

Research Article

Comparative Bioremediation of Crude Oil-Amended Tropical Soil Microcosms by Natural Attenuation, Bioaugmentation, or Bioenrichment

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Bioremediation is an efficient strategy for cleaning up sites contaminated with organic pollutants. In this study, we evaluated the effectiveness of monitored natural attenuation, bioenrichment, and bioaugmentation using a consortium of three actinomycetes strains in remediating two distinct typical Brazilian soils from the Atlantic Forest and Cerrado biomes that were contaminated with crude oil, with or without the addition of NaCl. Microcosms were used to simulate bioremediation treatments over a 120-day period. During this period, we monitored total petroleum hydrocarbons (TPHs) and n-alkanes degradation and changes in bacterial communities. Over time, we found the degradation rate of n-alkanes was higher than TPH in both soils, independent of the treatment used. In fact, our data show that the total bacterial community in the soils was mainly affected by the experimental period of time, while the type of bioremediation treatment used was the main factor influencing the actinomycetes populations in both soils. Based on these data, we conclude that monitored natural attenuation is the best strategy for remediation of the two tropical soils studied, with or without salt addition.

1. Introduction

The high demand for and use of petroleum and its derivatives worldwide has made petroleum hydrocarbon contamination a global problem with serious health and environmental consequences [1–3].

Contamination of soil and groundwater with petroleum compounds is frequently observed, necessitating the development of innovative technologies for remediation [4, 5].

Bioremediation is an efficient and environmentally friendly technology for long-term restoration of sites contaminated with petroleum hydrocarbons and derivatives [5, 6]. Several studies have focused on the composition of naturally occurring microbial populations that contribute to biodegradation of petroleum and its derivatives in different environments [7–10]. Monitored natural attenuation (MNA) uses the ability of the soil intrinsic

microbial community to degrade the contaminant. In cases where enhancement of the soil microbial community is deemed necessary, either bioaugmentation or bioenrichment is used. Bioaugmentation relies upon pollutant-degrading microorganisms found in the contaminated site, whereas in bioenrichment the microorganisms are exogenous [11, 12]. Regardless, studies of both approaches have demonstrated that the degrading organisms are not maintained in the contaminated environment after introduction [13, 14]. Therefore, molecular methods that rapidly survey the microbial community structure and function [15] are being employed extensively to monitor both the persistence of the microbial inocula added to the contaminated site and the effects of the introduced microorganisms upon the indigenous microbiota [16].

Actinomycetes have been widely used in bioremediation in soils contaminated with petroleum and derivatives

[5, 17, 18]. Actinomycetes have several characteristics that are essential for this ability: they are excellent metabolizers, they are able to survive in extreme conditions such as dry environments or under nutrient starvation, and they produce biosurfactants, which increase contaminant bioavailability and facilitate biodegradation process.

In a previous study, Alvarez et al. [19] isolated 14 halotolerant actinobacterial strains from a soil contaminated with crude oil and produced water sampled at Panelas, a terrestrial oil field in Brazil. The environment impact of the oil contamination was exacerbated by the presence of moderate salt concentration (around 7% NaCl) in the produced water. All 14 strains showed the ability to degrade crude oil and some oil derivatives [19]. However, these strains were not tested for their ability to degrade oil in a mixed microbial consortium. In this study, we consider the application of these strains for soil bioremediation after crude oil contamination in the Brazilian oil field area studied, Panelas and another site, Cantagalo. Specifically, we aim to (a) select a bacterial consortium from the previously isolated strains based on their growth capabilities and degradation profiles; (b) set up soil microcosms using two representative soils from Brazilian biomes, Cantagalo soil from the Atlantic Forest and Panelas soil from Cerrado, with or without the addition of salt; (c) compare the efficiency of monitored natural attenuation, bioaugmentation, or bioenrichment during a 120-day experiment; and (d) analyze the effect of each bioremediation treatment on total bacterial community and actinomycetes population by 16S rRNA-based PCR and denaturing gradient gel electrophoresis (DGGE). The results of this study will be of great use, given that oil spills may occur in the Brazilian areas studied, leading to environmental impacts in either Cerrado or Atlantic Forest biomes. Moreover, monitoring the persistence of the bacterial inocula added to the contaminated site and its effects upon the indigenous microbiota can provide main tools for future use of these bacteria.

2. Materials and Methods

2.1. Bacterial Strains. We chose seven of the 14 halotolerant actinobacterial strains identified in Alvarez et al. [19] for use in this study based upon their ability to degrade crude oil and petroleum derivatives in Bushnell-Haas (BH, Difco) medium containing 7% NaCl, their growth in mineral medium using crude oil as the sole source of carbon, and their ease of maintenance under laboratory conditions (Table 1).

2.2. Total Petroleum Hydrocarbons (TPH) and n-Alkanes Degradation Analyses. All seven selected strains were grown for 30 days at 28°C in 20 mL of BH medium with 7% NaCl and 1,000 ppm crude oil. TPH and n-alkanes concentrations were determined using the EPA 8015 C technique [20].

2.3. Soil Microcosms. Two 3-kg pristine soil samples were collected from the Atlantic Forest and Cerrado biomes in Brazil. The Atlantic Forest soil was sampled from a farm located in Cantagalo city at Rio de Janeiro state (latitude

21°58′52″ and longitude 42°22′05″—Southeast Brazil). The Cerrado soil sample was obtained near an oil field area in Panelas located at Carmópolis City, Sergipe state (latitude 10°38′53″ and longitude 36°59′20″—Northeast Brazil). Characteristics of each soil are detailed in Table 2.

In order to set up our microcosms of each soil, two portions of 684 g of each soil were contaminated with 36 g (50,000 ppm) of crude oil from the Panelas oil field. The first portion was then subdivided into two equal parts of 360 g each. One 360 g portion was supplemented with 21 mL of a 0.85% NaCl solution. The soil was then homogenized and distributed between three polyvinyl chloride (PVC) pots (25 cm in circumference, 8 cm in diameter and 12 cm high) lined with aluminum foil and plastic. These pots simulated a monitored natural attenuation (MNA) treatment in both soils without the addition of NaCl. The other 360 g portion was supplemented with 21 mL of a 0.85% NaCl solution and 10⁸ CFU/mL of the bacterial consortium. The soil was then homogenized and distributed between three PVC pots. To prepare the bacterial consortium inoculum, the strains *G. rubripectinta* DTSB 2.5, *R. equi* DLB 2.6, and *G. alkanivorans* DLB 3.22 were grown separately at 28°C for 72 h in 20 mL of LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) with crude oil (20 μL). Each strain was then inoculated in an Erlenmeyer containing 180 mL of LB medium and grown at 28°C for 72 h. The resultant cells were washed three times with saline (NaCl 0.85%) before inoculation, and 100 μL was used to determine the CFU/mL. The other 360 g portion of soil was prepared identically; however, a 7% NaCl solution was used instead. Microcosms were incubated for 120 days at room temperature. The pots were watered based on the water holding capacity of the soil and revolved for aeration twice a week.

In the case of soil from Panelas, the soil inoculated with bacteria represented bioaugmentation—BA (no salt) or BAS (salt). Conversely, soil from Cantagalo inoculated with bacteria represented bioenrichment—BE (no salt) or BES (salt). The microcosms submitted to MNA with salt were denoted MNAS.

2.4. Soil Petroleum Hydrocarbons and n-Alkanes Analyses. Composite samples were obtained for each bioremediation treatment by mixing 4 g of soil obtained from each triplicate. Total petroleum hydrocarbons (TPHs) and n-alkanes concentrations were determined by gas chromatography using the EPA 8015 D technique [22]. Percentages of degradation were calculated using the n-alkanes and TPH values obtained in T₀ and T₁₂₀ for each bioremediation treatment by the following expressions: percentage of TPH degradation = [(TPH_{T₀} - TPH_{T₁₂₀})/TPH_{T₀}] × 100 or percentage of n-alkanes degradation = [(alkanes_{T₀} - alkanes_{T₁₂₀})/alkanes_{T₀}] × 100. The efficiencies of bioenrichment and bioaugmentation were compared to those observed for monitored natural attenuation as follows: efficiency of BE/BA for TPH degradation = percentage of TPH degradation using BE/BA - percentage of TPH degradation using MNA Cantagalo/MNA Panelas.

TABLE 1: Growth of selected actinomycetes with different petroleum derivatives as the sole carbon sources and degradation of n-alkanes and total petroleum hydrocarbons (TPHs).

Bacterial strains	Petroleum derivatives ^a							Degradation of ^b	
	C6	C12	C16	DI	GA	TO	NA	n-alkanes	TPH
<i>Rhodococcus</i> sp. DTSB 2.3	-	+	+++	++	+	-	-	3	3
<i>Gordonia rubripertincta</i> DTSB 2.5	-	++	+++	+	-	-	+	2	3
<i>Rhodococcus</i> sp. DTSB 3.4	-	+	++	++	-	+	+	1	6
<i>G. alkaniivorans</i> DTSB 3.6	+	++	++	++	+	-	+	1	5
<i>R. equi</i> DLB 2.6	-	++	++	+++	+	-	+	4	3
<i>Rhodococcus</i> sp. DLB 3.4	-	+	++	+	-	-	-	1	7
<i>G. alkaniivorans</i> DLB 3.22	-	+++	+++	+	-	-	+	3	4

^a C6, hexane; C12, dodecane; C16, hexadecane; DI, diesel; GA, gasoline; TO, toluene; NA, naphthalene; (-), no growth observed (+), weak growth; (++) good growth; (+++) very good growth. Data from Alvarez et al. [19];

^bTPH and n-alkanes concentrations were determined using the EPA 8015 C technique [20]. Numbers represent the degradation range presented by the strains: (1) higher than 95%, (2) between 80 and 94%, (3) between 65 and 79%, (4) between 50 and 64%, (5) between 35 and 49%, (6) between 20 and 34%, (7) lower than 19%.

TABLE 2: Physical and chemical characteristics of Cantagalo and Panelas soils.

Soil property	Cantagalo/ RJ	Panelas/SE
pH	6.3	4.6
P (mg dm ⁻³)	13	6
K (mmol ^c dm ⁻³)	7	2.2
Ca (mmol ^c dm ⁻³)	43.3	27
Mg (mmol ^c dm ⁻³)	38.5	12
Al (mmol ^c dm ⁻³)	0	3
H + Al (mmol ^c dm ⁻³)	35.3	85
Na (mmol ^c dm ⁻³)	0.6	1.7
V (%) ^a	72	34
m (%) ^b	0	2.3
ISNa (%) ^c	0	1
Fe (mg dm ⁻³)	35.33	312
Cu (mg dm ⁻³)	0.42	0.8
Zn (mg dm ⁻³)	5.45	2
Mn (mg dm ⁻³)	98.97	3.8
Sand (%)	76	66
Silt (%)	8	12
Clay (%)	16	22
Classification ^d	Sandy-loam	Clay-sandy

^aBases saturation index; ^baluminum saturation index; ^csodium saturation index. ^dtrilinear soil classification was made according with the percentages of sand, silt, and clay [21].

2.5. DNA Extraction. The oil-contaminated soil microcosms were sampled at 0, 30, 60, 90, and 120 days for molecular analyses. Soil samples of 0.5 g were taken from each triplicate under different bioremediation treatments. Total DNA was extracted from these soil samples using the Fast DNA Spin Kit for Soil (Qbiogene, BIO 101 Systems, USA) according to the manufacturer's instructions. DNA was extracted from the bacterial strains used in the consortium using the methodology described by Seldin and Dubnau [23]. DNAs were visualized on 0.8% (w/v) agarose gels to assess their integrity and then stored at 4°C prior to their use in PCR reactions.

2.6. PCR Amplification of Bacterial 16S rRNA Genes. A 473 bp fragment of the bacterial 16S rRNA gene was amplified using PCR primers U968F-GC1 and 1401R [24]. The 50- μ L PCR reaction mix contained 1 μ L of template DNA (corresponding to 50–100 ng), 1x Colorless GoTaq Flexi Buffer, pH 8.5 (Promega, Madison, WI, USA), 5 mM MgCl₂, 200 μ mol of each dNTPs, 20 μ mol of each primer, 5 μ g of bovine serum albumin (BSA), and 2.5 U of *Taq* polymerase. Amplification conditions were previously described by Nübel et al. [25]. Negative controls without DNA were run in all amplifications. PCR products were visualized by electrophoresis on 1.4% agarose gels stained with ethidium bromide (2 μ g/mL). Amplicons were stored at -20°C until DGGE analysis.

2.7. PCR Amplification of Actinomycetes 16S rRNA Genes. For specific amplification of 16S rRNA gene fragments from

actinomycetes, the primers F243 (5' GGATGAGCCCGCG-GCCTA 3'), U968F-GC1 (5' ACCGCGAAGAACCTTAC 3') and 1401R (5' GCGTGTGTACAAGACCC 3'), were used in a two-step nested PCR [26]. The 25 μ L PCR reaction mixture contained 1x Colorless GoTaq Flexi Buffer (pH 8.5), 2.5 mM MgCl₂, 200 μ mol of each dNTPs, 100 nM of each primer (F243 and 1401R), 1 μ L of DNA template, and 1.25 U of *Taq* polymerase. Amplification conditions were as follows: initial denaturation for 5 min at 94°C; 35 cycles using 1 min of denaturation at 94°C, 1 min at 58°C for primer annealing, and 2 min at 72°C for primer extension; a final extension for 10 min at 72°C; cooling to 4°C. Expected fragments of 1158 bp were visualized by electrophoresis on 1.4% agarose gels stained with ethidium bromide (2 μ g/mL) and then used as templates for the second amplification. The second reaction was performed as described above using the primers U968F-GC1 and 1401R.

2.8. Denaturing Gradient Gel Electrophoresis (DGGE). DGGE using 16S rRNA gene PCR products obtained either from total bacterial communities or from the specific populations of actinomycetes was performed using a Dcode Universal Mutation Detection System (Bio-Rad, Calif, USA). PCR amplicons were loaded directly onto a 8% (w/v) polyacrylamide gel containing a denaturing gradient of urea and formamide varying from 40% to 70% in 1x TAE buffer (40 mM Tris-acetate (pH 7.4), 20 mM sodium acetate, and 1 mM disodium EDTA). DGGE was run for 16 h at 60°C and 75 V and then stained with SYBR-Green I (Invitrogen-Molecular Probes, SP, Brazil) for 40 min. Stained gel was visualized under UV light and digitized using STORM (Amersham Pharmacia Biotech, Munich, Germany). Clustering patterns of different profiles from DGGE were obtained using Gel-Compar II 4.06 software (Applied Maths, Saint-Martens-Latem, Belgium), Pearson's coefficient and the clustering method of unweighted pair-group method using arithmetic averages (UPGMA).

2.9. Canonical Correspondence Analysis (CCA). Canonical correspondence analysis (CCA) was used to correlate DGGE profiles (biological variables) with environmental variables (sampling time—T0, T60, and T120, bioremediation treatment, NaCl addition, and TPH and n-alkanes degradation). CCA was also used to cluster DGGE profiles obtained after 120 days of bioremediation experiment with the physical and chemical composition of soils used (Cantagalo and Panelas). CCA was performed using Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY).

3. Results

3.1. Consortium Selection. We chose to study seven of the 14 halotolerant actinomycetes strains previously isolated from Panelas, a tropical soil-containing area from the northeast of Brazil, which is described by Alvarez et al. [19]. Strains were chosen based on their profiles of degradation of petroleum derivatives (Table 1) and good growth and maintenance under laboratory conditions. They were first tested for

TABLE 3: Degradation rates of n-alkanes and TPH for each bioremediation treatment.

Soil	Bioremediation treatment ^a	n-alkanes degradation (%)	TPH degradation (%)
Cantagalo	MNA	56.5	15.6
	MNAS	64.8	32.5
	BE	60	24.8
	BES	67.7	36.2
Panelas	MNA	74	38.1
	MNAS	78.1	39.3
	BA	78.4	47.5
	BAS	77.3	51.3

^a MNA, monitored natural attenuation; MNAS, monitored natural attenuation in soil with the addition of NaCl; BE, bioenrichment; BES, bioenrichment in soil with NaCl; BA, bioaugmentation; BAS, bioaugmentation in soil with NaCl.

TPH and n-alkanes degradation after growth in medium containing 7% of NaCl. All strains tested showed n-alkanes degradation rates of at least 50% (Table 1). Strains DTSB 3.4, DTSB 3.6, and DLB 3.4 almost reached 100% degradation (Table 1). Conversely, TPH degradation rates were lower than those observed for n-alkanes, ranging from 10 to 70%. The best rates of TPH degradation (60 to 70%) were observed in the DLB 3.22, DTSB 2.3, DLB 2.6, and DTSB 2.5 strains (Table 1). These strains were previously identified as *Gordonia alkanivorans* (DLB 3.22), *Rhodococcus* sp. (DTSB 2.3), *R. equi* (DLB 2.6), and *G. rubripertincta* (DTSB 2.5) [19]. After initial testing, DLB 3.22, DTSB 2.3, and DLB 2.6 were chosen to represent the bacterial consortium due to their broad profiles of degradation of petroleum derivatives and good rates of n-alkanes and TPH degradation. Strain DTSB 2.3 was excluded from the consortium because it showed a degradation profile similar to DTSB 2.5, except that it lacked the ability to use naphthalene as a sole carbon source (Table 1).

3.2. Crude Oil Contamination Assay. We conducted three different bioremediation treatments in microcosms to evaluate the efficiency of crude oil degradation in artificially contaminated soils. We assessed the following treatments both in the presence or absence of salt: (a) monitored natural attenuation (MNA) of both soils; (b) bioenrichment (BE) used specifically in Cantagalo soil; (c) bioaugmentation (BA) used in Panelas soil. After 120 days, the degradation rates of n-alkanes were higher than the rates of TPH degradation in all treatment scenarios and in both soils (Table 3). However, degradation of n-alkanes and TPH in the Panelas soil microcosms were higher than those of the Cantagalo soil. Bioenrichment (BE and BES) and bioaugmentation (BA and BAS) resulted in a slight increase in the biodegradation rates of both n-alkanes and TPH when compared to monitored natural attenuation (MNA and MNAS) (Table 3). There was no considerable difference in the efficiency of n-alkanes degradation by bioenrichment treatment with or without the addition of salt. However, the addition of salt resulted in differences in TPH degradation efficiency. The efficiency of

bioenrichment treatment in Cantagalo soil decreased from 9% (BE) to 4% (BES) in the presence of salt, whereas the efficiency of bioaugmentation in Panelas soil increased from 9% (BA) to 12% (BAS) when salt was added (Table 3).

3.3. Molecular Analysis of Total Bacterial Communities and Actinomycetes. Native bacterial and actinomycetes consortium DNA was recovered from the soil microcosms at 0, 30, 60, 90, and 120 days (for each bioremediation treatment) and then subjected to PCR to amplify a 473 bp band corresponding to the 16S rRNA encoding gene. DGGE fingerprints were also generated for each sample producing consistent DGGE profiles among triplicate samples (data not shown). The composition of the bacterial communities from Cantagalo and Panelas soils changed throughout the course of the bioremediation treatments (Figures 1(a) and 1(b)) although DGGE bands corresponding to the bacterial consortium could be observed in all bioenriched or bioaugmented samples, with or without the addition of NaCl (Figures 1(a) and 1(b)). Clustering DGGE pattern profiles of the total bacterial communities showed that the duration of the bioremediation experiment was the main factor affecting total bacterial communities in both soils (Figures 1(c) and 1(d)). In Cantagalo soil, two main clusters were formed with 70% similarity: one formed by time 0 and the other from the 30-, 60-, 90-, and 120-day samples. The 30- and 60-day samples showed the most similarity to each other, 87%. In Panelas soil, two clusters showed 78% of similarity: one was formed by times 0, 30, and 60 days and the other by 90 and 120 days.

Actinomycetes populations present in both soils studied also varied throughout the bioremediation experiment (Figures 2(a) and 2(b)). However, in this case, the bioremediation treatment, rather than time as in the case of total bacterial community, was the main factor influencing changes in the actinomycetes populations from both soils. In Cantagalo soil, actinomycetes populations experienced an initial negative impact on their growth rate until day 60, likely due to oil contamination of soil. However, after 90 days (T90 and T120), the intensity of preexisting bands increased, and new bands were observed (Figure 2(a)). No significant changes were visualized between the BE and BES treatments of Cantagalo soil. The main difference between bioenrichment and MNA/MNAS was the presence of the bacterial consortium corresponding bands, plotted on a dendrogram (Figure 2(c)). DGGE profiles of BE and BES treatments were 78% similar to the MNA and MNAS treatment profiles. Moreover, the 90- and 120-day samples from MNA and MNAS were 72% similar to all of the remaining samples.

In Panelas soil, MNA and MNAS treatments did not vary much from each other during the entire 120-day experiment (Figure 2(b)). In BAS treatment conditions, the bacterial consortium corresponding bands were initially weak and not detectable at day 0 in DGGE. However, from day 30 to 120, the bacterial consortium bands became intense and were easily detectable (Figure 2(b)). The visual interpretation of these DGGE profiles was confirmed through plotting of a dendrogram of the actinomycetes populations in Panelas

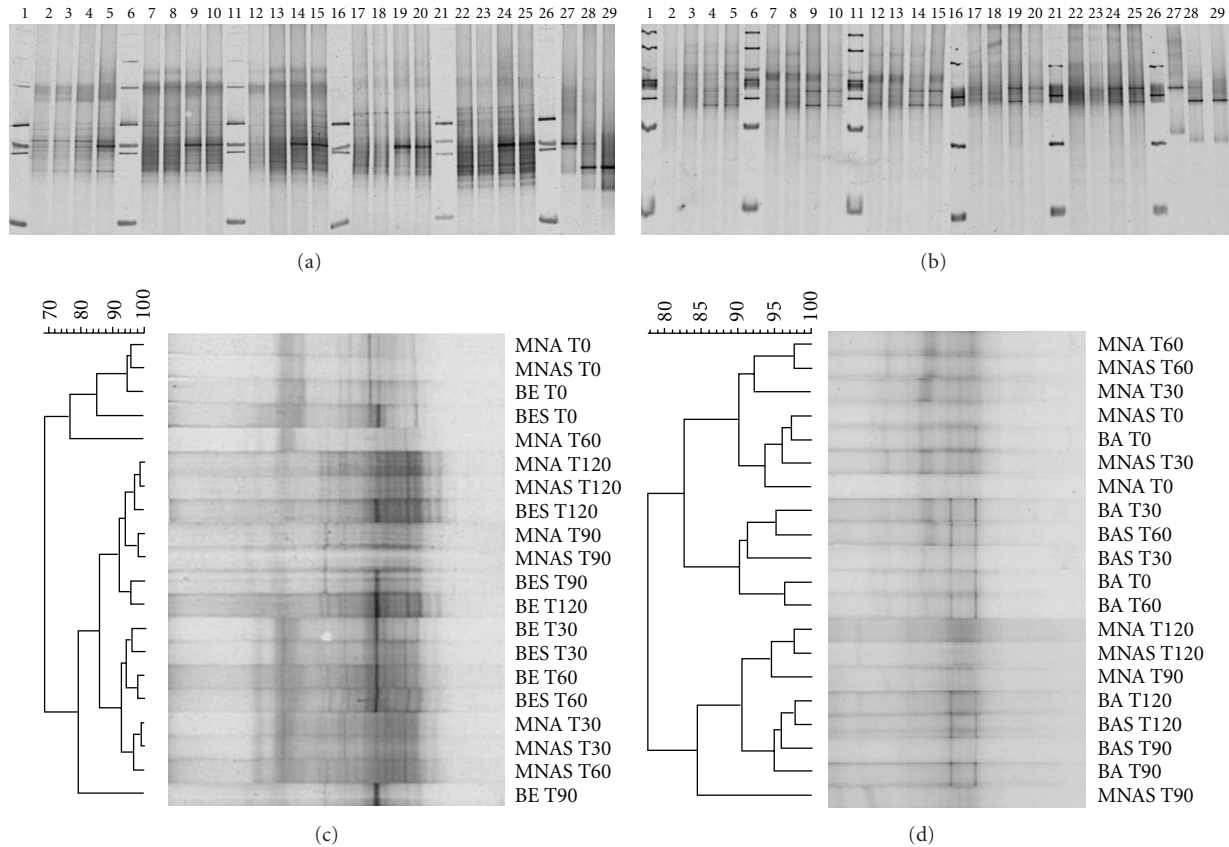


FIGURE 1: Denaturing gradient gel electrophoresis (DGGE) fingerprints of bacterial 16S rRNA gene fragments amplified from Cantagalo (a) and Panelas (b) microcosms submitted to different bioremediation treatments and sampled after 0, 30, 60, 90, and 120 days. Lanes: (1, 6, 11, 16, 21 and 26) 1 Kb ladder, (2) MNA T0, (3) MNAS T0, (4) BE/BA T0, (5) BES/BAS T0, (7) MNA T30, (8) MNAS T30, (9) BE/BA T30, (10) BES/BAS T30, (12) MNA T60, (13) MNAS T60, (14) BE/BA T60, (15) BES/BAS T60, (17) MNA T90, (18) MNAS T90, (19) BE/BA T90, (20) BES/BAS T90, (22) MNA T120, (23) MNAS T120, (24) BE/BA T120, and (25) BES/BAS T120. Strains used for the bacterial consortium: (27) *Gordonia alkanivorans* DLB 3.22, (28) *G. rubripertincta* DTSB 2.5, and (29) *Rhodococcus equi* DLB 2.6. GelCompar II 4.06 software was used to generate the dendrogram based on the DGGE clustering profiles of the soils from Cantagalo (c) and Panelas (d) using Pearson's coefficient and the clustering method of UPGMA.

soil (Figure 2(d)). This dendrogram shows that BAS T0 was 45% similar to the major cluster of all other samples. Furthermore, all samples from bioaugmentation (BA and BAS, except BAS T0) and all samples from monitored natural attenuation (MNA and MNAS) were 65% similar to each other. Further subgrouping formed between the treatments with and without the addition of NaCl (MNA \times MNAS and BA \times BAS) can be seen in Figure 2(d).

3.4. Canonical Correspondence Analysis. CCA analysis of both soils corroborated the results obtained from clustering the DGGE profiles. This analysis showed that the separation of these profiles was determined by the amount of time they were submitted to bioremediation treatment, as seen when these sample profiles were plotted by axis 1 with a high degree of variability, 87.6% in Cantagalo soil (Figure 3(a)) and 52.8% in Panelas soil (Figure 3(b)). In the Cantagalo soil CCA analysis, axis 2 separated DGGE profiles of microcosms with added NaCl from those without NaCl with a 12.4% variability. Moreover, the environmental variables of TPH

and n-alkanes degradation were correlated with the points corresponding to T60 and T120 in the soil from Cantagalo, particularly with those soils which had additional salt (BES T60 and BES T120, corresponding to points 11 and 12). In Panelas soil, axis 2 separated DGGE profiles according to the type of bioremediation treatment used. Points representing MNA (points 2 and 3) and MNAS (points 5 and 6) were separated from points representing BA (points 8 and 9) and BAS (points 11 and 12) with 47.2% variability. The environmental variables of TPH and n-alkanes degradation were correlated to points of 60 and 120 days (with the exception of points 2 and 5 representing MNAS T60 and MNA T60), similar to what was seen with Cantagalo soil.

CCA analysis was also used to correlate the DGGE profiles of all bacterial communities from T120 related to Cantagalo and Panelas soils with the physical and chemical properties of each soil (Figure 4). After 120 days of bioremediation, the points representing the Cantagalo soil profiles (points 1, 2, 3, and 4) were separated from points representing Panelas soil profiles (points 5, 6, 7,

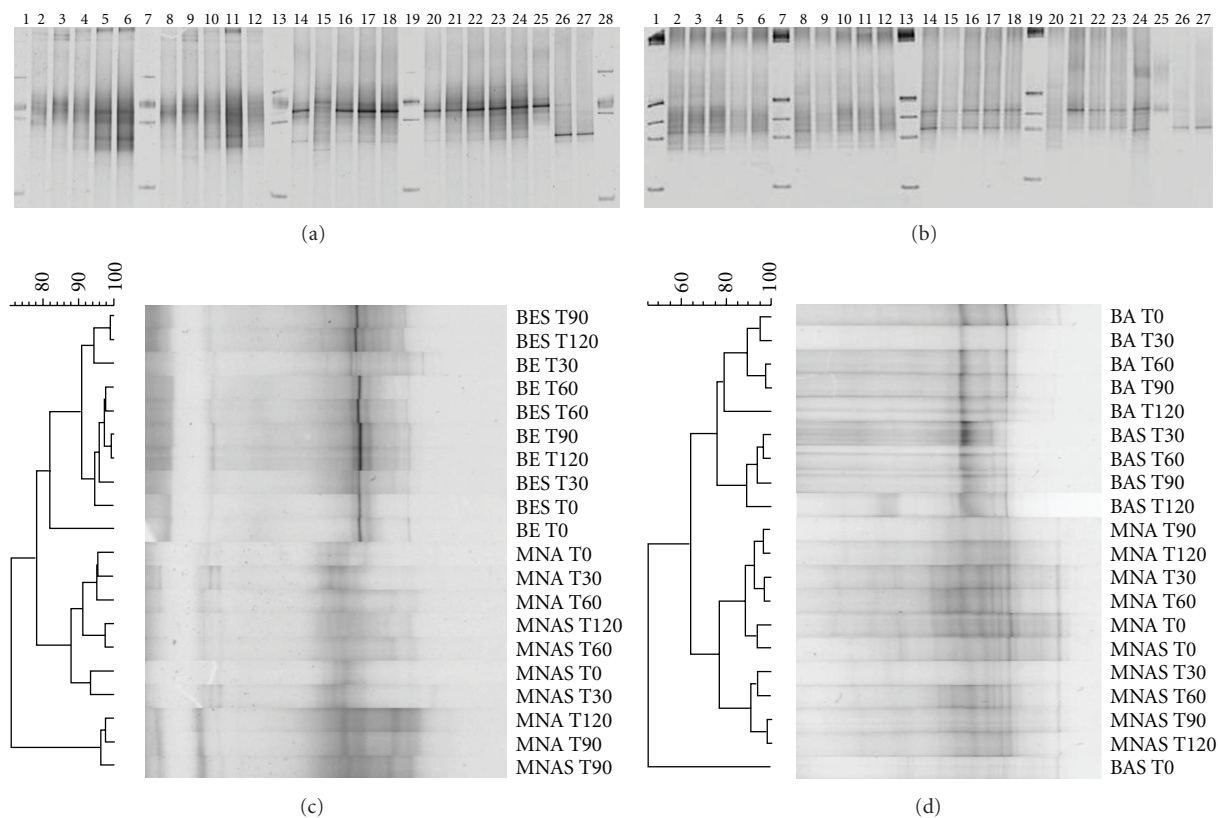


FIGURE 2: Denaturing gradient gel electrophoresis (DGGE) fingerprints of actinomycetes populations from Cantagalo (a) and Panelas (b) microcosms submitted to different bioremediation treatments and sampled after 0, 30, 60, 90, and 120 days. Lanes: (1, 7, 13, 19 and 28) 1 Kb ladder, (2) MNA T0, (3) MNA T30, (4) MNA T60, (5) MNA T90, (6) MNA T120, (8) MNAS T0, (9) MNAS T30, (10) MNAS T60, (11) MNAS T90, (12) MNAS T120, (14) BE/BA T0, (15) BE/BA T30, (16) BE/BA T60, (17) BE/BA T90, (18) BE/BA T120, (20) BES/BAS T0, (21) BES/BAS T30, (22) BES/BAS T60, (23) BES/BAS T90, and (24) BES/BAS T120. Strains used for the bacterial consortium: (25) *Gordonia alkanivorans* DLB 3.22, (26) *G. rubripertincta* DTSB 2.5, and (27) *Rhodococcus equi* DLB 2.6. Dendrogram based on the DGGE clustering profiles of the soils from Cantagalo (c) and Panelas (d) using Pearson's coefficient and the clustering method of UPGMA.

and 8), independent of the bioremediation treatment used. This separation pattern was influenced by environmental variables such as the physical and chemical composition of soils (represented by vectors). The separation of MNAS and BA treatments of Panelas soil (points 6 and 7) from the other treatments was due to a strong correlation with TPH and n-alkanes degradation variables.

4. Discussion

Bioaugmentation and bioenrichment are successful technologies for bioremediation in sites that lack significant microbial populations capable of degrading petroleum and its derivatives [27–29]. However, choosing efficient biodegraders for inoculation is not an easy task. In this study, three actinomycetes strains were chosen to be used for either bioaugmentation or bioenrichment of two different Brazilian soils that were artificially contaminated with crude oil and supplemented or not with NaCl. Two of the strains belonged to the genus *Gordonia* (*G. rubripertincta* and *G. alkanivorans*) and one to *Rhodococcus* (*R. equi*). Our choice was reinforced by data demonstrating that actinomycetes can

degrade n-alkanes and show great potential for bioremediation [10]. Specifically, *Gordonia* have the ability to degrade or modify aliphatic hydrocarbons [30], and *Rhodococcus* can degrade petroleum hydrocarbons and their derivatives [31, 32]. Moreover, actinomycetes are known to grow even under restrictive conditions and can persist in unfavorable environments for long periods of time [33].

During the bioremediation experiments, n-alkane and TPH degradation rates were determined under all conditions. Naturally occurring microbial communities from the Panelas soil sample were more effective in n-alkanes and TPH degradation than those found in the Cantagalo soil sample. Therefore, bioaugmentation using indigenous halotolerant bacterial strains may be a good strategy to treat oil-contaminated Panelas soil because indigenous microorganisms usually adapt more easily during reintroduction to their native soils [7]. Similarly, Zhuang et al. [34] demonstrated that bioremediation of saline soils and wastewaters were possible with the addition of halotolerant microorganisms.

We analyzed the bacterial community structure over a 120-day period of bioremediation by 16S rRNA gene-based PCR-DGGE and found that the length of treatment

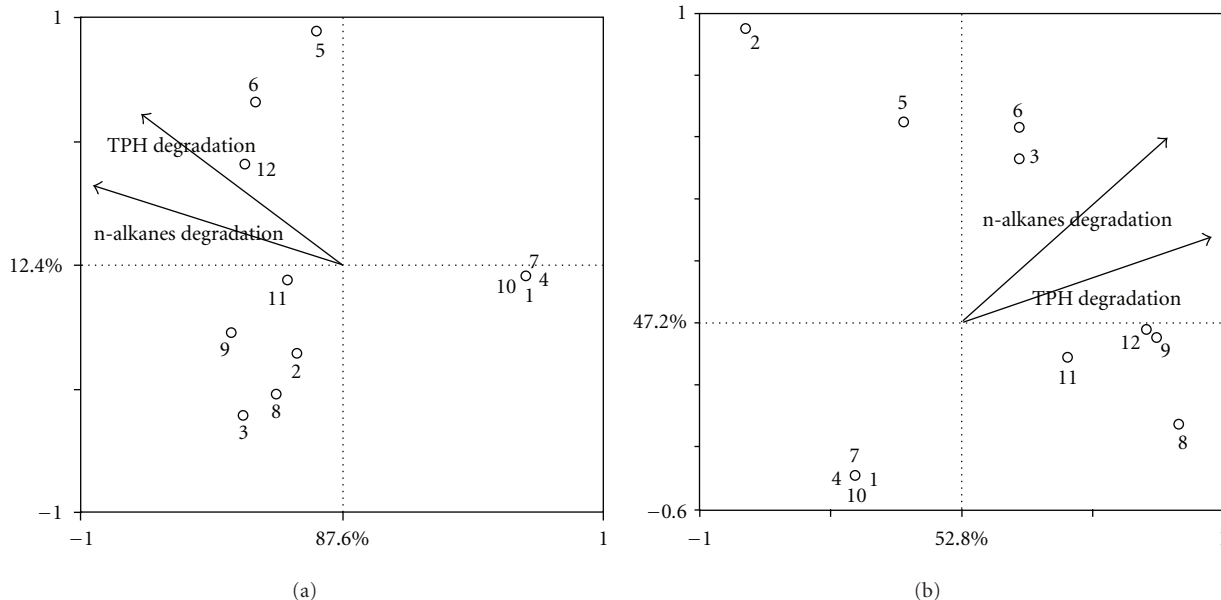


FIGURE 3: Ordination biplot displaying the first and second axis of a canonical correspondence analysis (CCA) using DGGE profiles (biological variables, open circles) and environmental variables (arrows) from Cantagalo (a) and Panelas (b) soil microcosms during bioremediation over time. (1) MNA T0, (2) MNA T60, (3) MNA T120, (4) MNAS T0, (5) MNAS T60, (6) MNAS T120, (7) BE/BA T0, (8) BE/BA T60, (9) BE/BA T120, (10) BES/BAS T0, (11) BES/BAS T60, and (12) BES/BAS T120.

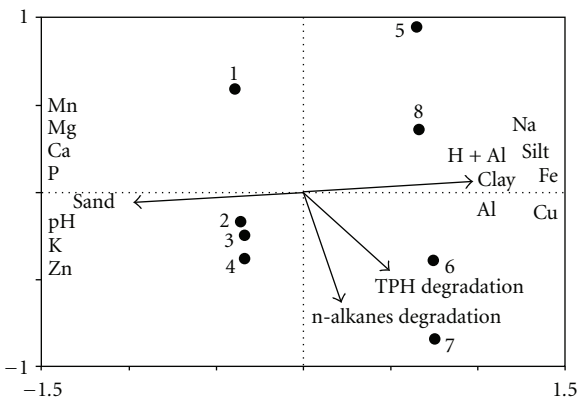


FIGURE 4: Ordination biplot displaying the first and second axis of a canonical correspondence analysis (CCA) using DGGE profiles (biological variables, open circles) and environmental variables (arrows) from Cantagalo and Panelas soils microcosms after 120 days of the bioremediation experiment. (1) MNA/Cantagalo, (2) MNAS/Cantagalo, (3) BE/Cantagalo, (4) BES/Cantagalo, (5) MNA/Panelas, (6) MNAS/Panelas, (7) BA/Panelas, and (8) BAS/Panelas.

was the main factor influencing the bacterial communities found in both soils we tested. This is consistent with previous work by Ciric et al. [16], which observed changes within the bacterial community during remediation in diesel-contaminated groundwater. Likewise, Piskonen et al. [35] observed the selection of specific bacterial populations during the biodegradation process in soil contaminated with polyaromatic hydrocarbons.

Through our monitoring of actinomycetes in this study, we found that the type of bioremediation treatment used was the main factor influencing the actinobacterial population in both soils. In Panelas soil, the addition of NaCl also resulted in changes within the actinomycetes population. This data is in line with previous work by De Azeredo et al. [36], which demonstrated that a community of mycolic-acid containing actinomycetes was affected by an increase in salinity during the treatment of industrial saline waste water.

Differences in the composition of both the soils used and the bioremediation treatment chosen contributed to the divergent results observed between Cantagalo and Panelas soil bacterial communities. CCA analysis clearly demonstrated these differences when DGGE profiles of total bacterial community of Cantagalo soil were clustered separately from the profiles of Panelas soil, likely influenced by physical and chemical compositions of each soil. Recent work by Hamamura et al. [10] suggests that the type and the concentration of nutrients in soil, as well as its physical composition and environmental parameters, influence the composition of microbial community and contribute to the variation observed in petroleum degrading populations. Thus, we believe that these soil characteristics may explain the difference in hydrocarbon mineralization rates during microbial degradation in different soils [3].

In summary, bioenrichment and bioaugmentation treatments showed slightly better results than monitored natural attenuation treatments in remediating Cantagalo and Panelas soils. Our added bacterial consortium persisted throughout the 120-day experiment and did not seem to disturb the indigenous soil microbiota, indicating these bacteria work well for bioremediation. However, the question remains of

whether the addition of the bacterial consortium is worthwhile due to the cost of bioenrichment or bioaugmentation. A recent study by Baek et al. [17] tested five different treatments (monitored natural attenuation, biostimulation, biosurfactant addition, bioaugmentation, and a combination of the later three treatments) for the bioremediation of a soil contaminated with crude oil. They found that the TPH concentration was similar in all treatments. Thus, monitored natural attenuation was considered the method of choice because it did not result in changes in the structure of the bacterial community. Other studies have also demonstrated that monitoring the microbial community and contaminant degradation in a polluted environment might be an efficient strategy for bioremediation [37, 38]. Indeed, Couto et al. [2] concluded that monitored natural attenuation was the most efficient and the cost effective treatment (among MNA, biostimulation, and bioaugmentation) for decontamination of refinery soil contaminated with crude oil.

In terms of environmental relevance to the biomes studied, the use of bioenrichment and bioaugmentation treatments yielded slightly better results in remediating Cantagalo and Panelas soils. However, monitored natural attenuation was considered the most cost-effective approach for bioremediation of the tropical soils when an oil spill occurs in these areas.

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