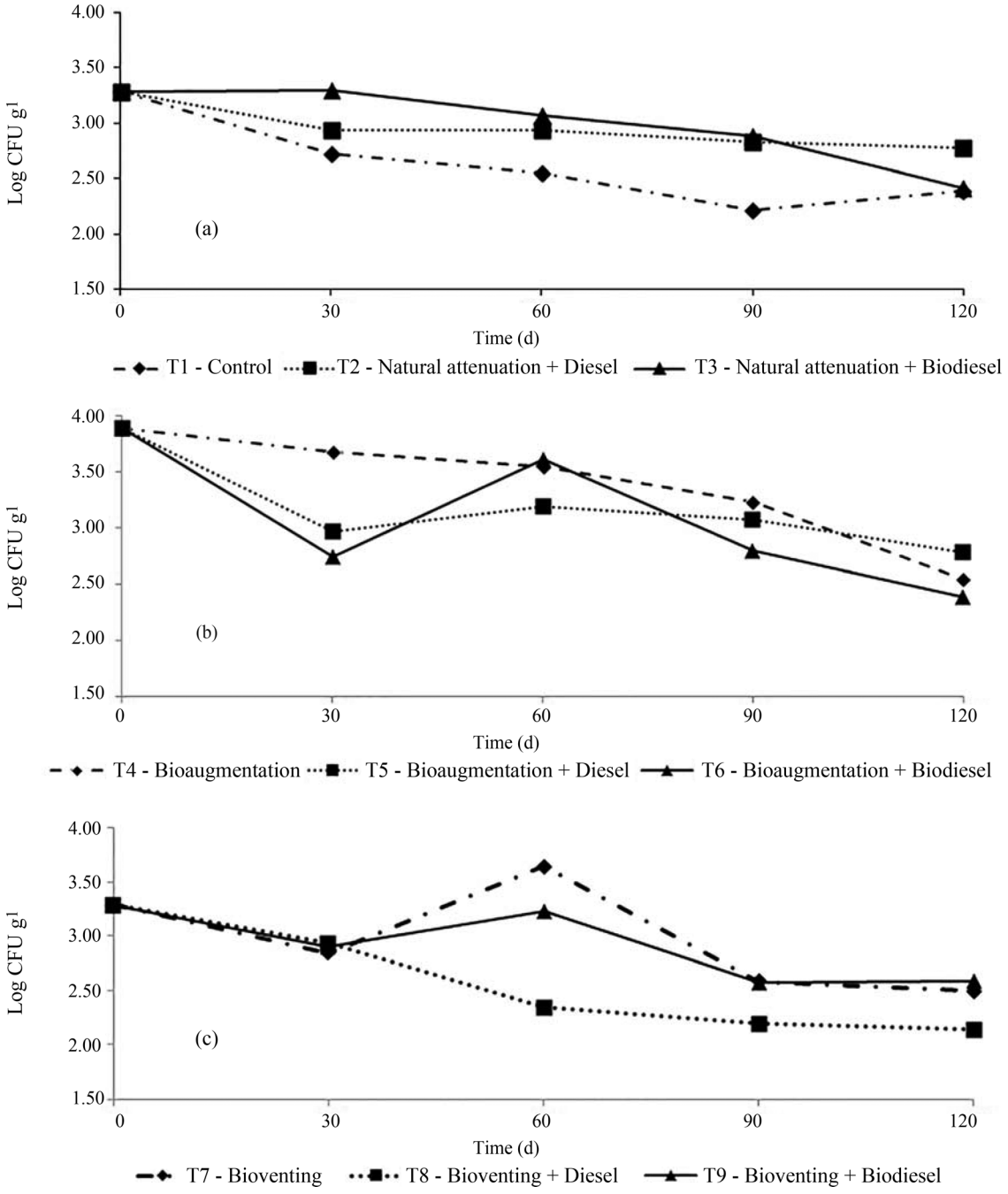

Figure 2 - Microbial density (CFU/g soil) of treatments over time. (a) Natural Attenuation, (b) Bioaugmentation and (c) Bioventing.

In bioremediation trials in which bioventing was used (Fig. 2c), there was a significant reduction ($p < 0.05$) in the concentration of microorganisms in the soil compared to the results obtained for treatments T7 (bioventing of contaminant-free soil) and T9 (bioventing of biodiesel-contaminated soil) at 30 days and at baseline. Even though microbial count decreased in T8 (bioventing of diesel-contaminated soil) relative to baseline values, no significant difference was found between these counts ($p > 0.05$). However, after 30 days of bioremediation, there was a significant reduction in microbial concentration in diesel-contaminated soils and in biovented in a period of up to 120 days, when counts are compared with those obtained in experiments T7 and T9. This may have been caused by a toxic effect on the metabolites produced during diesel bioremediation after 30 days of treatment (T8).

By comparing the control experiment in the natural attenuation treatment (T1) with the control experiment in the bioventing treatment (T7), microbial activity continues to increase up to 60 days under aerobic conditions whereas this activity begins to decline in the natural soil at 30 days. This increase in population density in treatment T7 indicates the presence of facultative aerobic/anaerobic microorganisms at this depth. These results are in agreement with those obtained by Österreicher-Cunha *et al.* (2004). They demonstrated that the natural soil when biostimulated with air (control + bioventing) had larger population density than the untreated natural soil (control), showing the presence of aerobic microorganisms. After 40 days, microbial population increased in all of the other treatments, confirming that microorganisms need some time to adapt to the bioremediation conditions imposed on the medium.

The rate of diesel and biodiesel degradation obtained by gas chromatography at the end of the experiment is shown in Fig. 3. The highest biodiesel biodegradation rates occurred in treatments T6 (bioaugmentation) and T9 (bioventing), amounting to 96.8% and 90.6%, respectively, followed by natural attenuation (T3), with a rate of 60.6%. Biodiesel biodegradation was easier than that of diesel in treatments T2, T5 and T9, as a consequence of the different chemical composition of contaminants. The results obtained in this work are in agreement with those found by Cyplik *et al.* (2011) and Lapinskiené *et al.* (2006). They found that the biodegradation caused by a microbial consortium relies on the type of fuel used and on the available aerobic conditions, and thus biodiesel degradation is faster than that of diesel under aerobic conditions. Biodiesel is much more biodegradable than fossil diesel due to its chemical features (Zhang *et al.*, 1998).

Figures 4 and 5 show the degradation rate of carbon chains for biodiesel and diesel, respectively. Biodiesel yielded 99% degradation of palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic (C18:1) acids in bioaugmentation; 97% for palmitic (C16:0) and oleic (C18:1) acids in bioventing, and 68% for oleic acid (C18:1) in natural attenuation.

The highest degradation rate for the light fraction (C12-C23) of TPH found in diesel (Fig. 4) was observed in bioaugmentation (78.5%), followed by bioventing (72%) and natural attenuation (26%). These findings are similar to those obtained by Bento *et al.* (2005) for bioremediation. These authors observed that bioaugmentation (63-84%) was more efficient in diesel degradation than were biostimulation (72%) and natural attenuation (47%). Also, according to Bento *et al.* (2003), bioaugmentation with

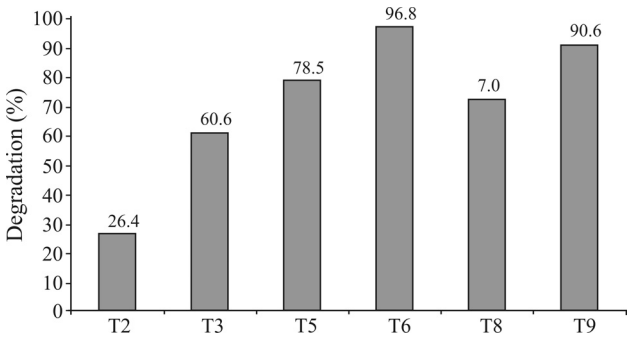

Figure 3 - Degradation rate for diesel oil and biodiesel in bioremediation treatments at 120 days.

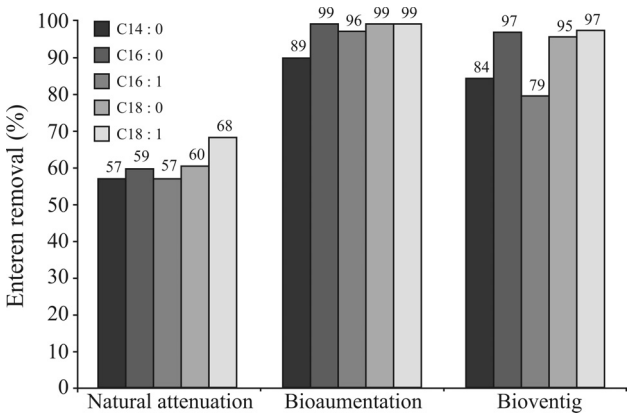

Figure 4 - Degradation rate for biodiesel esters.

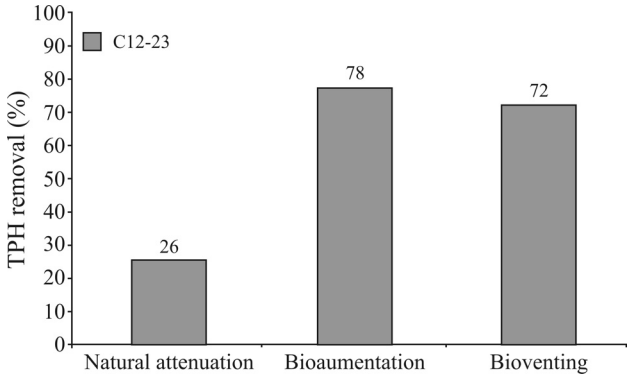

Figure 5 - Degradation rate for the light fraction of diesel (C12-23).

inocula from bacteria pre-selected from a contaminated medium provides higher degradation, as they recognize diesel oil hydrocarbons more easily, using them as source of energy for their growth. Bioremediation studies conducted in laboratory by Capelli *et al.* (2001) also demonstrated a reduction of over 70% of TPH when inoculated by pre-selected bacteria.

Other bioremediation studies carried out by Sayara *et al.* (2011) and Kauppi *et al.* (2011) demonstrated that biostimulation with nutrients and oxygen is more efficient than bioaugmentation. Sayara *et al.*, 2011 assessed the degradation of several aromatic polycyclic hydrocarbons (APHs) in the soil by applying bioaugmentation, biostimulation, and natural attenuation. After 30 days, analyses showed that the inoculum of fungus *T. versicolor* did not improve the degradation of APH significantly. However, biostimulation degraded 89% of APHs while natural attenuation degraded 29.5% (carried out by microorganisms of the indigenous soil).

As presented in Table 4, the 120 days of studied bioremediation techniques do not show statistical differences in the evaluation of microorganisms, however, it can be observed in Fig. 3, that there is a great difference in the percentage of degradation among the techniques, being the degradation of 26.4% for T2 (Natural Attenuation + diesel), while the degradation for the technique T6 (Bioaugmentation + biodiesel) was 96.8%.

4. Conclusion

This study assessed the biodegradation potential of a residual clayey soil (Oxisol) contaminated by diesel and biodiesel, comparing natural attenuation, bioaugmentation and bioventing processes, allowing to identify differences in contaminant degradation.

The highest degradation took place in the treatments of biodiesel-contaminated soil, compared to those contaminated by diesel, due to the chemical characteristics of contaminants.

Bioaugmentation was the bioremediation procedure with the best test results owing to the inoculum of microorganisms pre-selected from the residual soil, with 78.5% degradation of the light fraction of diesel (TPH) and 96.8% degradation of fatty acid esters of biodiesel.

Bioventing yielded higher biodegradation rates than natural attenuation because of the presence of aerobic bacteria in the residual soil.

Microorganisms of the genera *Bacillus circulans* and *Pseudomonas aeroginosa* have shown to be efficient organic pollutant degraders in residual clayey soil (oxisols).

The inoculum of degrading microorganisms, associated with the addition of nutrients (oxygen, nitrogen, phosphorus and/or potassium) allows increasing the degradation of soil contaminants by reducing time and costs of remediation processes.

Acknowledgments

The authors wish to express their gratitude to the Brazilian Research Council (CNPq/MCT and CAPES/MEC) and Rio Grande do Sul State Research Foundation (FAPERGS) for their financial support.

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