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RESEARCH ARTICLE

Bioremediation of diesel oil-contaminated soil using native hydrocarbon-degrading bacterial strains under microcosm study.

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Abstract

In gas stations, leaks occur as a result of fuel supply, storage and transport. These leaks are particularly expensive to remediate if site closure is required for conventional *ex situ* treatments. Here, we evaluated the effectiveness of soil decontamination by less costly techniques of 'bioremediation', which can be performed *in situ*. We compared natural attenuation (i.e., maintaining soil hydration) with 'successive biostimulation' (nutrient addition) alone or combined with 'successive bioaugmentation' of contaminated soil via the addition of degradative microorganisms. Initially, 34 bacterial strains were isolated from soil with historical diesel contamination removed from a gas station in southern Brazil. A group of 5 strains was then selected to form the 'consortium'used in bioremediation experiments, based on diesel oil (B5) biodegrading ability (measured using tetrazolium chloride reduction), biosurfactant production (rhamnolipid quantification and emulsification index) and species identification (16S rRNA sequencing).CO_2 evolution demonstrated no significant difference in soil microbial activity between treatments. The increase in the heterotrophic microorganism population in contaminated soil was not altered by any of the bioremediation strategies, while the population of degrading microorganisms increased in all treatments. The degradation of hydrocarbons was monitored by analysis total petroleum hydrocarbon (TPH). Within the experimental period of 60 days, the initial TPH concentration in the contaminated soil (133 mg kg soil^{-1}) was reduced by 84% via natural attenuation, 90% by bioaugmentation/biostimulation, and 91% by biostimulation alone, showing that the treatments displayed positive effects on biodegradation, with a substantial reduction in TPH levels.

INTRODUCTION

Soil contamination with hydrocarbons is a worldwide problem and a permanent environmental concern. Diesel spills usually occur during manufacturing, storage and transportation, causing serious environmental pollution problems (Das and Chandran, 2011). Oil companies are important sources of contamination, because of numerous accidental spills; however, gas stations functioning in irregular conditions have a high pollution potential, through small and

continuous casting. In Brazil, fuel leakage at petrol stations is often caused by the use of old storage tanks, although Brazilian legislation requires the exchange of old oil tanks for internally-coated modern ones that are leak resistant.

Internal leak detection systems are also required, besides the remediation of degraded areas. There are several forms of remediation for contaminated areas; however, the most commonly used is the physical removal of soil for treatment *ex situ*, which is expensive, because gas stations remain inoperative during soil removal. Cost-effective remediation techniques that are compatible with social and environmental aspects require further evaluation to expand their use as substitutes for conventional decontamination methods such as incineration, solidification/stabilization, and soil steam extraction and washing (Xu and Lu, 2010).

The use of biological methods for the remediation of contaminated areas (or 'bioremediation') is a promising and effective strategy, in terms of both results and costs (Szulc et al., 2014). Bioremediation techniques are characterized by the addition of appropriate microorganisms ('bioaugmentation') and the adjustment of abiotic factors ('biostimulation'), such as the incorporation of nutrients, pH correction, moisture and aeration. Biostimulation factors may be applied independently or in association (Evans et al., 2004). Bioremediation eliminates the need for gas stations to stop their activities during soil treatment, since remediation can be applied *in situ*.

Bioaugmentation leads to the degradation of toxic contaminants by microorganisms with the metabolic and physiological ability to use hazardous organic compounds as a source of carbon and energy (Meyer et al., 2014). The increase in initial cell biomass levels improves biodegradability and soil treatment (Mishra et al., 2001; Mukherjee and Bordoloi, 2011). Also, compared with conventional decontamination methods, bioaugmentation offers the advantage of recycling nutrients into natural metabolites (Fantroussi and Agathos, 2005).

However, contaminant reduction rates are not always satisfactory after bioaugmentation. Lin et al. (2011) reported that biodegradation kinetics analysis showed an initial period of intense activity, followed by a reduction in degradation rates. On the other hand, Tyagi et al. (2010), showed that the imbalance of abiotic factors may make the process unfeasible under natural conditions, as the exposure of the population inoculated with natural predation conditions would be reduced. Furthermore Tahhan et al. (2011) showed that competition among microorganisms can also decrease the biodegradation efficiency. Also, further studies are necessary to determine the ideal bacterial strains for bioaugmentation, including the testing of native strains removed directly from the contaminated soil, and which might have had their decontamination potential naturally 'optimized'.

Thus, this study aimed to evaluate different bioremediation treatments - natural attenuation, bioaugmentation combined with biostimulation and biostimulation alone - in soil contaminated with hydrocarbons removed from a gas station in the state of Rio Grande do Sul (Brazil). For bioaugmentation/biostimulation treatment, successive additions of a bioprospected microbial consortium composed of bacterial strains isolated from the contaminated soil were applied and were used in the same soil treatments in order to ascertain whether the addition of microorganisms already 'acclimatized' to the contaminated environment would be more effective compared to natural attenuation.

2. Materials and Methods

2.1. Soil sampling

Contaminated soil used in bioprospecting and bioremediation tests were removed from a gas station located in the city of Porto Alegre, Rio Grande do Sul, State, Brazil (Latitude - 30.0277, Longitude: -51.2287 30 ° 1'40 "South, 51 ° 12'43" West). The soil had historical diesel oil contamination, and more recent contamination with diesel-biodiesel mixtures. The soil collected served as a base for fuel tanks and was removed from a depth of 2 to 3 meters, during the replacement of leaking, old tanks (with 20 years of use). After collection, samples were placed in hermetically closed sterile bags and kept at 4 °C until use in experiments.

Initial and final concentrations of total petroleum hydrocarbons (TPH) in the contaminated soil were determined by gas chromatography (see "Degradation of hydrocarbons" below). Physico-chemical soil analyses were performed according to the methods described in Tedesco et al. (1995) (Table 1).

2.2. Bioprospecting

The enrichment microbial isolation method described by Bento et al. (2005) was used. Erlenmeyer flasks were inoculated with 1g of contaminated soil and 1% diesel oil (B5, 95% petroleum diesel and 5% biodiesel) in a final volume of 100 mL of mineral medium- MM1 (0.7 g L^{-1} of KCl, 2.0 g L^{-1} of KH_2PO_4, 3.0 g L^{-1} of Na_2HPO_4, 1.0 g L^{-1} of NH_4NO_3 and micronutrient solution with 4.0 mL L^{-1} of $MgSO_4$, 0.2 mL L^{-1} of $FeSO_4$, 0.2 mL L^{-1} of $MnCl_2$, and 0.2 mL L^{-1} of $CaCl_2$).To prevent photo-oxidation, fuels were stored in glass vials at room temperature, and in the dark, and were sterilized by vacuum filtration (though a 0.22-µm pore-size membrane) prior to use in

experiments. Inoculated flasks were kept at 30 ºC, and under agitation at 190 rpm. Every 7 days, cultures were passaged (at a dilution rate of 1:10) into fresh 10 -medium with fuel. After three transfers, grown cultures were serially diluted, and plated on nutrient agar (5 g/L peptone, 3 g/L meat extract, 15 g/L agar). The microorganisms isolated were kept refrigerated at 4 ºC. Unless stated otherwise, bacterial cultures were grown in nutrient broth (5 g/L peptone, 3 g/L meat extract) for 24h, at 30 ^{o}C (and with agitation at 180 rpm) before use in experiments.

2.3. Degradation assay

The hydrocarbon degradation ability of the isolates was evaluated using the redox indicator 2,3,5-triphenyltetrazolium chloride (TTC), according to Braddock and Catterall (1999). TTC is a colorless substance, but when in contact with a microorganism capable of using oil as the main source of carbon and energy, it reacts by reducing triphenyl formazan (TPF), producing a reddish coloration (Casida, 1977). Bacterial cells (10^8 CFU/mL) from each isolate tested were inoculated in 5mL of mineral medium 1 (MM1) containing TTC and 2% diesel oil (B5) as a carbon source, and samples were incubated at 30 °C for up to 120 h. As a negative control, sterile water replaced the inoculum. Results were scored as "positive" when the medium colour changed from colourless to red. Isolates that changed in coloration within this period were selected for further tests.

2.4. Biosurfactant production assays

2.4.1. Emulsification index

Biosurfactants are substances that improve hydrocarbon biodegradation by microorganisms by increasing the bioavailability of these substances; therefore, they are considered as adjuvants in the degradation of heavy oils. To quantify the emulsification index of each isolate, bacterial cells were inoculated (in triplicates) in Erlenmeyer flasks with 50 mL of nutrient broth, at a final concentration of A_{600nm} = 0.07, and incubated at 30 °C with agitation at 180 rpm, for 24 h. Then, 25 mL of the supernatant and 5 mL of lubricant oil (B5) were mixed by vortexing for 2 minutes. After 24 h, the height of the emulsion layer (water in oil) was measured, and used to calculate the percentage of emulsion volume relative to the total volume – defined as the 'emulsification index' – as described in Cooper and Goldenberg (1987).

2.4.2. Rhamnolipid production

To test for rhamnolipid biosurfactant production, bacterial cells were plated onto methylene blue plates (10 g/L peptone, 10 g/ L lactose, 2 g/L monobasic potassium phosphate, 0.065 g/L methylene blue chloride and 15 g/L agar, 10 g/L of CTAB) and incubated at 30 °C, for 24 hours. The formation of halo around the colonies indicates a positive result for the production of biosurfactants of the rhamnolipid type.

2.5. Bacterial identification

Bacterial genomic DNA was extracted according to Sambrook and Russell (2001). Polymerase chain reaction tests (PCR) for the amplification of the 16S rRNA gene of each bacterial isolate were conducted using universal oligonucleotides corresponding to the 27F (5' AGA GTT TGA TCM TGG CTC AG 30) and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') positions. The reactions were prepared using Go Taq Reaction Buffer 1x (1.5 mM $MgCl_2$), 0.2 mM dNTP, 10 pmol of each oligonucleotide, 1.25 U Go Taq DNA polymerase (Promega) and 1 µl genomic DNA (25-µl reactions). The following PCR cycle conditions were used: 95 ºC for 5 min, followed by 35 cycles at 95 ºC for 1 min, 65 ºC for 30 seconds and 72 ºC for 1 min, with a final extension step at 72 ºC for 5 min.

PCR products were analyzed by 1% agarose gel electrophoresis, and amplicons were purified from agarose gel slices using the PureLink™ Quick Gel Extraction kit (Invitrogen), following the manufacturer's instructions. DNA quantification was performed by fluorometric analysis using a Qubit fluorometer (Invitrogen) and the DNA quantification kit Quant-iT, following the manufacturer's instructions. Sequencing was performed in a MegaBACE 1000 sequencing platform, using 200 ng of genomic DNA per reaction. Nucleotide sequence similarity searches were conducted using GenBank nucleotide collection BLAST searches.

2.6. Biodegradation experiments

The microcosms were set up in triplicate using 1-L hermetically sealed glass flasks that contained 300 g of soil. During 60 days of experimental analysis, the systems were maintained at controlled temperature (28 °C±1) and protected from light. The inoculum and nutrient solution volumes added to the treatments were adjusted to maintain the soil field capacity at 80 %. In treatments with no addition of nutrients and consortium, sterile distilled water was used to maintain the soil moisture. The purpose of the addition of nutritional solution to the bioaugmentation and

biostimulation treatments was to correct the C/N/P ratio to 100:10:1. During the inoculation and contamination processes, microcosms were individually homogenized, using a sterilized spatula.

For treatment of natural attenuation samples were maintained in a moist environment; biostimulation, characterized by the successive introduction of a nutrient solution consisting of 250 mg kg^{-1} of NH_4SO_4 and 100 mg kg^{-1} KH_2PO_4, as suggested by Jiménez et al. (2007); and bioaugmentation/biostimulation where the successive addition nutrient was combined with successive inoculation of a 'consortium' of five bacterial isolates, at a final concentration of 10^8 colony forming units (CFU)/mL. For the negative control we used a soil without contamination. Microbial activity was evaluated during the degradation process, and the most probable numbers (abundance) of the total heterotrophic and degrading population was estimated. Fuel degradation was estimated by CO_2 evolution and by gas chromatography (GC) analysis of TPH.

2.6.1. Microbial growth

Microbial population growth monitoring was carried out at 0, 30, 45, 54, 60 experimental days. The total numbers of heterotrophic and degrading microorganisms were estimated using the MPN method in microtiter plates according to the method described by Bento et al. (2005). The microbial population was determined using MPN tables (APHA 1995).

2.6.2. CO_2 evolution

This method aims to determine microbial activity directly by measuring carbon dioxide produced during microbial respiration, as an indirect estimate of the biodegradation of organic contaminants (Stotzky, 1965). The carbon dioxide produced by microbial activity was captured using a 0.2 M KOH solution (10 mL) located next to the biometer flasks. Periodically, the KOH solution was removed, 1 mL of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added to the solution, and the residual KOH was titrated with a 0.1 M HCl standardized solution.

The amount of carbon dioxide produced was calculated using the formula:

$$\textbf{CO}_2\textbf{ generated (mg/Kg soil)} = (V_B - V_A) \cdot (M_C/2) \cdot M_{HCl} \cdot FC/m$$

Where V_B and V_A are the volume of 0.1 M HCl (in mL) used to titrate the blank and the treatment, respectively; M_C is the molar mass of carbon dioxide in g/mol; M_{HCl} is the molar concentration of HCl standard solution in mol/L; FC is the correction factor for acid/base molarity (M_{HCl}/M_{KOH}), and m is the mass (in Kg) of dry soil in the flask.

2.6.3. Chromatography

The levels of total petroleum hydrocarbons (TPH) were determined using the EPA 8015-B-96 technique, by gas chromatography (AGILENT 6890 Series GC System), with a mass selective detector (AGILENT 5973 Network Mass Selective Detector). Samples (1-ul aliquots) were analyzed in 'splitless' mode, in silica columns with 5% phenyl and 95% dimetilpolysiloxane (30m x 0.35mm x 0.25 mM), using the following conditions: 290 ºC injection temperature, 40 ºC initial temperature for 1 min, followed by an increase in temperature of 6 ºC/min, up to 300 °C, which was maintained for 30 min.

2.7. Statistical analysis

Descriptive statistics (mean and standard deviations) were utilized. ANOVA was used to test for differences among treatments, and treatment means were evaluated by Boneferroni's test, with a confidence level of 95%.

3. Results

3.1.Microorganism isolation, selection and identification

Ideally, bioremediation should be performed using the native bacterial strains found in the contaminated soil. Thus, our initial step was to isolate and perform sequence-based species identification of the bacterial strains found in the soil used here. Bacterial isolates were obtained from soil with historic hydrocarbon contamination, and which was collected from a gas station in the city of Porto Alegre (Rio Grande do Sul State, Brazil). The physico-chemical characteristics of the contaminated soil are described in Table 1. We isolated 34 different species of soil bacteria, using an enrichment technique in a liquid mineral medium, with diesel oil as the sole carbon source (Table 2).

The selection of bacterial isolates was based on preliminary assays of the biodegradation of a redox indicator (TTC) and the production of biosurfactant rhamnolipids. Biodegradability tests using TTC were positive for 24 of the isolated microorganisms (Table 2), suggesting not only the existence of adequate diesel-degrading microorganisms, but also varying degradation capabilities among the microorganisms studied, as indicated by the time for medium color transition (from colourless to red).

Isolates that tested positive in the TTC assay were subjected to molecular identification. Partial 16 S rRNA gene analyzes showed that 18 isolates belonged to the genus *Pseudomonas*, including 12 isolates from the pathogenic species *P. aeruginosa*, (Table 3). Five isolates belonged to the *Bacillus* genus (*B. cereus* and *B. subtilis*), and one isolate was identified as *Acinetobacter calcoaceticus* species (Table 3).

Isolates were then tested for the production of rhamnolipid biosurfactant, by the colorimetric method using methylene blue and CTAB (cetyltrimethylammonium bromide). This method was originally developed to select species that produce the rhamnolipid biosurfactant type ion or to select any anionic biosurfactant of low molecular weight that can be produced by *Pseudomonas* species. These biosurfactants form an insoluble pair with the cationic surfactant (CTAB) and methylene blue basic dye, which are added to mineral medium agar. The colorimetric assay for the detection of rhamnolipids was positive for 20 of the isolates (Table 2). Among these species, we chose not to work with *P. aeruginosa* due to its pathogenicity; thus, the 12 remaining isolates were subjected to further testing.

3.2.Emulsifier production

To select microorganisms for use in bioremediation, the production of biosurfactants of high-molecular weight (also known as emulsifiers) was evaluated in the 12 previously selected microorganisms, and an emulsification index was calculated for each isolate. The isolates demonstrated an emulsification index of up to 31% in the absence of cells (Fig.1). With the exception of BPB 1.1 (*Pseudomonas pseudoalcaligenes*), emulsification index of all selected microorganisms was significantly higher when compared with that observed in the negative control.

3.3. Bioremediation experiments

Five microorganisms isolates - *P. mendocina* (BPB 1.6), *B. cereus* (BPB 1.15), *A. calcoaceticus* (BPB 1.22), *B. subtilis* (BPB 1.13) and *B. cereus* (BPB 1.26)- were selected to compose the 'consortium' used in the bioaugmentation/ biostimulation experiments. The isolated bacterial strains were grown separately, and then 10^8 CFU/mL of each isolate were added to a mixture used in bioaugmentation/biostimulation treatments. For biostimulation alone, the same nutrient solution used in bioaugmentation/biostimulation experiments was added to the soil, but without the addition of bacteria. The consortium application as well as the nutrient solution was applied successively (once a week) for 60 days. Finally, in natural attenuation experiments, samples were kept in controlled humidity, with no addition of cells or nutrients as well as in the negative control.

3.3.1. Heterotrophic and diesel-degrading bacteria

Bacterial growth throughout the 60-day treatment period was estimated using the Most Probable Number (MPN) technique. The number of heterotrophic microorganisms increased significantly all treatments compared with the negative control, from 45 days of incubation (Fig. 2A). Furthermore, bacterial numbers in the negative control remained constant (at ~10^5 MPN/mL) until the end of the experiment, whereas we observed growth to 10^7 and 10^8 MPN/mL in all other treatment conditions.

In the evaluation of diesel-degraders (Fig. 2B), natural attenuation increased the number of degrading microorganisms relative to the other treatments, mainly at 45 days and 54 days (10^9 MPN/mL); however, the number of degrading organism drastically decreased by treatment day 60 (10^3 MPN/mL). However, biostimulation reduced MPN counts after 30 days, compared with the negative control, reaching 10^2 MPN/mL after 60 days of treatment. Bioaugmentation/biostimulation maintained small variations in diesel-degrading populations during the 60 days of treatment, and this treatment yielded the highest number of degrading microorganisms among all treatments at day 60 (10^5 MPN/mL). We concluded that there were no significant differences between the general populations and the populations of heterotrophic decomposers.

3.3.2. Microbial activity (CO_2)

All treatments increased the levels of respiration activity compared with the negative control ($p < 0.001$), over the time course (Fig. 3), suggesting that there is a capacity to metabolize microbial hydrocarbon contaminants. Natural attenuation, bioaugmentation/biostimulation and biostimulation yielded similar CO_2 production values, with no statistically significant difference between the respirometry results of the different treatments.

We also observed that all treatments showed a similar CO_2 evolution profile, with an adaptive phase that occurred after 7 days, and a fall in at the end of the experimental period.

3.3.3.Degradation of hydrocarbons

The effect of different treatments on soil contamination was assessed by measuring the levels of total petroleum hydrocarbons (TPH) using gas chromatography. Contamination corresponded to fractions of aliphatic and aromatic hydrocarbons with C_{13} to C_{27} chains, and concentrations of pristane and phytane. The initial contamination of the soil sample was 133 ± 2 mg kg^{-1} soil (Table 4), which fits the " alert value " limit ("T", indicative of soil alterations that require further investigation) of 2.525 mg kg^{-1} soil (Dutch List, Table 6530-2; CETESB, 1999). After being subjected to natural attenuation, biostimulation or bioaugmentation/biostimulation, soil contamination reached a "reference value", Reference value (S): indicates a level of quality of soil and groundwater that allows considering them "clean", considering its use for any purpose, where the limit is 50 mg kg^{-1} soil (Table 4), according to the same literature.

Biostimulation treatment reduced soil contamination significantly within the first 40 days of treatment, while reductions in contamination occurred later, within 60 days of treatment, after natural attenuation and bioaugmentation/biostimulation treatments (Table 4). Nevertheless, all treatments led to similar final decreases in contaminant degradation, with no statistically significant difference between TPH level reduction from day 0-60 by natural attenuation (84%), biostimulation (90%) and bioaugmentation/biostimulation (91%). Thus, the microbial consortium promoted the degradation of hydrocarbons at virtually the same ratio as the native microbial community, with or without the addition of nutrients.

Table 1. Physico-chemical characteristics of the soil with historic al diesel oil- contamination removed from a petrol station in southern Brazil.

Parameters	Depth (2-3m)
Organic matter (g Kg^{-1})	11
Clay (%)	9
Fine sand (%)	41
Silt (%)	8
Organic carbon (g Kg^{-1})	5
Total nitrogen (g Kg^{-1})	0.5
P (mg dm^{-3})	0
K (mg dm^{-3})	65
CTC ($mmol_c$ dm^{-3})[1]	2.4
CTC pH7 ($mmol_c$ dm^{-3}) [2]	5.2
Al ($mmol_c$ dm^{-3})	0.8
Ca ($mmol_c$ dm^{-3})	0.6
Mg ($mmol_c$ dm^{-3})	0.2
H + Al ($mmol_c dm^{-3}$)	3.9
pH (H_2O)	4.5

Table 2. Rhamnolipid production and potential for biodegradation (using the redox indicator TTC) by 34 isolates, for a period of 120 hours.

Isolate	Rhamnolipid	production TTC
BPB 1.1	+	+
BPB 1.2	+	-
BPB 1.3	+	+
BPB 1.4	+	+
BPB 1.5	-	-
BPB 1.6	-	+
BPB 1.7	-	-
BPB 1.8	-	+
BPB 1.9	+	+
BPB 1.10	+	+
BPB 1.11	+	+
BPB 1.12	+	+
BPB 1.13	-	+
BPB 1.14	+	+
BPB 1.15	-	+
BPB 1.16	-	-
BPB 1.17	-	+
BPB 1.18	-	+
BPB 1.19	-	+
BPB 1.20	-	+
BPB 1.21	+	+
BPB 1.22	+	+
BPB 1.23	+	-
BPB 1.24	+	+
BPB 1.25	-	+
BPB 1.26	-	+
BPB 1.27	+	+
BPB 1.28	+	-
BPB 1.29	-	-
BPB 1.30	+	+
BPB 1.31	+	-
BPB 1.32	+	-
BPB 1.33	+	-
BPB 1.35	+	+

+ positive reaction; - negative reaction

Table 3. Molecular identification of bacterial strains isolated from contaminated soil.

Isolate	Identification	Identity (%)
BPB 1.1	*Pseudomonas pseudoalcaligenes*	98
BPB 1.3	*Pseudomonas mendocina*	99
BPB 1.4	*Pseudomonas aeruginosa*	99
BPB 1.6	*Pseudomonas mendocina*	99
BPB 1.8	*Pseudomonas mendocina*	99
BPB 1.9	*Pseudomonas aeruginosa*	99
BPB 1.10	*Pseudomonas aeruginosa*	99
BPB 1.11	*Pseudomonas aeruginosa*	99
BPB 1.12	*Pseudomonas aeruginosa*	99

BPB 1.13	*Bacillus subtilis*	99
BPB 1.14	*Pseudomonas aeruginosa*	99
BPB 1.15	*Bacillus cereus*	99
BPB 1.17	*Pseudomonas aeruginosa*	99
BPB 1.18	*Bacillus subtilis*	99
BPB 1.19	*Pseudomonas aeruginosa*	99
BPB 1.20	*Bacillus cereus*	99
BPB 1.21	*Pseudomonas aeruginosa*	99
BPB 1.22	*Acinetobacter calcoaceticus*	99
BPB 1.24	*Pseudomonas aeruginosa*	99
BPB 1.25	*Pseudomonas aeruginosa*	99
BPB 1.26	*Bacillus cereus*	99
BPB 1.27	*Pseudomonas putida*	99
BPB 1.30	*Pseudomonas aeruginosa*	99
BPB 1.35	*Bacillus sphaericus*	99

Table 4. Levels of total petroleum hydrocarbons (TPH) present in the contaminated soil after treatment by different bioremediation strategies.

Original sample	Time (days)	Natural attenuation	Bioaugmentation/ bioestimulation	Biostimulation
133 ± 20	40	121 ± 84 Aa	103 ±9 Aa	44 ± 13Ab
	60	17 ± 13 Bb**	9 ± 4Bb**	8 ± 3 ABb**

All values are in mg TPH/Kg soil^{-1}. Values followed by the same capital letters are not significantly different among treatments, and values followed by the same lowercase letters are not significantly different to those obtained at different time-points (based on Boneferroni's test, with $p < 0.05$).**, $p < 0.05$, between the original sample and the treatments.

Fig. 1

Biosurfactant production of bacterial isolates found in soil with historical hydrocarbon contamination. Bacteria were grown for 24h, and then conditioned medium was mixed with lubricant oil, and allowed to settle for 24h before estimation of the emulsification index, according to Cooper and Goldenberg (1987). NC, negative control. Data are expressed as mean ± standard deviation values for triplicates, $p < 0.001$, when compared to the negative control and < 0.05 when compared to BPB 1.27.

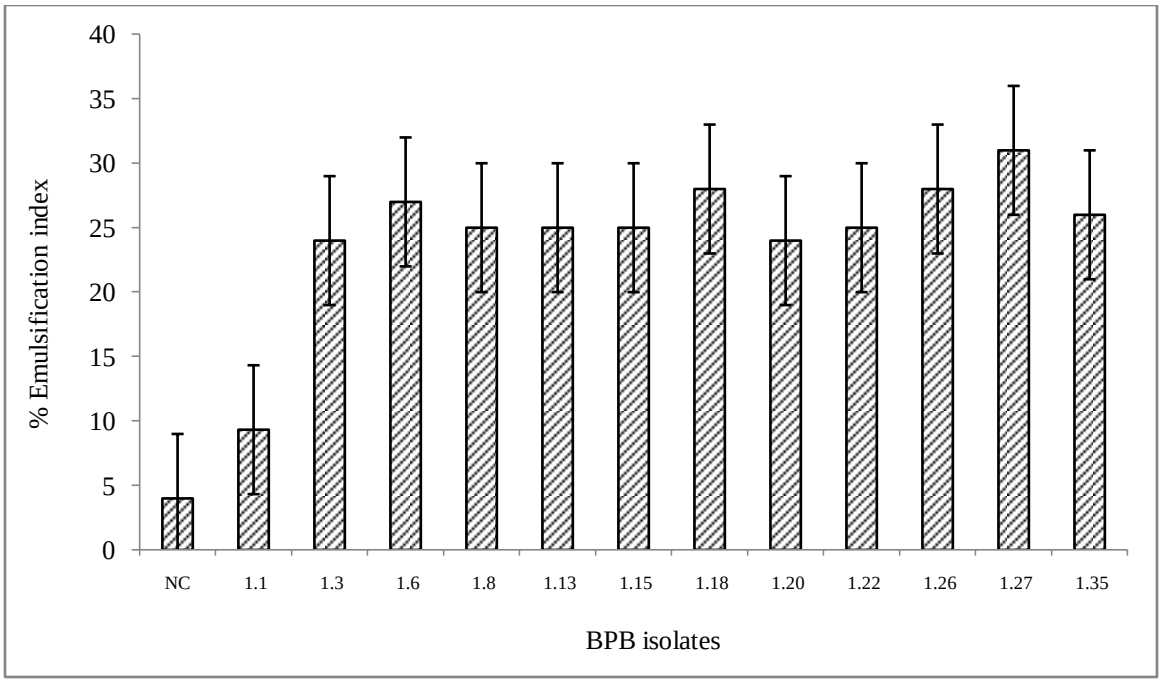


Fig. 2
Heterotrophic (A) and degrading (B) microorganisms most probable number estimative in bioremediation of hydrocarbon-contaminated soil during 60 days of incubation.

Fig. 2A

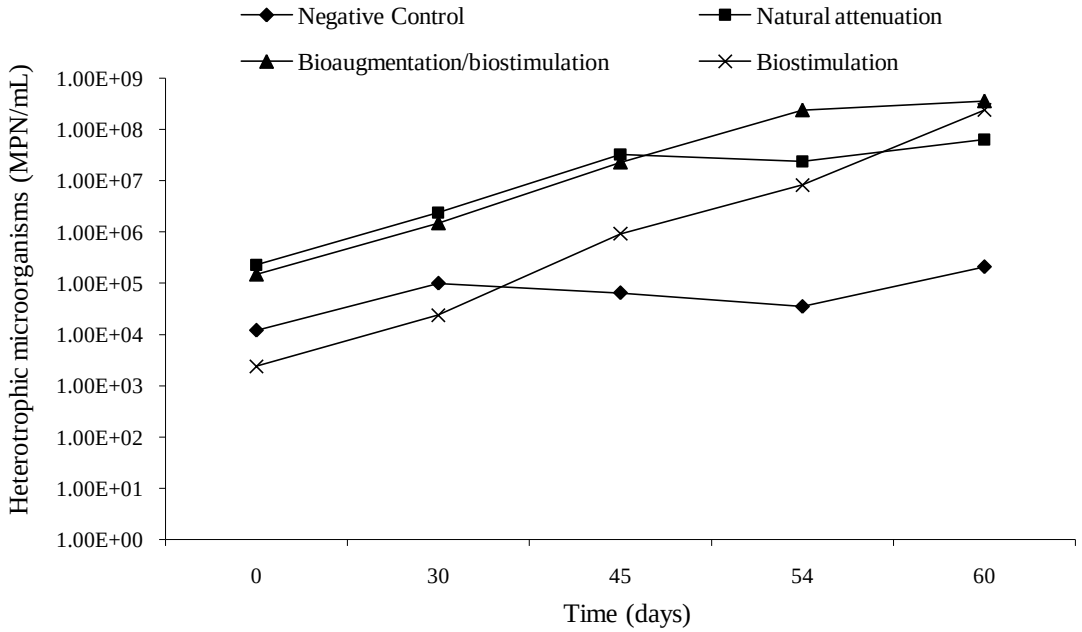


Fig. 2B

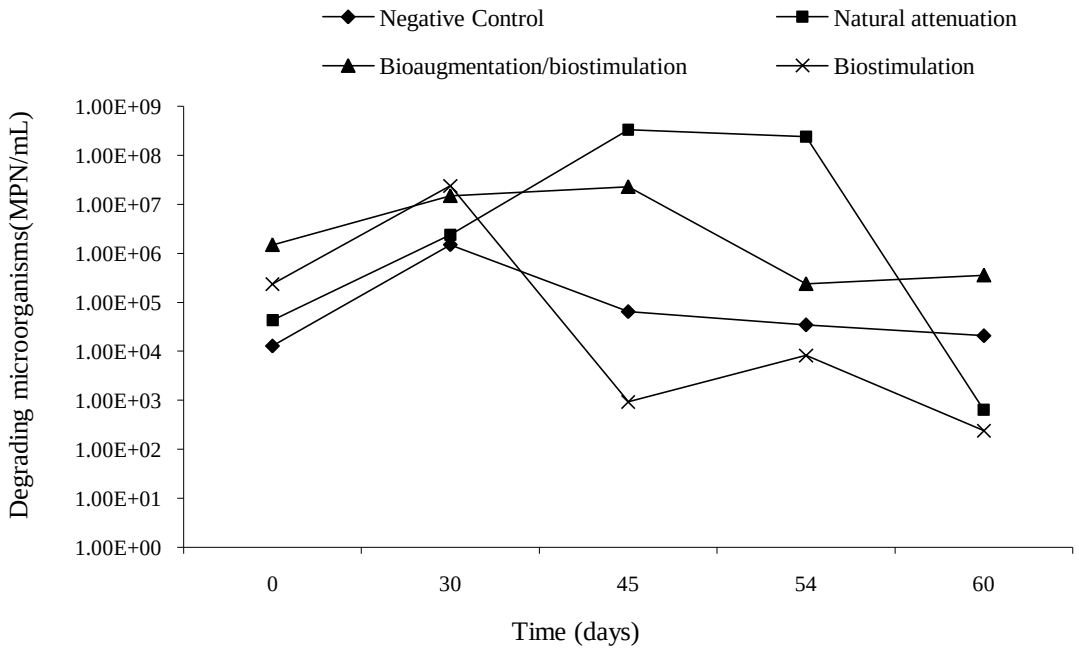
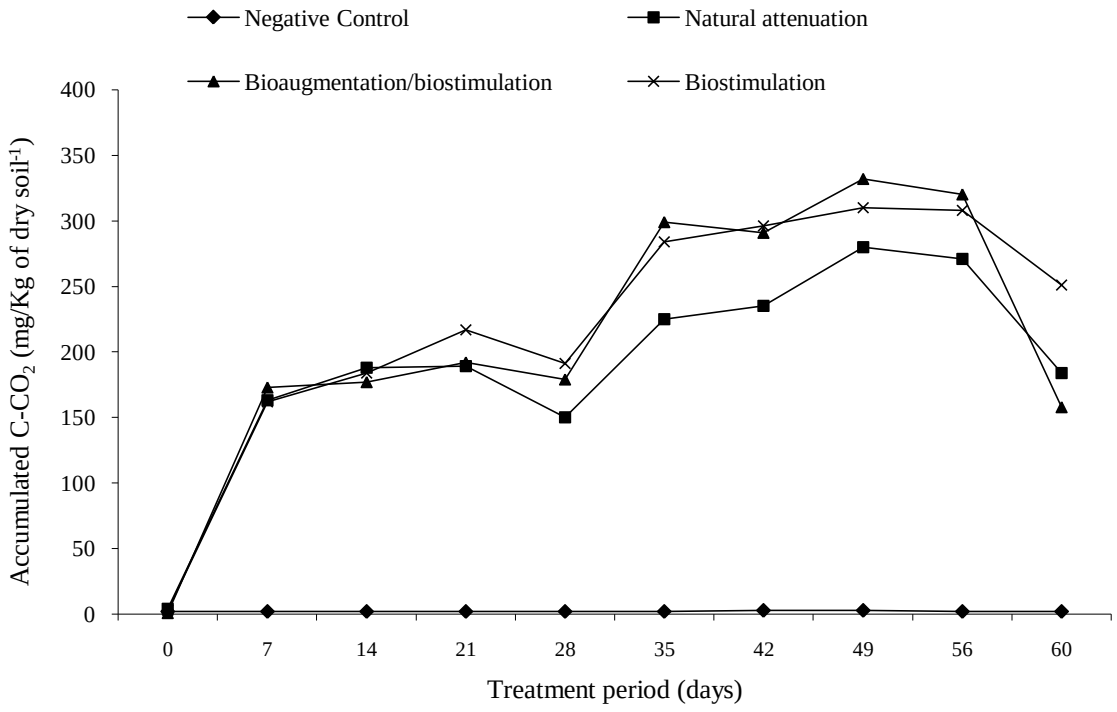


Fig. 3
C-CO_2 accumulated release quantification by soil microbial population from contaminated soil measured during 60 days of bioremediation assay.



4. Discussion

In the treatment of areas contaminated with complex compounds - such as diesel oil - which are difficult to degrade, the use of microorganisms with the ability to both degrade contaminants and produce biosurfactants (by using contaminants as a carbon sources) is both economically and environmentally interesting. This alternative offers great advantages over the use of bacteria with degradative capacity only (Cameotra and Singh, 2008).

Analyses of the emulsifying activity demonstrated that all isolates described here were able to produce biosurfactants containing diesel oil as the sole carbon source. The isolates demonstrated an emulsification index of up to 31% in the absence of cells (Fig.1), and the emulsification index was higher than that observed in the negative control for all isolates ($p < 0.001$), with the exception of the BPB 1.1. There was no significant difference in the production of emulsion among the isolates. These results were lower than those reported in other studies. Dhote et al., (2009) reported emulsification rates of 46% and 57% for two bacterial isolates from oily sludge identified as *Bacillus* sp. and *Pseudomonas* sp., whereas Cerqueira et al. (2011) obtained 55.1%, when cells were kept in the measuring mixture. Yu et al. (2014) evaluated the emulsification capacity of *Acinetobacter* strain for use in pyrene degradation and the results showed a 15% rate of emulsifier production. As we can see the literature describes that there is a great variation between emulsifying capacities of the various microorganisms described above, but in most cases there is an increase of the degradation of different contaminants when these are used bioemulsificadores. Thus, our results confirm the production of bioemulsificants tested by microorganisms, which may be assisting in the use of hydrocarbons by microorganisms.

Thus, the isolates showed efficiency in the used of carbon source used (diesel oil). All isolates showed similar behaviour as the color changed as a function of time, showing a‘turning point’ from colorless to red in the first 24 hours.

Several studies have employed the rapid TTC test to predict the presence of oil degrading microorganisms. Kluber et al. (1995) isolated bacteria capable of oxidizing hydrocarbons directly from soil; approximately 90% of these showed the ability to reduce the redox indicator TTC. Cerqueira et al. (2011) used TTC in a preliminary analysis of degradation of petrochemical waste by 45 bacterial isolates, and showed that the vast majority of isolates were capable of degrading hydrocarbons from 18 to 48 h. These data are consistent with our results showing that 24 out of the 34 isolates described here had biodegradability potential (according to the TTC test) when incubated in the presence of diesel and thus were capable of using diesel oil efficiently as a carbon source.

Rhamnolipids have the ability to form emulsions and solubilize components immiscible in water, which makes them suitable for various industrial and biotechnological applications (Costa et al., 2010). In bioremediation, they can be used as washing agents for contaminated soils, as well as in the remediation of oil spills in aquatic and terrestrial environments (Maier and Soberon-Chavez, 2000). Al-Tahhan et al. (2000) point out that these biosurfactants have features related to the assimilation of insoluble substrates, especially hydrocarbons. Moreover, Cameotra and Singh (2008) reported improved hydrocarbon degradation (95% in 4 weeks) by a microbial consortium when a mixture of rhamnolipids was added to the medium.

Additionally, Rahman et al. (2003) evaluated the n-alkane degradation rate of oil residues deposited inside storage tanks, using a microbial consortium with rhamnolipid added, which yielded positive effects in the bioremediation of these hydrocarbons. Thus, the production of biosurfactant, specifically rhamnolipids, is widely used to check the efficiency of hydrocarbon degradation by microorganisms. In our study, 20 isolates produced rhamnolipids, suggesting that these isolates have potential for use in the remediation of environments contaminated with hydrocarbons.

The consortium used in these experiments was chosen based on TTC test results, the production of biosurfactants and on species identification by 16S rRNA partial sequencing. *P. mendocina* (BPB 1.6), *B. cereus* (BPB 1.15), *A. calcoaceticus* (BPB 1.22), *B. subtilis* (BPB 1.13) and *B. cereus* (BPB 1.26).

Bacteria from the genera *Pseudomonas* and *Bacillus* isolated from hydrocarbon contaminated environments are able to grow and degrade aliphatic and aromatic chains (Assih et al., 2002; Fernández-Luqueño et al., 2011; Thavasi et al., 2011; Tahhan et al. 2011). Particularly, *Pseudomonas* species are commonly found in soils polluted with hydrocarbons and, as described by Gunther et al. (2005), are the main sources of rhamnolipid production. *P. aeruginosa* is considered a primary rhamnolipid producer, this makes the process more attractive biodegradation, whereas biosurfactants increase the contaminant bioavailability (Nitschke and Pastore, 2002; Chrzanowski et al., 2012).

Coutinho et al. (2013) isolated species of *P. aeruginosa* that exhibited emulsification abilities from a site contaminated with refined oil, and identified the species as being of great potential use for the bioremediation of oil wastewater to promote environmental recovery and/or degradation of oil. However, in the present study, we chose not to use *P. aeruginosa* due to its pathogenicity.

The measurement of CO_2 production in soil is an indirect form of estimating the decarboxylation of organic compounds degraded in the system (Margesin et al., 2001). The microorganisms identified in this study are capable of assimilating hydrocarbons as a carbon source, liberating CO_2.

All soil treatments tested here yielded CO_2 production levels higher than those observed in the negative control (NC) ($p < 0.001$), suggesting that there is a microbial capacity to metabolize hydrocarbon contaminants. However, there was no significant difference in CO_2 production among the treatments (Figure 2).

Alisi et al. (2009) show that the microbial community requires an adaptive phase to begin CO_2 production, and this ceases as soon as the availability of the carbon source is exhausted. In agreement with these data, our results show that all treatments had similar adaptive phase profiles (from experimental day 7), and we observed a decrease in CO_2 production at the end of the 60-day experiment.

We also observed an increase in bacterial population over time, reaching a maximum of 10^7 MPN/mL during the bioaugmentation/biostimulation treatment. The bacterial population increase indicated that the diesel oil, used as the sole carbon source, stimulated the growth and metabolism of different isolates. Silva et al. (2012) demonstrated similar results for biodiesel contaminated soil, where an "adaptation phase" occurred within 10 days of the start of the experiment and decreased after 45 days.

The biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and amount of oil and hydrocarbons present in the contaminated area. The low availability of these contaminants to microbial attack affects the efficiency of biodegradation (Cerqueira et al., 2011). Although we did not detect significant differences in biodegradation between the bioremediation strategies of natural attenuation, bioaugmentation/biostimulation and biostimulation in this study, all TPH degradation rates were relatively high, with reduction in TPH between days 0 and 60 days of ~90% (Table 4).

Biostimulation treatment reduced significantly the levels of contaminants after 40 days of treatment, implying that degradation of hydrocarbons by a given native microbial population is improved by the presence of the required nutrients in the contaminated site. Whereas in most environments the presence of nitrogen and phosphorous is limited, biostimulation accelerates the decontamination rate, as the addition of one or more rate-limiting nutrients to the system improves the degradation potential of the inhabiting microbial population (Tyagi et al. 2010).

The timing and degradation rates observed here were higher than those reported in other studies. Colla et al. (2013) obtained TPH degradation values of 35.7 and 32.2 % for biostimulation and successive bioaugmentation treatments, respectively. Bento et al. (2005) also showed that natural attenuation resulted in further degradation of the light fraction of TPH in soil, and the authors attributed this effect to indigenous adaptation that allows organisms to become physiologically compatible with their habitat, in contrast to transient autochthonous organisms that do not occupy a functional niche. This was also demonstrated in a study by Couto et al. (2010), where natural attenuation was as efficient as the treatments of bioaugmentation and biostimulation in soil contaminated by refinery petroleum.

Tahhan et al. (2011) suggested that more effective bioaugmentation is achieved by repeating the application of the microbial consortium during the degradation period, because of the tendency of the autochthonous population to overcome the exogenous inoculum, when microbial communities in contaminated soil express their degrading potential. However, the results presented in this study show that even with successive additions of the consortium, there was no significant difference between the attenuation and natural treatments and bioaugmentation/biostimulation.

According to Sabaté et al. (2004), natural attenuation in soils with historic contamination may improve bioremediation, because the native microorganisms are already adapted to the contaminant; however, this is not consistent with the results shown here, since the microorganisms inoculated in the treatment of bioaugmentation/biostimulation were isolated from the soil itself used in the treatment. Thus, these microorganism strains were already adapted to the contaminants. Overall, our results show that all treatments tested here are promising *in situ* bioremediation techniques, reinforcing the notion that bioremediation treatments are effective and offer cost-benefit in relation to physical and chemical treatments.

5. Conclusions

The microbial consortium selected, in combination with macronutrients successively added to the system, effectively accelerated soil contaminant degradation, with high biodegradation rates (~ 90%). The results of this study also show that soils with old history of contamination, harbours microbial communities capable of expressing biodegrading potential when suitable stimulus. Further research is now required under field conditions, to evaluate the full potential of bioremediation for effective treatment of hydrocarbon-contaminated soil.

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6. References

Alisi,C., Musella,R., Tasso,F., Ubaldi,C., Manzo,S., Cremisini,C., Sprocati,A.R. (2009): Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Sci. Total Environ. 407, 3024-3032.

Al-Tahhan, R. A., Sandrin, T. R., Bodour, A.A., Maier, R.M. (2000): Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa:* effect on cell surface properties and interaction with hydrophobic substrates. Appl.Environ.Microb.v. 66, n. 8, 3262-3268.

APHA. (1995): Standard methods for the examination of water and waste-water, 19th ed. American Public Health Association, Washington, DC.

Assih, E.A., Ouattara, A.S., Thierry, S., Cayol, J.L., Labat, M., Macarie, H. (2002): Stenotrophomonas acidaminiphila sp. nov., a strictly aerobic bacterium isolated from an up flow anaerobic sludge blanket (Uasb) reactor. Int. J. Syst. Evol.Microbiol. 52, 559-568.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger,W.T. (2005): Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource Technol. 96, 1049-1055.

Braddock, J. F., Catterall, P. H. (1999): A Simple Method for Enumerating Gasoline- and Diesel-Degrading Microorganisms. Bioremediation J. 3, 81-84.

Cameotra, S.S., Singh, P.(2008):Bioremediation of oil sludge using crude biosurfactants. Int. Biodeter. Biodegr. 62, 274–280.

Casida, L. (1997): Microbial metabolic activity in soil as measured by dehydrogenase determinations. Appl. Environ. Microb.v. 34, n. 6, 630-636.

Cerqueira,V.S., Hollenbach,E.B., Maboni,F., Vainstein,M.H., Camargo,F.A.O., Peralba,M.C.R., Bento,F.M. (2011): Biodegradation potential of oily sludge by pure and mixed bacterial cultures. Bioresource Technol. 102, 11003-11010.

CETESB. (1999):Companhia de Tecnologia de Saneamento Ambiental. Lista holandesa de valores de qualidade do solo e da água subterrânea, Valores STI, Available from: http://www.cetesb.sp.gov.br/solo/areas_contaminadas/anexos/download/6530.pdf (accessed 10.06.11).

Chrzanowski,L., Ławniczak,L., Czaczyk,K. (2012): Why do microorganisms produce rhamnolipids? World J. Microb. Biot. 28, 1-9.

Colla,T.S., Andreazza,R., Bücker,F., De Souza,M.M., Tramontini,L., Prado, G.R., Frazzon,A.P.G., Camargo,F.A.O., Bento,F.M. (2014): Bioremediation assessment of diesel-biodiesel-contaminated soil using an alternative bioaugmentation strategy. Environ. Sci. Pollut. R. 21, 2592-2602.

Cooper, D.G., Goldenberg, B. G. (1987): Surface-active agents from two *Bacillus* species. Appl.Environ. Microb. 53, 224-229.

Costa, S. G., Nitschke, M., Lepine, F., Déziel, E., Contiero, J. (2010): Structure, properties and applications of rhamnolipids produced by Pseudomonas aeruginosa L2-1 from cassava wastewater. Process Biochem.v. 45, n. 9,1511-1516.

Coutinho,J.O.P.A., Silva,M.P.S., Moraes,P.M., Monteiro,A.S., Barcelos,J.C.C., Siqueira,E.P., Santos,V.L.(2013): Demulsifying properties of extracellular products and cells of *Pseudomonas aeruginosa* MSJ isolated from petroleum-contaminated soil. Bioresource Technol. 128, 646-654.

Couto, M. N. P. F. S., Monteiro, E., Vasconcelos, M. T. S. D. (2010): Mesocosm trials of bioremediation of contaminated soil of a petroleum refinery: comparison of natural attenuation, biostimulation and bioaugmentation. Environ. Sci. Pollut. R. 17, 1339-1346.

Das,N., Chandran,P. (2011): Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. R. Int., 1-13.

Dhote,M., Juwarkar,A., Kumar,A., Kanade,G.S., Chakrabarti,T. (2009): Biodegradation of chrysene by the bacterial strains isolated from oily sludge. World J. Microb. Biot. 26,329-335.

Evans, F.F., Rosado, A.S., Sebastian, G.V., Casella, R., Machado, P.L.O.A., Holmström, C., Kjelleberg, S., Van Elsas, J.D., Seldin, L. (2004): Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. FEMS Microbiol. Ecol. 49, 295-305.

Fantroussi, S., Agathos, S.N. (2005): Is bioaugmentation a feasible strategy for pollutant removal and site remediation? Curr. Opin. Microbiol.8, 268-275.

Fernández-Luqueño, F., Valenzuela-Encinas, C., Marsch, R., Martínez-Suárez, C.,Vázquez-Núñez, E., Dendooven, L.(2011): Microbial communities to mitigate contamination of PAHs in soil – possibilities and challenges: a review. Environ.Sci. Pollut. R. 18, 12-30.

Gunther, N.W., Nuñez,A., Fett,W., Solaiman,D.K.Y. (2005):Production of rhamnolipids by *Pseudomonas chlororaphis*, a nonpathogenic bacterium. Appl. Environ. Microb. 7, 2288-2293.

Jiménez, N., Viñas, M., Bayona, J.M., Albaiges, J., Solanas, A.M. (2007): The Prestige oil spill: bacterial community dynamics during a field biostimulation assay. Appl.Microbiol. Biot. 77 (4), 935-945.

Kluber, H. D.; Lechner, S., Conrad, R. (1995): Characterization of populations of aerobic hydrogen-oxidizing soil bacteria. FEMS Microbiol. Ecol.v. 16, n. 2, p. 167-176.

Lin, T.C., Pan, P.T., Young, C.C., Chang, J.S., Chang, T.C., Cheng, S.S. (2011): Evaluation of the optimal strategy for ex situ bioremediation of diesel oil-contaminated soil. Environ. Sci. Pollut. R. 18,1487-1496.

Maier, R. M. and Soberon-Chavez G. (2000): Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. Appl. Microbiol. Biot.v. 54, n. 5, p. 625-33.

Margesin, R., Schinner, F. (2001): Bioremediation (Natural Attenuation and Biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. Appl. Environ. Microbiol. 67, 3127-3133.

Meyer, D.D., Beker, S.A., Bücker, F., Peralba, M.C.R., Frazzon, A. P.G., Osti, J.F., Andreazza, R., Camargo, F.A.O., Bento, F.M. (2014): Bioremediation strategies for diesel and biodiesel in oxisol from southern Brazil. Int. Biodeter. Biodegra. 95, 356-363.

Mishra, S., Jyot, J., Kuhad, R.C., Lal, B.(2001): Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge contaminated soil. Appl. Environ. Microb. 67,1675-1681.

Mukherjee, A.K., Bordoloi, N.K. (2011): Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously seeded bacterial consortium: a pilot-scale study. Environ. Sci. Pollut. Res. 18, 471-478.

Nitschke, M., Pastore, G.M.(2002): Biossurfactantes: propriedades e aplicações. Quím. Nova. 25, 772-776.

Rahman, P. K. S. M. and Gakpe, E. (2008):Production, Characterization and Applications of Biosurfactants. Rev. Biotechnol. v. 7, n. 2, p. 360-370.

Sabaté, J, Viñas, M, Solanas, A.M. (2004):Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. Int. Biodeter. Biodegra.54, 19–25.

Sambrook, J. and Russell, D.W. (2001): Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Silva, G.S., Marques, E.L.S., Dias, J.C.T., Lobo, I. P., Gross, E., Brendel, M., Da Cruz, R.S., Rezende, R.P.(2012):Biodegradability of soy biodiesel in microcosm experiments using soil from the Atlantic Rain Forest. App. Soil Ecol. 55, 27-35.

Stotzky, G. (1965): Microbial respiration. In: Black, C.A. (Ed.), Methods in Soil Analysis. SSSA, Madison, 1550-1572.

Szulc, A., Ambrozewicz, D., Sydow, M., Lawniczak, L., Piotrowska-Cyplik , A., Marecik, R., Chrzanowski, L.(2014): The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. J. Envir. Manag.132, 121-128.

Tahhan, R.A., Ammari, T.G., Goussous, S.J., Al-Shdaifat, H.I. (2011): Enhancing the biodegradation of total petroleum hydrocarbons in oily sludge by a modified bioaugmentation strategy. Int. Biodeter. Biodegra. 65, 130-134.

Thavasi, R., Jayalakshmi, S., Banat, I.M. (2011): Effect of biosurfactant and fertilizer onbiodegradation of crude oil by marine isolates of Bacillus megaterium,Corynebacterium kutscheri and Pseudomonas aeruginosa. Bioresource Technol.102 (2), 772-778.

Tedesco, M.J., Gianello, C., Bissani, C.A., Bohnen, H., Volkweiss, S.J. (1995): Análise de solos, plantas e outros materiais (Analysis of soils, plants and other materials), 174. Departamento de Solos, UFRGS, Porto Alegre.

Tyagi, M., Da Fonseca, M.R., De Carvalho, C.C.C.R. (2010): Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation. 22, 231-241.

USEPA METODO 8015B Orgânicos Nonhalogenados usando GC/FID [Nonhalogenated organics using GC/Fid]. (1996): Available at: http://www.caslab.com/EPAMethods/ PDF/8015b.pdf (accessed 11.05.12).

Yu, Y., Zhang, W., Chen, G., Gao, Y., Wang, J.(2014): Preparation of petroleum-degrading bacterial agent and its application in remediation of contaminated soil in Shengli Oil Field, China . Environ Sci Pollut Res 21: 7929–7937.

Xu, Y., Lu, X. (2010): Bioremediation of crude oil-contaminated soil. Comparison of different biostimulation and bioaugmentation treatments. J. Hazard. Mater. 183, 395-401.