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Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms

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Abstract

The aim of this study was to analyse the effect of oil contamination and biostimulation (soil pH raise, and nitrogen, phosphate and sulphur addition) on the diversity of a bacterial community of an acidic Cambisol under Atlantic Forest. The experiment was based on the enumeration of bacterial populations and hydrocarbon degraders in microcosms through the use of conventional plating techniques and molecular fingerprinting of samples directly from the environment. PCR followed by denaturing gradient gel electrophoresis (DGGE) was used to generate microbial community fingerprints employing 16S rRNA gene as molecular marker. Biostimulation led to increases of soil pH (to 7.0) and of the levels of phosphorus and K, Ca, and Mg. Oil contamination caused an increase in soil organic carbon (170–190% higher than control soil). Total bacterial counts were stable throughout the experiment, while MPN counts of hydrocarbon degraders showed an increase in the biostimulated and oil-contaminated soil samples. Molecular fingerprinting performed with 16S rRNA gene PCR and DGGE analysis revealed stable patterns along the 360 days of experiment, showing little change in oil-contaminated microcosms after 90 days. The DGGE patterns of the biostimulated samples showed severe changes due to decreases in the number of bands as compared to the control samples as from 15 days after addition of nutrients to the soil. Results obtained in the present study indicate that the addition of inorganic compounds to soil in conjunction with oil contamination has a greater impact on the bacterial community than oil contamination only.

Keywords: Atlantic forest; Bacterial diversity; Biostimulation; Cambisol; Crude oil; DGGE; 16S rRNA gene; Soil microcosms

1. Introduction

Bioremediation of oil- or petroleum-contaminated soil is an interdisciplinary technology involving microbiology, engineering, ecology, geology, and chemistry, and is based on the ability of soil microorganisms to degrade oil compounds [1,2]. Microbiological decontamination of oil and derivatives in polluted environments is claimed to represent an efficient, economic and versatile alternative to physicochemical treatment [1,3]. In situ treatment is one of the most attractive advantages of this technology, and several reports have already demonstrated the use of bioremediation in the treatment of petroleum-contaminated sites [2,4]. Adding nutrients to a contaminated site to stimulate the growth of the indigenous soil microbial community is known as

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biostimulation. Biostimulation has been studied in contaminated marine shorelines and studies have indicated that biostimulation can efficiently promote oil biodegradation [5]. However, current knowledge of the impact of this process on the ecosystem is limited. Therefore, a detailed characterization of the contaminated site in relation to the pollutant, environmental conditions and the microbial community is still necessary for in situ bioremediation and/or biostimulation to be considered reliable and safe cleanup technologies [1].

Several studies have focused on the presence of degradative capacities of bacterial population in polluted environments [6–8]. One important objective was the determination of physiology and function of such diverse catabolic populations in the bioremediation process. However, these studies were hampered because great part of environmental bacteria cannot be cultured yet by conventional laboratory techniques [9]. Molecular ecological information is useful for the analysis of the diversity of pollutant-degrading microorganisms, and for the development of strategies to improve bioremediation [2,10]. The use of microorganisms to clean up a polluted environment has also raised questions about the impact these treatments may have on the ecosystem. Microbial communities can adapt to oil compounds after prolonged exposure by changing their composition. Hence, assessment of the structure of microbial communities is an important step to determine possible indicators of petroleum hydrocarbon degradation. In this aspect, some studies investigated the changes in the indigenous bacterial community structure for addressing the impact of oil contamination on the microbiology of ecosystems [11,12].

Molecular markers, such as 16S rRNA gene, have been extensively applied to detect and identify microbial diversity in environmental samples [9]. Polymerase chain reaction, in combination with methods that generate fingerprints such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and single-strand conformational polymorphism (SSCP), has been commonly used in analysis of bacterial communities [13]. DGGE has gained preference in many research groups to profile microbial communities in environmental samples [14–16]. It is an effective method that enables analysis of many samples simultaneously, and can show changes in bacterial community structure that are not detected by methods based on bacterial culture alone. On the other hand, major bands in DGGE gels may not represent major populations in the original environment due to bias in DNA extraction and PCR [12,17]. Therefore, it is important to use more than one analytical method to provide valuable information about the changes in the indigenous bacterial community structure for assessing the impact of biodegradation.

In this study, the influence of oil contamination and biostimulation on the diversity of indigenous bacterial community in a tropical soil with no previous history of oil pollution was determined by culture-dependent and culture-independent methods. For that purpose, soil microcosms were set up for a long-term experiment (360 days) and four different treatments (bulk soil, oil-contaminated soil, biostimulated bulk soil and oil-contaminated biostimulated soil) were compared using conventional microbial counts and PCR-DGGE employing 16S rRNA gene primers.

2. Material and methods

2.1. Site and soil characteristics

The soil was collected from a site with no history of previous oil contamination, located at the Biological Reserve of Poço das Antas (22°28′S; 42°12′W), Rio de Janeiro State, Brazil. This region is of great importance, as it is one of the last remains of the Atlantic forest, an endangered tropical ecosystem. The soil was a sandy loam Cambisol (595 g sand kg^{-1}; 246 g silt kg^{-1}; 182 g clay kg^{-1}) with low pH (5.5).

2.2. Soil biostimulation and oil contamination – experimental design

Bulk soil (160 kg) was collected from the upper soil layer (0–20 cm) at Poço das Antas and divided in two containers of 80 kg. One container was biostimulated with nutrients to reach a C:N:P ratio of 100:10:1. Nitrogen and sulphur as $(NH_4)_2SO_4$ (11.3 g kg^{-1} soil), and phosphorus and potassium as KH_2PO_4 (1.05 g kg^{-1} soil) were added to the soil and mixed thoroughly to distribute the salts through the soil particles. Soil pH was determined by the saturated paste method [18] and the pH was adjusted to 7.0 (±0.2) with $CaCO_3$ (1.8 g kg^{-1}). The other container was maintained untreated. Both soil portions were subdivided into two lots of 40 kg. Thus, half of the samples of the biostimulated and untreated soils (40 kg each) were treated with 5% (w/v) of Arabian light oil. The treated samples were thoroughly homogenized by manual mixing to distribute the oil through the soil particles and to enhance aeration whilst the other samples were mixed to distribute the nutrients as well as to aerate. The four portions of 40 kg were used to fill microcosms (plastic pots of 18 cm diameter) with 1.5 kg of soil each. Microcosms were kept at room temperature, and pots regularly watered to substitute the evaporated water. The microcosms were sampled at time zero, and 15, 30, 90, 180, 270 and 360 days after the start of the experiment. Triplicate microcosms were used per treatment for each sampling as follows: (1) untreated soil; (2) oil-contaminated soil; (3)

biostimulated soil; (4) biostimulated and oil-contaminated soil. Thus, in total 84 microcosms were prepared at the beginning of the experiment.

2.3. Enumeration of total bacterial community and oil degraders in soil

From a composite sample of each treatment, consisting of 500 g of soil from three different microcosms mixed thoroughly to produce 1.5 kg samples, 10 g of soil were mixed with 90 ml of 0.85% NaCl and the resulting suspensions were shaken for 20 min at 120 rpm. Serial 10-fold dilutions of these suspensions were plated on Plate Count Agar (Merck, Darmstadt, Germany) and incubated at 30 °C for 48 h to enumerate total indigenous bacterial CFUs at zero, 15, 30, 90, 180, 270 and 360 days after the start of the experiment. The most probable number (MPN) technique was also used to count total bacteria in the same medium and the hydrocarbon consumers in Bushnell–Haas broth (Difco Lab., MI, USA), according to Brown and Braddock [19]. Counts of total bacteria and hydrocarbon consumers were plotted in curves along the 360 days of experiment and these data were compared non-parametrically with unpaired two groups. For statistical analyses, the Mann–Whitney test was applied using the StatView 4.01 package. Furthermore, enumerations of total bacteria and those grown in Bushnell–Haas broth were also analyzed according to the time course. The same statistical test described above was applied to compare data obtained in the beginning (1, 15 and 30 days) versus data obtained in the end (180, 270 and 360 days) of the experiment.

2.4. Soil DNA extraction

At the beginning of the experiment (time zero and 15 days), total microbial community DNA from microcosms corresponding to the four treatments – bulk soil, oil-contaminated soil, biostimulated soil and biostimulated and oil-contaminated soil was obtained and analyzed separately. Direct extractions of total microbial DNA from soil samples were performed using the FastPrep System and the FastDNA SPIN Kit for soil (BIO 101, CA, USA). Soil DNA was analyzed by electrophoresis in 1% (w/v) agarose gels in Tris–Borate–EDTA buffer as well as in a spectrophotometer at 260 nm absorbance (Beckman DU-600) to check its amount, purity and molecular size. The final DNAs obtained from soil samples (corresponding to the four treatments) were not coloured, of large molecular size (>10 kb) and could be amplified by PCR using 16S rDNA gene based primers. DNA extracts were amplified by PCR using 1 μl of the extract (5–20 μg of DNA g soil^{-1}) per 50 μl of reaction. As banding patterns obtained in DGGE were very reproducible among triplicate samplings, a composite sample of each treatment, consisting of 500 g of soil from three different microcosms mixed thoroughly to produce 1.5 kg samples, was then used for further molecular analysis as described here.

2.5. PCR amplification

The 16S rRNA gene based primers used in the PCR reactions were 968F with 1401R [20]. A GC clamp [7] was added to the forward primer (F). All PCR amplifications were performed using a PCR Sprint or a ThermoHybaid PCR cycler (Molecular Biology Instrumentation, MA, USA). PCR mixtures were prepared with 5 μl of *Taq* buffer 10×, 2.5 mM of $MgCl_2$, 200 μmol of each deoxynucleoside triphosphate, 20 pmol each primer, 5 μg of bovine serum albumin, 1% of formamide and 2.5U *Taq* polymerase (Roche Molecular Biochemical, Mannheim, Germany) and sterile filtered milliQ water to a final volume of 50 μl. The PCR program was as follows: denaturing step of 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. The amplification products were routinely analyzed by electrophoresis in 1.4% agarose gels in 1× Tris–Borate–EDTA buffer [21].

2.6. DGGE analysis

PCR products were run on a 6% polyacrylamide gel in a 45–65% denaturing gradient of urea and formamide for 16S rDNA analysis. DGGE was carried out using a BioRad DCode Universal Mutation Detection System at 100 V at 60 °C for 15 h, in 1.0× TAE buffer (20 mM Tris, 10 mM acetate, 1 mM EDTA pH 7.4). After electrophoresis, gels were stained for 30 min with SYBR gold nucleic acid gel stain (1:10000 dilution; Molecular Probes, USA). Stained gels were photographed under UV light with the Gel Doc 2000 system (Bio-Rad Laboratories, CA, USA). The digitized images of DGGE gels were analyzed by Image Quant (ver. 5.2) to generate a densitometric profile. Bands were considered when the peak height relative to total peak height exceeded 1% according to Iwamoto et al. [22]. The calculation of similarities was based on the Pearson (product–moment) correlation coefficient and resulted in a distance matrix. The Pearson correlation is an objective coefficient that does not suffer from typical peak/shoulder mismatches as often found when band-matching coefficients are applied and is recommended for use with data originated from DGGE profiles [23]. The clustering algorithm of Ward was used to calculate the dendrograms of each DGGE gel using the software package Statistica (ver. 5.1, StatSoft).

For sequencing of selected DGGE fragments, bands were extracted from the gels by the method described by Duarte et al. [15]. The excised DNA was amplified

according to the 16S rRNA-based PCR reactions described above, with reduction of the PCR cycle number to 30 and elimination of formamide in all reactions. After that, DGGE was run again to check the purity of the band. PCR products of the extracted bands were purified by the QIAquick PCR purification kit (Quiagen Inc., CA, USA) and, then, sequenced using an ABI PRISM model 373 automatic sequencer with a BigDye Terminator Cycle sequencing kit (PE Biosystems, CA, USA). Sequence identification was performed using the BLAST-N facility of the National Center for Biotechnology Information. The sequences from excised DGGE bands have been deposited in the GenBank database under Accession Number AY500280 and AY500281.

3. Results

3.1. Changes in soil chemical properties

Table 1 summarizes the results obtained from the 360 days of the experiment. The pH values of the control soils decreased slightly during the study, but this did not result in a decrease in phosphorus level. Oil affected the content of organic carbon in soil. At time zero, the amount of organic carbon of the oil-contaminated soil was 170% higher than that in the control soil and on day 360 the organic carbon content in this treatment was 190% higher. Biostimulation caused an increase of soil pH to 7.0–7.3. The content of phosphorus and basic cations (K, Ca, and Mg) also increased by biostimulation and remained higher than those of control soil throughout the study. Similar changes were observed when biostimulation was combined with the addition of oil (D treatment), except for the organic carbon level, which was higher than in the A and C treatments.

3.2. Enumeration of bacterial cells

Viable cell counts in oil-contaminated and/or biostimulated soil samples are presented in Fig. 1. Data obtained from both plate count agar and MPN were similar (Fig. 1(a) and (b)). With one exception (see below), the total bacterial counts did not show any significant difference ($P > 0.0495$) among the treatments and throughout the experiment, and were generally between 10^6 and 10^8 CFU g^{-1} of dry soil. Bacterial populations were more sensitive in oil-contaminated soil than in other soils as shown by plate assays as, after 90 days of oil contamination, the CFU counts had declined about two orders of magnitude (Fig. 1(a)). However, this difference in cell numbers was lower during the remaining samplings. MPN assessments did not show any significant differences among the treatments and over the 360 days of the experiment (Fig. 1(b)). Counts of hydrocarbon consumers were more variable over time

Table 1
Physical and chemical characteristics[a] of soil in microcosms under different treatments

Soil treatments[b]	pH (water)			Organic carbon (g dm^{-3})			P (g dm^{-3})			K^+ (g dm^{-3})			Ca^{2+} (meq/100 g soil)			Mg^{2+} (meq/100 g soil)		
	0	180 days	360 days	0	180 days	360 days	0	180 days	360 days	0	180 days	360 days	0	180 days	360 days	0	180 days	360 days
A	5.5	4.9	4.9	10	10	11	8	7	9	117	106	109	0.8	0.9	0.8	0.5	0.6	0.6
B	5.9	5.2	5.1	27	25	29	9	6	7	55	78	94	0.7	0.9	0.7	0.2	0.5	0.6
C	7.3	7.0	7.0	10	11	10	200	119	114	359	403	337	18.0	18.0	20.0	3.5	2.8	2.4
D	7.3	7.0	7.0	24	22	23	170	106	100	297	370	344	14.0	17.0	19.0	2.9	2.6	2.9

[a] All soil analyses were carried out using methods suggested by Embrapa [31].
[b] A: Control soil; B: Soil contaminated with oil; C: Soil with nutrients (biostimulated soil); D: oil contaminated and biostimulated soil.

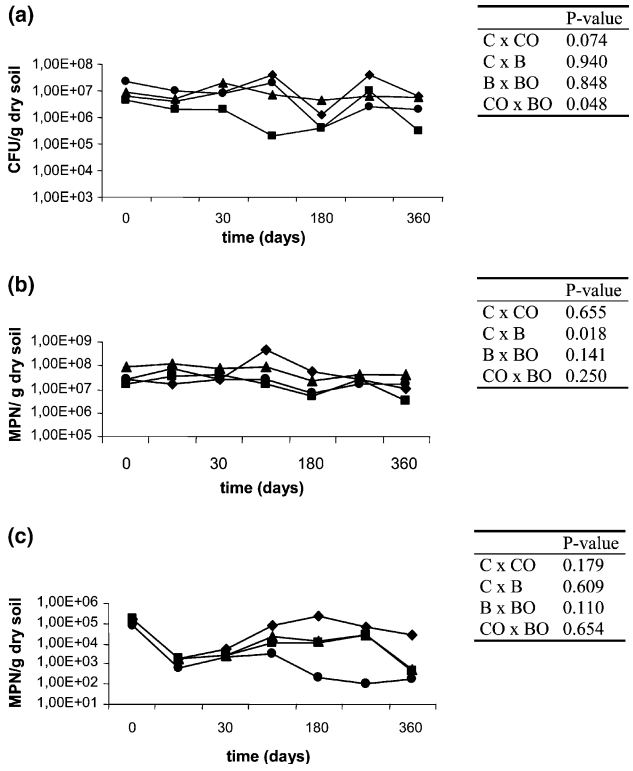

Fig. 1. Enumeration of bacterial cells in microcosms during 360 days of experiment. (a) number of heterotrophic bacteria grown in plate count agar; (b) MPNs of heterotrophic bacteria – plate count medium; (c) most probable number of oil degrading bacteria – Bushnell–Haas medium. *P*-values were obtained using the Mann–Whitney test. ● control soil (C), ■ oil-contaminated soil (CO), ▲ biostimulated soil (B), ◆ oil-contaminated biostimulated soil (BO).

and an increase of this population was clearly seen in oil-contaminated and biostimulated soil, from 90 days up to the end of the experiment, when compared to other treatments (Fig. 1(c)). This observation was supported statistically ($P < 0.0495$). In addition, curves representing different treatments and the time course were also compared using the Mann–Whitney test. The obtained *P*-values are shown in Fig. 1. In general, the differences between treatments were not statistically significant, when all points in the curves were considered as two unpaired groups. The only two exceptions were the counts in oil-contaminated (CO) × oil-contaminated biostimulated (BO) soil samples (Fig. 1(a)) and the MPN counts in bulk (C) × biostimulated (B) soil (Fig. 1(b)). Point deviations in bacterial counts in both cases could be responsible for the significant *P*-values obtained.

3.3. Total bacterial DNA extraction and DGGE analysis

Total community DNA was extracted from all microcosms using the FastPrep system. Suitable yields of high-molecular weight DNA (usually 5–20 μg g^{-1} soil) were achieved for all soil samples. PCR products were obtained from all 84 soil DNA samples, corresponding to the four treatments at 0, 15, 30, 90, 180, 270 and 360 days. Hence, the oil used in soil microcosms did not affect the quality of DNA extracted from soil. Fingerprints of the most dominant populations were obtained after separation of PCR products in DGGE. Banding patterns obtained in DGGE from samples at time zero and 15 days were very reproducible among the triplicates (data not shown); therefore we decided to analyze composite samplings using three microcosms per treatment. This considerably reduced the DNA samplings, and extractions from 84 to 28.

DGGE fingerprinting was thus used to compare the structural diversity of bacterial communities in soil microcosms containing: (i) bulk soil versus oil-contaminated soil, (ii) biostimulated soil versus biostimulated and oil contaminated soil and (iii) oil contaminated soil versus biostimulated and oil contaminated soil.

3.4. Bulk soil versus oil-contaminated soil

A comparison of 16S rRNA gene-based DGGE fingerprints of bulk soil and oil-contaminated soil showed relative stability of the bacterial community profiles between treatments and over time (0–360 days). Dominant bands in DGGE appeared in both soils, with or without oil contamination. Furthermore, both samplings typically showed many faint bands (Fig. 2(a)). In general, oil introduced into soil did not affect the main bacterial community structure, however, two bands became more intense after 90 days of incubation of oil-contaminated soil microcosms. These might be from increased growth of specific groups of bacteria existing in bulk soil and able to utilize the added oil as a carbon source. DGGE patterns were further clustered according to the Ward algorithm and the Pearson coefficient. The obtained dendrogram is shown in Fig. 2(a). Two main clusters were observed, one made up of oil-contaminated samples obtained after 180, 270 and 360 days and the other of the remaining samples (control and oil-contaminated soil samples from zero to 90 days and control samples from 180 to 360 days). This second cluster was further split into two groups, one formed by samples obtained in the beginning of the experiment (control time 0, 15 and 30 days and oil-contaminated soil 0 and 15 days) and the other of later samples of the control. The dendrogram is in agreement with the observation that the effect of the introduction of oil in bulk soil on bacterial community structure is slow in development and can only be detected after 90–180 days of experiment.

3.5. Biostimulated soil versus biostimulated and oil-contaminated soil

Fig. 2(b) shows the 16S rRNA gene-based DGGE fingerprints comparing the bacterial communities

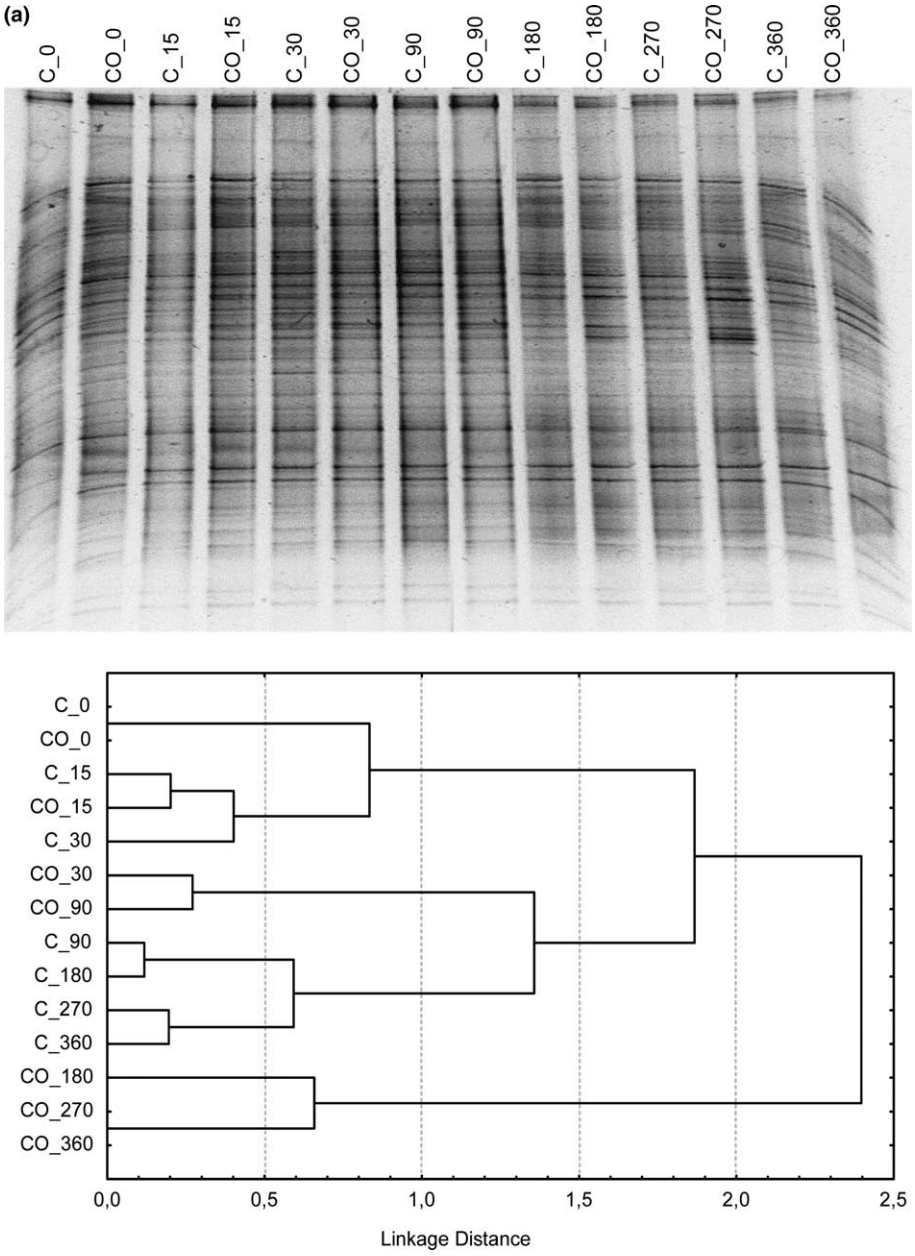

Fig. 2. DGGE patterns and respective dendrograms comparing bacterial communities in samples of (a) non-treated soil (lanes C_0, C_15, C_30, C_90, C_180, C_270 and C_360) and oil-contaminated soil (lanes CO_0, CO_15, CO_30, CO_90, CO_180, CO_270 and CO_360); (b) biostimulated soil (lanes B_0, B_15, B_30, B_90, B_180 and B_270) and biostimulated oil-contaminated soil (lanes BO_0, BO_15, BO_30, BO_90, BO_180 and BO_270) and (c) oil-contaminated soil (lanes CO_0, CO_15, CO_30, CO_90, CO_180, CO_270 and CO_360) and biostimulated oil-contaminated soil (lanes BO_0, BO_15, BO_30, BO_90, BO_180, BO_270 and BO_360). C: control soil, CO: oil-contaminated soil, B: biostimulated soil and BO: oil-contaminated biostimulated soil. Numbers after the letters correspond to days of sampling. Bands *1 and *2 were excised, re-amplified and sequenced.

between microcosms containing biostimulated soil and those containing biostimulated and oil-contaminated soil. In the beginning of the experiment, profiles of both systems were very similar. However, the stability of the population was disturbed after 15 days of treatment. Two strong bands (Fig. 2(b), lanes BO_15 and BO_30) could be observed in samples with oil in the microcosms sampled after 15 and 30 days, indicating a possible selection of both bacterial types caused by the addition of oil to the biostimulated soil microcosm. After 90 days, a drastic shift could be observed in the bacterial population in oil-contaminated soil microcosms, indicating that the oil contamination had a great influence in biostimulated soil. Probably, the bacterial population selected by the introduction of nutrients was more responsive to oil contamination than the population

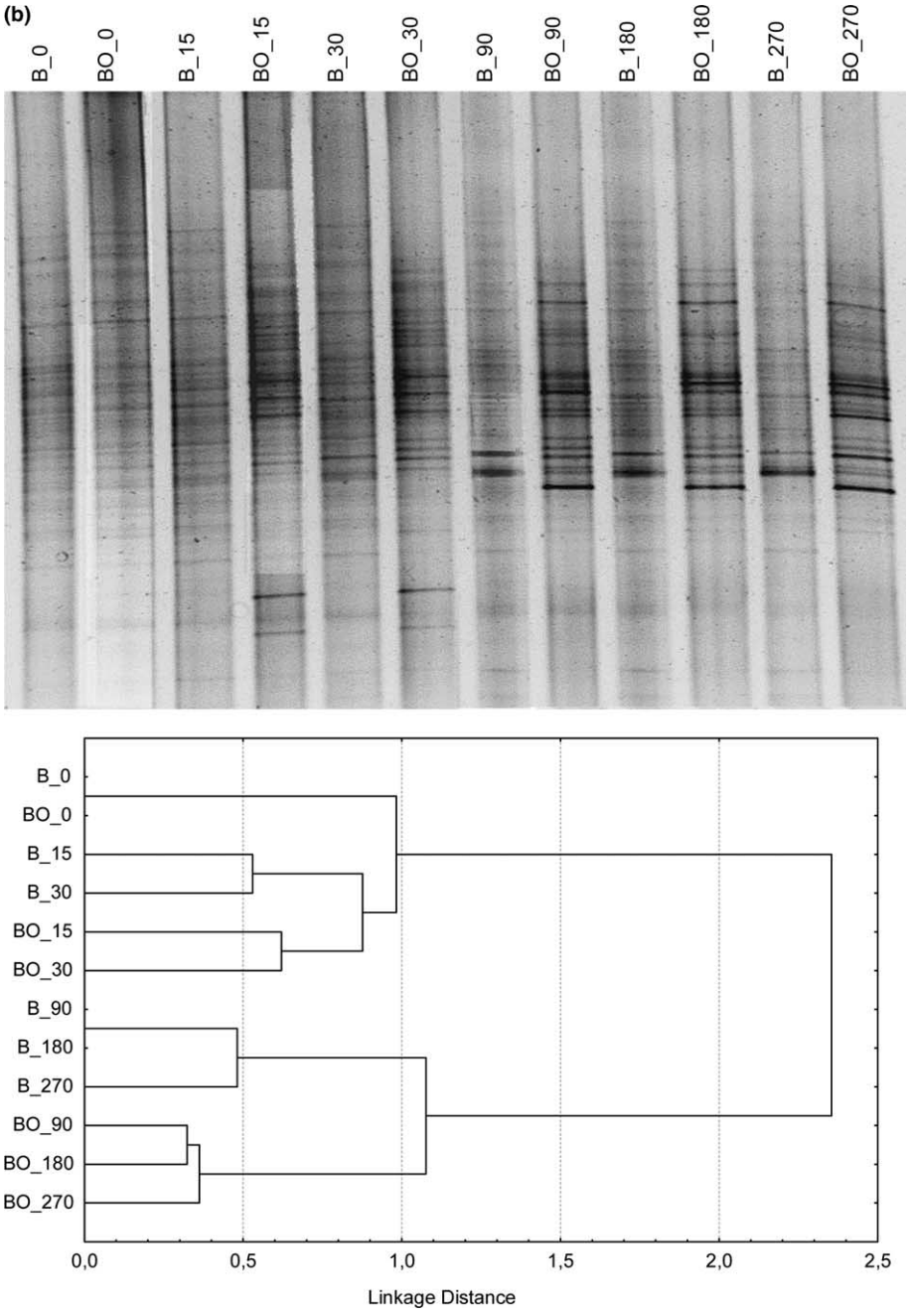

Fig. 2. (*continued*)

previously established in the soil. This could explain the stability of DGGE profiles previously demonstrated in bulk soil contaminated with oil (Fig. 2(a)). After 90 days of biostimulation and oil contamination, novel bands could be observed in the DGGE gels (Fig. 2(b), lanes BO_90, BO_180 and BO_270). Moreover, after 360 days, the same profile was observed in both treatments (data not shown). When a dendrogram was constructed based on the DGGE pattern shown in Fig. 2(b), two main clusters could be detected. One cluster was formed by samples (with and without oil) up to 30 days, while the second cluster was formed by samples from 90 to 270 days. This cluster division also indicated that oil contamination and time course had a great effect in biostimulated soil, since shifts could be observed in the bacterial populations present in biostimulated and biostimulated oil-contaminated soil microcosms.

3.6. Oil-contaminated soil versus biostimulated oil-contaminated soil

In the oil-contaminated samples, including those biostimulated with nutrients, the DGGE profiles (Fig. 2(c)) confirmed those shown in Figs. 2(a) and (b).

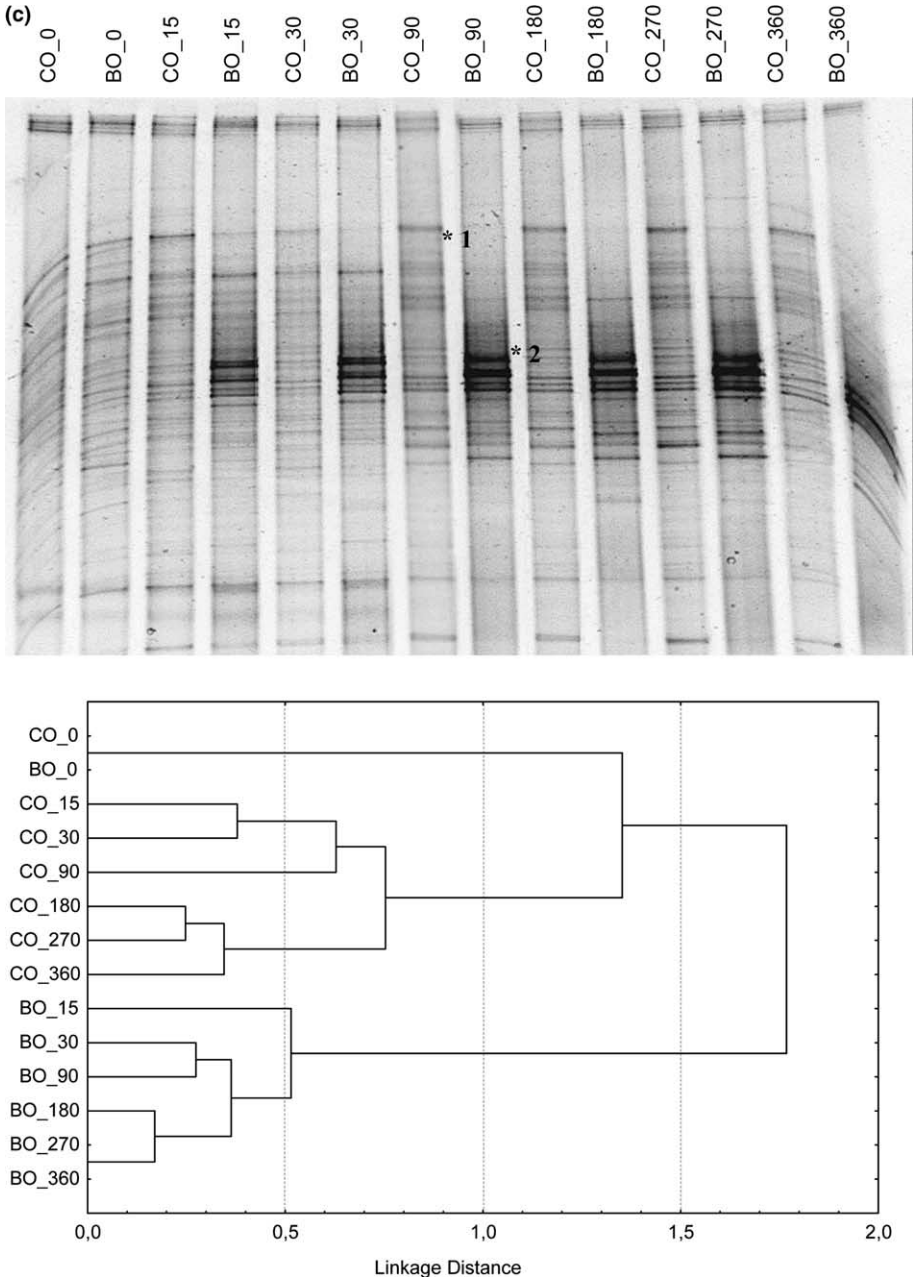

Fig. 2. (*continued*)

Bacterial populations present in soil microcosms, which received only oil, were roughly stable throughout the 360 days of experiment while from the fifteenth day of the experiment onwards, severe changes in DGGE profiles were observed in the oil-contaminated soil, which had also been biostimulated. Changes in band intensities were also detected over time in the biostimulated oil-contaminated soil samples, indicating that changes in the bacterial community structure were predominantly induced by the addition of nutrients rather than oil. The dendrogram that is based on the DGGE profile (Fig. 2(c)) clearly confirms the change in the bacterial community structure by the addition of nutrients. The dendrogram is composed of two main clusters, one consisting of the biostimulated oil-contaminated samples (15–360 days) and the other made up predominantly of soil samples that were treated only with oil.

Two electrophoretic bands (marked in Fig. 2(c)) were excised from the denaturing polyacrylamide gel, re-amplified, purified and sequenced. Band 1 was present in both control and oil-contaminated soils and disappeared after 30 days of biostimulation, while band 2 was observed only in biostimulated oil-contaminated soil after 90 days of experiment. From sequence comparisons

using BLAST-N, the phylogenetic affiliations of the fragment sequences showed that DGGE band 1 was 81% similar to a 16S rRNA gene sequence found in *Bacillus* sp. CPB9 [24] and band 2 was most similar to a 16S rRNA gene sequence of *Planococcus* (86% similarity with *P. southpolaris* and *P. psychrotoleratus*). However, because of the small size of the analyzed sequences, the phylogenetic affiliations and sequence similarity values need to be interpreted with caution.

4. Discussion

In the present study, DGGE fingerprints based on 16S rDNA fragments amplified from bacterial DNA were combined with traditional cultivation techniques to study the dynamics of microbial communities in soil contaminated with oil for over one year. Moreover, the effect of biostimulation through the utilization of inorganic nutrients and management of other parameters, like aeration and pH, was evaluated. The primary objective was to determine whether oil contamination enriches specific bacterial populations as a result of the addition of extra carbon sources. A second objective was to monitor if the addition of nutrients to soil before oil contamination, as part of the biostimulation treatment, exerts a positive effect on the number and diversity of indigenous bacterial population.

Traditional cultivation methods have been previously used to isolate bacteria involved in the degradation of petroleum hydrocarbons, even though only a minority of the microorganisms in soil can be cultivated [9]. This includes organisms that are able to degrade oil in situ [11]. In the present work, populations of hydrocarbon-degrading bacteria showed a decrease in number, in the control or oil-contaminated soil samples. On the other hand, counts of the hydrocarbon degraders showed a large increase (from 10^2 to 10^4 cfu g^{-1} soil) in the biostimulated oil-contaminated soil after 30 days of incubation, reaching the highest counts after 180 days (Fig. 1(c)). Song and Bartha [25] also detected an accentuated increase of the number of oil degrading bacteria four weeks after the addition of oil in soil. However, in their study, the increase of the number of oil-degrading bacteria was not maintained throughout the experiment. The major difference between the approach of Song and Bartha [25] and the one in the present study is that the soil we used does not show any previous contamination with hydrocarbons. It can be assumed that selection for oil-degrading bacteria occurred in soils with previous history of contamination, which explains the quick response of the bacterial community after re-contamination with oil compounds. In contrast, bacterial populations in our soil showed a stable pattern throughout the entire experiment, although the total bacterial cell numbers in the biostimulated microcosms were expected to increase following the addition of nutrients. We believe that either some members of the community increased in number after biostimulation, making the total bacterial numbers remain the same, or the stable CFU counts do not reflect the dramatic changes, which may be occurring in non-cultured viable cell counts. Similar results, i.e. stability over time of bacterial cell counts in soil treated with petroleum, have been reported by Duarte et al. [15].

PCR-DGGE profiles obtained with primers based on 16S rRNA gene usually yield complex patterns that reflect the composition of the most dominant soil microbial populations, including the non-culturable fractions [26]. However, care should be taken with interpretation of PCR-DGGE results, since bias can occur within all molecular steps employed (i.e. soil DNA extraction, PCR amplification and denaturing gels) [14,27]. DGGE patterns obtained in this study with total community DNA from soil under different treatments showed shifts in the composition of dominant bacterial populations related to oil pollution in combination with biostimulation. Molecular profiling data (Fig. 2(a)) did not point to a strong selection of any specific bacterial population throughout 360 days, although a separated phylogenetic cluster made up of oil-contaminated samples of 180, 270 and 360 days was formed. This general observation of stability is in accordance with the numbers of cultivated bacteria present in oil-contaminated samples (Fig. 1(a) and (b)), since no increase in any specific populations, which might replace bacteria sensitive to oil hydrocarbons, was observed. Nevertheless, it is important to consider that the light Arabian crude oil used was applied at a constant concentration of 5% in all microcosms. Recently, Duarte et al. [15] have shown with DGGE analysis that bacterial community of soil with previous history of contamination showed significant changes as compared to less polluted soil, with the selection of specific bacterial populations. Furthermore, we focused on one particular type of soil, considering its specific physical and chemical characteristics when contaminated with crude oil. Juck et al. [7] studied changes in bacterial communities adapted to low temperatures in two different contaminated soils from the north of Canada. Their study revealed that enrichment of oil-degrading bacteria occurred only in one soil, whilst the other one showed a stable bacterial profile as evidenced with DGGE analysis.

In contrast, biostimulation itself had a substantial impact on bacterial diversity in the environment studied (Fig. 2(b) and (c)). Changes in the number of bands after 15 days of biostimulation were observed in both contaminated (oil + biostimulation) and control soils (biostimulation). At 90 days, the banding pattern in the DGGE gels again showed a shift, remaining quite stable until the end of the experiment at 360 days. Those results were corroborated in both dendrograms produced

by clustering algorithm of Ward and the Pearson coefficient. The stability of bacterial community from 90 to 360 days contrasts other reports. Ogino et al. [12] evaluated bioremediation of a marine site contaminated in 1997 by the *Nakhoda* tanker in Japan. The authors reported that the bacterial community in the treated polluted site became similar to the non-treated contaminated site, used as negative control, within 98 days of nutrient addition. However, since their study was undertaken under different conditions than our experiments, a common point between the studies is the strong selection pressure that biostimulation represents. Other studies, such as the recent report of Kasai et al. [28], support the use of biostimulation to promote the degradation of petroleum hydrocarbons. Residual petroleum compounds were degraded through stimulation of microbial community with inorganic nutrients such as nitrogen and phosphorous. Our results lead to the conclusion that bacterial community shifts occurred as a result of biostimulation and that the addition of nutrients possibly has an irreversible effect on the communities. This could be due the activation of bacterial community through nutrient input, favouring changes for adaptation, and enhancing (when present) biodegradation capabilities in the autochthonous degraders previously selected. However, other factors (e.g. the presence and activity of predators, soil texture, etc.) can affect microbial community in a particular environment, as discussed earlier [7]. Therefore, it is advisable to use polyphasic analysis to appreciate the complexity of behaviour of microbial communities during bioremediation in contaminated environments.

In order to understand which populations were selected with biostimulation or were tolerant to the applied oil, two bands from the 16S-DGGE gels were sequenced. As mentioned earlier, because of the small size of those selected sequences, their assignment in a specific genus is not clearly delineated. However, the information is useful as it provides a suggestion that obtained sequences are close to *Bacillus* and *Planococcus*, genera that are both closely related at 16S rRNA gene level. Strains belonging to the genus *Bacillus* have been previously isolated from an oil reservoir of Brazil [29], while a new species of *Planococcus* (*P. alkanoclasticus*) has been recently isolated from intertidal beach sediment and characterized as a hydrocarbon-degrading bacterium [30]. It is, thus, possible that the organism identified as being similar to *Planococcus* sp. can have a role in the biodegradation of light Arabian oil and is stimulated by added nutrients. On the other hand, Juck et al. [7] found that actinomycetes were predominant in petroleum hydrocarbon-contaminated soils from two northern Canadian environments. In future work, to evaluate the ecological behaviour of hydrocarbon-stimulated organisms in soil, one should attempt to isolate the organisms, and assess the physiological characteristics of these, as only the direct study of the whole organisms will enhance our understanding of biostimulation.

Combined application of culture-dependent and dependent methods in this study suggests that biostimulation has a great impact on microbial community structure of oil-polluted soil. Further studies including RNA-based characterizations (e.g. by use of RT-PCR-DGGE), more sequence analyses, screening for catabolic genes and establishing links between function and community structure will provide clues on environmental functioning of bacterial populations and the strains found therein.

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