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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s00253-015-6919-0

Appl Microbiol Biotechnol (2015) 99:10815–10827
DOI 10.1007/s00253-015-6919-0

ENVIRONMENTAL BIOTECHNOLOGY

Evaluation of soil bioremediation techniques in an aged diesel spill at the Antarctic Peninsula

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Received: 24 March 2015 / Revised: 30 July 2015 / Accepted: 5 August 2015 / Published online: 20 August 2015

Abstract Many areas on the Antarctic continent already suffer from the direct and indirect influences of human activities. The main cause of contamination is petroleum hydrocarbons because this compound is used as a source of energy at the many research stations around the continent. Thus, the current study aims to evaluate treatments for bioremediation (biostimulation, bioaugmentation, and bioaugmentation + biostimulation) using soils from around the Brazilian Antarctic Station "Comandante Ferraz" (EACF), King George Island, Antarctic Peninsula. The experiment lasted for 45 days, and at the end of this period, chemical and molecular analyses were performed. Those analyses included the quantification of carbon and nitrogen, denaturing gradient gel electrophoresis (DGGE) analysis (with gradient denaturation), real-time PCR, and quantification of total hydrocarbons and polyaromatics. Molecular tests evaluated changes in the profile and quantity of the *rrs* genes of archaea and bacteria and also the *alk*B gene. The influence of the treatments tested was directly related to the type of soil used. The work confirmed that despite the extreme conditions found in Antarctic soils, the bacterial strains degraded hydrocarbons and bioremediation treatments directly influenced the microbial communities present in these soils even in short periods. Although the majority of the previous studies demonstrate that the addition of fertilizer seems to be most effective at promoting bioremediation, our results show that for some conditions, autochthonous bioaugmentation (ABA) treatment is indicated. This work highlights the importance of understanding the processes of recovery of contaminated environments in polar regions because time is crucial to the soil recovery and to choosing the appropriate treatment.

Keywords Soil · Bioremediation · Antarctica · Diesel

Introduction

The Antarctic continent has areas with high levels of contamination of anthropogenic origin and areas of chronic hydrocarbon contamination (Vázquez et al. 2009). Accidental spills around stations represent a major form of pollution caused directly by humans in Antarctica (Bargagli 2008). Oil spills can lead to reproductive failure and bioaccumulation in tissues of birds and fish that come into contact with these contaminants (McDonald et al. 1992). Because of the small number of animals and plants in Antarctica, such effects can be quickly recognized and quantified. Analyses of Antarctic fish and crustaceans have already shown considerable transfer and accumulation of heavy metals (Bargagli et al. 1996; Bargagli et al. 1998) in penguins and other birds (Corsolini 2009).

Moreover, oil spills into soil can lead to increased numbers of degrading microorganisms and a reduction of microbial diversity (Aislabie et al. 2004; van Dorst et al. 2014). This phenomenon is caused by toxicity, combined with an unbalanced C/N/P ratio. The latter can be rebalanced by adding nutrients to the soil (biostimulation), stimulating the ability

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6919-0) contains supplementary material, which is available to authorized users.

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of microorganisms to degrade the pollutant (Santos et al. 2010). Although contaminant removal can be increased by the addition of living organisms with degradation capacity (bioaugmentation), this method requires the speedy development and maintenance of a high biomass of microbial populations (Xu and Lu 2010), beyond the amount needed for contaminant degradation (Ruberto et al. 2003; Jacques et al. 2008). These treatments are examples of bioremediation, a way to decrease the concentration of toxic contaminants using natural biological activity (Robles-González et al. 2008).

The current work proposes to evaluate the changes caused by an accidental spill of diesel oil around a Brazilian station in Antarctica (Antarctic Station Comandante Ferraz, EACF) in 1986. The traffic of diesel-powered machines and small oil spills during frequent refueling and transport also contribute to the site contamination. The contamination is visible decades later particularly during the soil thaw periods, when the water helps disperse the diesel oil and is accompanied by the strong smell of oil on site.

The persistence of the contaminant is probably related to adverse environmental conditions as the low temperatures restrict the selection and diversity of microorganisms (Ruberto et al. 2008), which are essential in the process of biodegradation. Additionally, the cycles of freezing and thawing, directly related to the temperature fluctuations, influence the dispersion of contaminant in the soil (Aislabie et al. 2004) and alter degradation patterns (Yang et al. 2009). Furthermore, polar soils are nutritionally poor (Tarnocai and Campbell 2002), where natural levels of nitrogen and phosphorus are often low (Ruberto et al. 2008). This restricts the microbial activity in the soil by limiting the supply of nutrients, electron donors and acceptors, amino acids, and vitamins (Yang et al. 2009).

The natural biodegradation may also have been affected by the lack of moisture, especially during winter, when the humidity of Antarctic soils is reduced (Tarnocai and Campbell 2002). The annual precipitation is low and occurs mostly in the form of snow (Filler et al. 2001), which limits the availability of water and consequently microbial growth. Moreover, the soils are mainly formed by rocks, and constant winds leave the soil fairly oxygenated, which is a positive factor for bioremediation. Because this contaminant is being widely used as an energy source for research stations of several countries, a permanent strategy for soil recovery using the metabolic activity of bacteria to naturally degrade these compounds will be necessary for future accidents.

To address these factors, we established an experimental microcosm in situ for 45 days to evaluate possible changes in the microbial profile of the soil front using some strategies for bioremediation (bioaugmentation—adding degrading bacteria to contaminated soil; biostimulation—adding fertilizer to the soil contaminated; and bioaugmentation + biostimulation). The activities developed in this study are the results of two experiments, where the first was the previous soils collection and screening of the strains of bacterial capable to degrade petroleum hydrocarbons for later to be used on the in situ microcosm. In this experiment, we evaluate the molecular changes in the bacterial and archaeal gene *rrs* (16S) and a gene responsible for the degradation of hydrocarbons (*alkB*) using gene quantification and analytical tests.

Materials and methods

Sampling

The soil used in this study was collected from the Admiralty Bay, located in the central part of King George Island, which belongs to the archipelago of the South Shetland Islands, situated 130 km northwest of the Antarctic Peninsula, between latitudes 61°S and 63°30′S and longitude 53°55′W and 62°50′W. The Comandante Ferraz Antarctic Station (EACF) is located on the Keller Peninsula in Admiralty Bay (Electronic supplementary material (ESM) Fig. S1).

The first soil collection was conducted during the austral summer of 2009/2010, with the aim to isolate bacterial strains to be used on consortium. These soil samples were preserved at 4 °C until they were moved to Brazil. A second soil sampling was performed for the in situ microcosm experiment. Samples were collected from five points near EACF in the austral summer of 2011/2012. Three of these points were taken from the area around the fuel storage tanks and were considered contaminated by diesel oil (points 3, 4, and 5) and the other two were considered uncontaminated (points 9 and 10) (ESM Fig. S2). Approximately 3 kg of each samples point (0–15 cm deep) was exhaustively mixed during 2 h with the aid of shovels. The mixtures were performed using plastic boxes and shovels previously sterilized, taking in account contaminated and uncontaminated soils separately. The soils used to assembly the microcosm were the mixture of uncontaminated or uncontaminated soils.

MPN and bacterial isolation

In the Laboratory of Molecular Microbial Ecology, UFRJ, Brazil, 5 g of soil (sampling 2009/2010) was added to 45 ml of saline (0.85 % NaCl) in an Erlenmeyer flask and was kept stirring at 2×*g* for 1 h. Then, serial dilutions were made (10^{-2} to 10^{-7}), in BH medium (Bushnell Haas Broth: magnesium sulfate 0.2 g, calcium chloride 0.02 g, mono-potassium phosphate 1 g, diammonium hydrogen phosphate 1 g, potassium nitrate 1 g, ferric chloride 0.05 g, distilled water 1000 ml)(Brown et al. 1991). The inoculation to total heterotrophs (TH) and heterotrophic degraders (HD) were made in BH medium as well but with different carbon source. To estimate the TH, 2 % yeast extract was added to the BH medium, while to quantify the HD, 2 % (*v*/*v*) diesel oil was added to the

BH medium (the same used for energy generation in EACF). The material was incubated at 4 °C for 3 days with agitation at 2×*g*.

After 3 days of incubation at 4 °C, the most probable number (MPN) of organisms was estimated (Peixoto et al. 2011). Then, 100 μl of the suspension containing 10^7 cells was diluted in saline (0.85 % NaCl) and then diluted until reach the dilution 10^{-4}, using one tube per dilution. The dilutions were plated on BH medium plus 2 % of diesel oil (*v*/*v*) containing 15 g of agar per liter and incubated for 2 weeks at 4 °C.

Analytical results and total petroleum hydrocarbons analysis

The samples of soil were maintained at −20 °C until arriving in Brazil. Falcon tubes of 15 ml each were filled with the samples in triplicates and then sent for analysis. Ten grams of soil was used from each sample to perform the phosphorus and nitrogen analyses in accord to the Standard Methods for the Examination of Water and Wastewater (SMEWW 4500-P and SMEWW 4500-N) and Ascorbic Acid Method and, respectively. The potassium analyses were performed according to the USEPA 3050B method for Acid Digestion of Sediments, Sludges, and Soils and the SMEWW 3111B Direct Air-Acetylene Flame Method. Total petroleum hydrocarbons (TPH) results were obtained after extraction from the samples using an Accelerated Solvent Extractor model 200 (Dionex, USA), as described in a previous study (Cury et al. 2015). After, the material was concentrated in a Turbovap Model II to a volume of 1 ml. The concentrated material was applied in a HP5890 gas chromatograph (equipped with a HP-5 column), as described in the USEPA 8015B manual (USEPA-8015B, 1996).

DNA extraction

The extraction of genomic DNA from the bacterial strains, when in pure culture, was performed with the Wizard Genomic DNA extraction kit (Promega). The extraction of DNA from soil samples was performed using the method of direct extraction with the FastDNA SPIN Kit for Soil from BIO101 (California, USA) according to the manufacturer's protocol. The purity and quality of the DNA obtained was checked by gel electrophoresis in 0.8 % agarose with electric current of 80 V in 1× TBE buffer (89 mM Tris, 2.5 mM EDTA, 89 mM H_3BO_3) for approximately 2 h using a running buffer (50 % glycerol, 20 mM EDTA, pH 7.5, 0.05 % bromophenol blue, 0.05 % xylene cyanol). After the run, the gels were stained with ethidium bromide, exposed to UV light, and then photographed in an image capture system (Fisher Science, Term life).

BOX-PCR

The DNA obtained from bacterial isolates was subjected to BOX-PCR using the oligonucleotide BOXA1R (5′-CTACGCCAAGGCGACGCCTGACG - 3′), with the aim of generating BOX profiles (Versalovic et al. 1994). The reactions made up 50 μl total, containing 1× DreamTaq Green Buffer, $MgCl_2$ 2.0 mM, mixed dNTPs 0.2 mM, 2.5 μl 100 % DMSO; 10 pmol BOX primer; 2.5 U Taq polymerase; and 10 ng of sample DNA from each strain. The PCR program comprised a 7 min of denaturation step at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 8 min at 65 °C, and a final extension at 65 °C for 16 min. After the PCR, an aliquot of 15 μl of the sample was subjected to electrophoresis at 80 V through agarose gel (1.2 %) in 1× TBE for 5 h. The gel was then stained for approximately 15 min in a solution of ethidium bromide (2 mg/ml) and then photographed under UV light in an image capture system (Fisher Science, Term life).

Sequencing

The bacterial isolates with different band patterns revealed by BOX-PCR were then subjected to *rrs* gene sequencing. First, the gene was amplified by PCR, using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) (Lane 1991) and 1492 (5′- GGTTACCTTGTTACGACTT-3′) (Turner et al. 1999). The reactions in a final volume of 50 μl was composed of a mixture of 1× Dream Taq Green buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, 2.5 U Taq polymerase (Fermentas), and sterile Milli-Q water. Approximately 5 ng of DNA from each strain was amplified. The PCR program included 1 cycle of denaturation for 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. To verify the success of the amplification, an aliquot of 5 μl of the sample was subjected to electrophoresis at 80 V in a 1.2 % agarose gel for 2 h. The gel was stained for approximately 15 min in a solution of ethidium bromide (2 mg/ml).

DNA sequencing of the selected strains was performed from the products of PCR amplification using primers from the ends 27f (5′-AGAGTTTGATCATGGCTCAG-3′) (Lane 1991) and 1492R (5′-GTT TAC CTT GTT ACG ACT T-3′) (Turner et al. 1999) in addition to the intermediate primers 532F (5′-GTGCCAGCAGCCGCGGTAA-3′) (Weisburg et al. 1991) and 907R (5′- CCGTCAATTCMTTTRAGTTT-3′) (Weisburg et al. 1991). The amplicons were purified with the PCR Purification System kit (Qiagen). Each sequencing reaction was performed in a MegaBACE automated sequencer. The sequences obtained were analyzed using BLASTn (http://www.ncbi.nlm.nih.gov/) and Genbank to align the sample sequences with those in the database.

Antagonism test

The bacterial strains that grew up faster in culture medium containing 2 % of diesel oil and had different colony polymorphism (e.g., different colors and shape) were chosen to assembly the bacterial consortium. In this regard, strains 3F, 5A, 5E, 9B, 3C, 9A, 10B, and 3A were submitted to the antagonism evaluation. This test consisted in figuring out if the bacterial strains chosen presented inhibition against each other, which could disturb the consortium propose when applied into the microcosm. For this, the strains were pre-incubated for 2 days at 12 °C in LB liquid medium (10 g Luria-Bertani-Tripitona mix, 5 g yeast extract, 5 g NaCl, 1000 ml distilled water (Martínez-Rosales and Castro-Sowinski 2011)). Then, 100 μl of each strain was plated (one strain per plate) on LB agar plates using a Drigalski handle to obtain a "carpet" of bacteria. Soon after, a drop of 10 μl of each remained strains was plated in the same Petri dish (in different areas), except for the initially inoculated strain that was forming a "bacterial carpet" in whole plate. The plates were incubated at temperatures of 4 and 25 °C for 2 weeks to know if the antagonism effect could change depending of the temperature. The result should reveal the inhibition effect through the non-growth of the inoculated strains (in drop way) or the non-growth halo formed around the colonies incubated in drop way, indicating the non-growth of the bacterial strain that was inoculated on the whole plate.

Transport and application of the consortium

After growing the individual strains isolated in 15 ml Falcon tubes in LB medium at 12 °C for 3 days, 10 ml of the cultures was centrifuged at 4500×*g* for 10 min and the supernatant was discarded. Then, the precipitate was homogenized in 2 ml saline (0.85 % NaCl) and kept at nearly 4 °C for 1 week. The strains were transported from the laboratory (Rio de Janeiro State, Brazil) to the Brazilian Antarctic Station (EACF) in a thermal bag containing ice sheets that were changed along the way.

At the time the strains were introduced to the in situ microcosm, distilled water was added to the tubes to reach 10 ml of solution, to reach the desired cell concentration (10^5 cells/ml). The consortium was then added to the microcosms containing the soil that were used for bioaugmentation treatments.

Mounting of the microcosm

During Operation Antarctica XXIX (January 2011 to March 2011), we assembled the microcosm experiments using cylindrical polyvinyl chloride (PVC) bottles 8.2 cm in height and 7.1 cm in diameter, each containing 400 g of soil previously collected and mixed according to "Sampling" section. The PVC bottles containing soils that were used in the experiment were placed on a stable surface under environmental conditions, according to those present in EACF. During the experiment period, the temperature was approximately 2 °C.

Three soil conditions were used for the experiment: soil with a history of contamination (C)—points 3, 4, and 5; soil with a history of contamination plus 2 % (*v*/*v*) of the diesel oil used in EACF (C + O)—points 3, 4, and 5; and soil without historical contamination plus 2 % (*v*/*v*) of the diesel oil used in EACF (N + O)—points 9 and 10 (ESM Fig. S2).

These soils were subjected to four treatments: biostimulation (BS), biostimulation + bioaugmentation (BS + BA), bioaugmentation (BA), and control (Ctr) (ESM Fig. S3). For the biostimulation treatments, a commercial mono-ammonium phosphate (MAP) soil fertilizer was added at a concentration of 250 mg N/kg, whereas for the bioaugmentation treatments, soil was added to cultures of bacterial strains previously selected from Antarctic soils and transported to Antarctica (strains 3F, 5A, 5E, 9B, 3C, 9A, 10B, and 3A). After 45 days, the experiment was collected in triplicate and the PVC bottles were placed in plastic bags and stored at −20 °C until they were returned to Brazil for analysis (1 month).

PCR-DGGE of *rrs* bacterial and archaeal gene and *AlkB* gene

We generated four denaturing gradient gel electrophoresis (DGGE) gels for each gene analyzed, one gel from each model of soil studied: soil with a history of contamination by diesel oil (C); soil with a history of contamination by diesel + 2 % diesel oil (C + O), soil with no history of contamination by diesel + 2 % diesel oil (N + O), and a control gel that also contained samples from the three soil models. All DGGE analysis of the four treatments tested: control, biostimulation, bioaugmentation, and biostimulation + bioaugmentation, was performed in triplicate.

For the *rrs* bacterial gene, the reactions were performed using primers U968f (5′-AAC GCG AAG AAC CTT AC-3′) containing a GC clamp (5′-CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G-3′) added at the 5′ terminus and the primer 1401r (5′-CGG TGT GTA CAA GAC CC-3′) (Nübel et al. 1999). The final volume was 50 μl, containing 2.5 μl of 10× PCR buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.5 U recombinant Taq DNA polymerase (Fermentas), 5 ng of DNA total; 10 pmol of each primer, 1 % BSA, 1 % formamide, and sterilized Milli-Q water. The PCR program used included a 5-min cycle of denaturation at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and a final extension period at 72 °C for 3 min.

The PCR amplification of archaeal *rrs* required two steps. For a final volume of 50 μl, the first reaction primers containing 21F (5′-TTCCGGTTGATCCYGCCGGA-3′) (DeLong 1992) and ARCH915r (5′-GTGCTCCCCCGCCAATTCCT-

3′) (Luna et al. 2009) consisted of a mixture of 1× DreamTaq Green Buffer, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 20 pmol of each primer, 2.5 U Taq polymerase (Fermentas), and sterile Milli-Q water. Approximately 5 ng of DNA from each strain was amplified. The second reaction was performed using the same PCR mix and the following primers: ARC344f + GC (5′-CGCGCCGCCGCCGCGGGCGGGGCGGCAGGGGGC GGG GGC AGC GGG YGC G CGC AGC AGG GA-3′) and 519r (5′-GWATTACCG CGGCKGCTG-3′) (Yu et al. 2008). The PCR program used for both reactions included 1 cycle of denaturation for 5 min at 95 °C, followed by 30 cycles for 1 min at 94 °C, 1 min at 55 °C and 1 min 30 s at 72 °C, and a final extension at 72 °C for 10 min.

*Alk*B gene amplification was obtained using the primers *Alk*B F + GC (5′ - CGC CCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-GCIGTIGGICIIAIICACG ACAAGGAGA - 3′) and *Alk*B R (5′ - IGCITGITGTCAGTGIIIICGIAGCTG - 3′)(Chénier et al. 2003). The reactions in a final volume of 50 μl were composed of a mixture of 1× DreamTaq Green Buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, 2.5 U Taq polymerase (Fermentas), sterile Milli-Q water, and approximately 5 ng of the soil DNA. The PCR program used comprised a cycle of denaturation for 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and extension at 72 °C for 3 min.

To verify the successful amplification of the three genes, a 5-μl aliquot of the samples was subjected to electrophoresis at 80 V through an agarose gel (1.2 %) for 2 h. The gel was stained for approximately 15 min in a solution of ethidium bromide (2 mg/ml) and was visualized under UV light in a Fisher Science imaging system.

The PCR products were separated by polyacrylamide gel electrophoresis with a denaturing gradient (DGGE). DGGE gels were prepared with a polyacrylamide solution (6 % for the 16S rRNA and 8 % for *Alk*B and Archaea) in Tris-acetate buffer (pH 8.3). A 45 to 65 % gradient of chemical denaturants (urea and formamide) was established for the 16S rRNA gels, and a 45 to 70 % gradient was created for the *Alk*B gels. The run time was 16 h at 75 V. The gels were stained with a 1:10, 000 *v*/*v* solution of SYBR Green (Molecular Probes) diluted in 1× TAE buffer according to the manufacturer's specifications for approximately 40 min in the dark. Then, the gel was observed under UV light and photographed in a STORM snapshot system (Amersham Pharmacia).

The DGGE gels were analyzed to create dendrograms based on profiles of the bands observed using the Jacard similarity coefficient and the UPGMA method, with the BioNumerics V6.0 software (Applied Maths) (Nübel et al. 1999; Clegg et al. 2003). The matrix obtained after analyzing the DGGE gels with the BioNumerics V6.0 software was used to generate graphs in the PC-ORD 5.0 software (MjM Software, Gleneden Beach, OR), where we used non-metric multidimensional ordination (NMS). Depending on the values obtained by the matrices generated in BioNumerics, the PC-ORD generated graphics in two or three dimensions, according to the best significance set by software.

Real-time PCR

PCR assays were performed in real time in polypropylene plates (96 wells) using the ABIPrism 7500 detection system (Applied Biosystems) to quantify the total abundance of the gene encoding the 16S ribosomal RNA according to the manufacturer's instructions. The amplification reaction was performed in a volume of 25 μl containing 12.5 μl Platinum SYBR Green qPCR SuperMix-UDG 2X (60 units/ml Platinum Taq® DNA Polymerase, SYBR® Green I dye, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 mM dATP, 400 mM dCTP, 400 mM dGTP, 800 mM dUTP, 40 units/ml UDG; Invitrogen), 200 nM of each primer, 0.5 μl of ROX reference dye (50 mM; Invitrogen), 0.5 μl BSA (1 mg/ml), 5.5 μl H_2O, and 5 ng of DNA. The bacterial *rrs* gene sequences were amplified using the primer pair 357F (5′-GRSCTACGGGCAG-3′) and 529R (5′-AGCTGGTGCGGCCGC-3′) (modified from Muyzer et al. 1993). All amplifications were performed in triplicate. Standard DNA samples from a previously used clone containing the gene coding for 16S ribosomal RNA subunit were included to construct the standard curve, and water was used as negative control. The PCR conditions were 94 °C for 3 min, followed by 30–40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 45 s.

Statistical analysis

The variance and significant differences of TPH, *n*-alkane, and analytics data among various treatments were analyzed by ANOVA and Tukey's test. The statistical significance in this analysis was defined at $p < 0.05$.

Results

Processing and screening

MPN analysis used to quantify the total number of heterotrophic (HT) cells revealed that all samples contained greater than or equal to 10^8 cells/g soil. For hydrocarbon-degrading heterotrophs (HD), the results showed points 3, 9, and 10 had 10^7 cells/g soil, and points 4 and 5 contained 10^6 and 10^8 cells/g soil, respectively (Table 1).

Plating on BH medium plus 2 % diesel yielded 29 bacterial isolates whose genomic DNA was extracted and subjected to BOX-PCR. Some similar profiles were observed; strains 5C and 6A were similar, as were 11A, 12D, 16A, 17C, 9C, 13E,

Table 1 Most probable number (MPN) results

Site	TH Cells per g soil	DH
3	1.1×10^{8}	1.1×10^{7}
4	$>1.1\times10^{8}$	$>1.1\times10^{8}$
5	$>1.1\times10^{8}$	4.6×10^{6}
9	1.1×10^{8}	2.9×10^{7}
10	$>1.1\times10^{8}$	4.6×10^{7}

The value refers to the total heterotrophs (TH) and degrading heterotrophs (DH)

and 15B (data not shown). This analysis decreased our strain collection to 17 strains, of which 10 were chosen to compose a consortium. The criteria used for the selection were based on morphological differences and fast growth rates during incubation in medium containing diesel oil as the sole carbon source.

The tests for antagonism between the strains on two culture media (LB and BH) and at two incubation temperatures (4 and 25 °C) showed no zone of inhibition among the ten strains chosen (data not shown). This observation allowed the use of all of these strains in the assembly of a bacterial consortium.

Strain identification

The 16S rRNA gene sequences generated were deposited and compared to reference species in the GenBank database (Table 2). The isolated bacterial strains have been deposited at Coleção de Bactérias do Ambiente e Saúde, CBAS (http://cbas.fiocruz.br) under the following accession codes: CBAS 577, CBAS 578, CBAS 580, CBAS 582, CBAS 576, CBAS 581, CBAS 583, CBAS 575 (strain 3F to 3A, Table 2).

Soil analyses

The soil nitrogen, phosphorus, and potassium levels were greatly different among the treatments. In general, the potassium amount was low, whereas nitrogen and phosphorus fluctuated depending on the treatment type. The samples that received fertilizer revealed increases in phosphorus and nitrogen of at least 1 log unit, in comparison to the control soil (Table 3).

The treatments that received fertilizer (BS and BS + BA) in contaminated soil with 2 % diesel oil (C + O) revealed greatly enhanced amounts of phosphorus and nitrogen. This result was not observed in the other two soils studied (Table 3).

Quantification of hydrocarbons in soil

We analyzed the total polyaromatic hydrocarbon (TPH) levels, comprising unresolved complex mixture (MCNR) and hydrocarbons resolved from petroleum (HRP), and found degradation rates of approximately 86.5 and 85.3 % in the BS in contaminated soil (C) and in the uncontaminated soil that received 2 % of oil (N + O), respectively. Similarly, the BS + BA treatments presented similar degradation rates. Although the BA treatment did not stimulate the degradation in chronically contaminated (C) and non-contaminated plus 2 % (*v*/*v*) of diesel oil (N + O) (Fig. 1), the profile of degradation was not repeated when the soil had a higher oil concentration (C + O). In this case, bioaugmentation alone appeared to affect degradation to a greater extent than did the other treatments (Fig. 1a).

By analyzing only the *n*-alkane fraction, a similar degradation rate of 85.3 % was observed for BS treatment in contaminated soil (C) and of 100 % for BS treatment in non-contaminated soil with 2 % diesel oil (N + O). However, the contaminated soil with 2 % diesel oil (C + O) had a different profile as all three treatments (BS, BA, and BS + BA) resulted in efficient degradation rates (73,5, 87.5, and 60.8 %, respectively), wherein the bioaugmentation treatment showed the best results (Fig. 1b). In general, the samples from experimental controls (Ctr) showed a slight increase of hydrocarbon compared with time zero control (Ctr.T0), which is likely related to the PVC used to perform the experiment, once that material is derivate from petroleum.

Table 2 Bacterial strain identification, similarity index with reference species in GenBank, number of bases analyzed, and Culture Collection Accession Numbers

Strains/access number (GenBank)	Reference species/access number "Genbank"	Similarity	Number of bases	Culture bank CBAS[a]
3F/KR024326	*Pseudomonas frederiksbergensis*/JX122166.1	99 %	1260	CBAS 577
5A/KR024327	*Pseudomonas grimontii*/AB698742.1	99 %	1196	CBAS 578
5E/KR024329	*Sphingomonas* sp./AF177917.1	93 %	633	CBAS 580
9B/KR024331	*Pseudomonas graminis*/KM434253.1	94 %	425	CBAS 582
3C/KR024325	*Sphingomonas glacialis*/NR_117270.1	99 %	1179	CBAS 576
9A/KR024330	*Acinetobacter lwoffii*/KF317877.1	95 %	1340	CBAS 581
10B/KR024332	*Acinetobacter guillouiae*/AP014630.1	96 %	318	CBAS 583
3A/KR024324	*Pseudomonas fluorescens*/KM453978.1	93 %	516	CBAS 575

[a] Coleção de Bactérias do Ambiente e Saúde, CBAS (http://cbas.fiocruz.br)

Table 3 Soil characteristics (mean±standard deviation) to each treatment (n=3)

Samples[a]	Moisture (%)[b]	Potassium(mg/kg)[c]	Phosphorus(mg/kg)[b]	Nitrogen(mg/kg)[b]
(C)T0	0.8±0.8	181.0±11.6	1331.5±208.0	3540.2±189.6
(C)Ctr	12.0±2.9	205.0±53.5	1296.4±37.7	175.5±41.6
(C)BS	12.5±1.2	308.1±103.4	9861.7±139.4	5627.3±620.1
(C)BA	13.5±1.1	153.5±24.1	1818.6±120.6	184.7±46.3
(C)BS+BA	11.8±1.5	168.2±43.3	5933.1±2100.8	4164.8±233.3
(C+O)T0	1.1±0.8	170.2±110.5	2112.5±533.7	3490.5±366.6
(C+O)Ctr	8.1±1.2	444.8±62.6	1816.7±110.8	190.2±8.8
(C+O)BS	12.5±0.7	394.4±38.5	18,724.8±2666.0	4330.1±467.8
(C+O)BA	10.8±0.5	219.8±120.8	1671.0±1466.9	142.1±66.7
(C+O)BS+BA	12.5±2.1	1.3±2.3	12,288.3±1752.3	5350.3±409.4
(N+O)T0	4.2±1.4	169.1±109.5	3216.8±768.0	105.5±30.7
(N+O)Ctr	13.3±4.4	452.9±27.4	1352.7±163.9	1896.3±16.5
(N+O)BS	11.2±1.8	213.3±15.6	7092.8±1512.1	4665.2±51.2
(N+O)BA	7.8±0.7	88.8±7.8	2227.9±401.2	135.8±15.6
(N+O)BS+BA	9.8±3.6	253.0±16.3	7943.7±325.5	7111.8±848.1
(NC)T0	7.6±1.9	197.8±28.6	2471.9±512.6	132.8±27.4

All samples that are not indicated from time zero were collected at the end of experimental period (45 days). The treatments used are indicated for: *Ctr* Control, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation

[a] Soils samples ID indicating the type of soil used: (C) contaminated soil, (C+O) contaminated soil added 2 % diesel oil, (N+O) non-contaminated soil added 2 % of diesel oil, (C)T0 control from contaminated soil at time zero, (NC) control from non-contaminated soil at time zero, (C+O)T0 control from contaminated soil added 2 % diesel oil at time zero, (N+O)T0 control from non-contaminated soil added 2 % diesel oil at time zero

[b] Values showed significance difference by ANOVA (p<0.05).

[c] No significant difference was observed by ANOVA (p>0.05).

Analysis of the abundance and the diversity of the soil microbial communities

DGGE profiles with replicates of the same treatments were fairly similar (sometimes represented for less than 3 points in the graphic, which means that replicates have identical positions) and those of samples from the different treatments were different (Figs. 2 and 3). We thus observed significant differences in most of the profiles; however, there were no substantial differences in the archaeal profiles (ESM Fig. S4). One determining factor was the addition of fertilizer, causing strong changes in the communities, such as the complete elimination of some bands and the appearance of others. In general, BA treatment revealed strong bands compared to the control, next to some newly emerging DNA bands (data not shown).

The graphical analysis of the *rrs* bacterial gene in the gels revealed well-defined groups. In the graphs of the C soil, axis 1 was primarily responsible for the separation of samples, once the effect of this axis in the grouping was high as indicated by the high percentage value (68.3 %, p=0.0196). The Ctr samples were closer to the BS treatment group, whereas the BA and BS + BA treatment groups were distanced (Fig. 2a). The replicates from C + O soil grouped separately. In the N + O soil, the BS + BA replicates were distanced from all others. Axis 3 was more important in the separation of the BS-treated samples and also for BS + BA in comparison to control and BA.

In the analysis performed with the control treatment for the three soil models, good separation was also achieved. When we analyzed axis 1, C + O replicates were closer to N + O soil samples. However, when axis 2 was analyzed, the C soil was nearest to the C + O soil. These results suggest that for the bacterial gene *rrs*, the samples of soil with a history of contamination have an intermediate profile between the other two models studied (C + O and N + O; Fig. 2d). The graphs generated for this gene were considered statistically significant for p values ≤0.042.

In the profiles generated by communities of Archaea in the different soil models and treatment conditions, the triplicates of the soil with historical contamination did not group with any treatment. The groups formed did not show relationships that could be consistently elucidated, and the band profile could not be associated with any of the remediation techniques employed (ESM Fig. S4). This result was the same for C + O and N + O. Although graphics generated for each soil type did not show differences among the replicates, the control soil was able to form defined groups (ESM Fig. S4). This result may

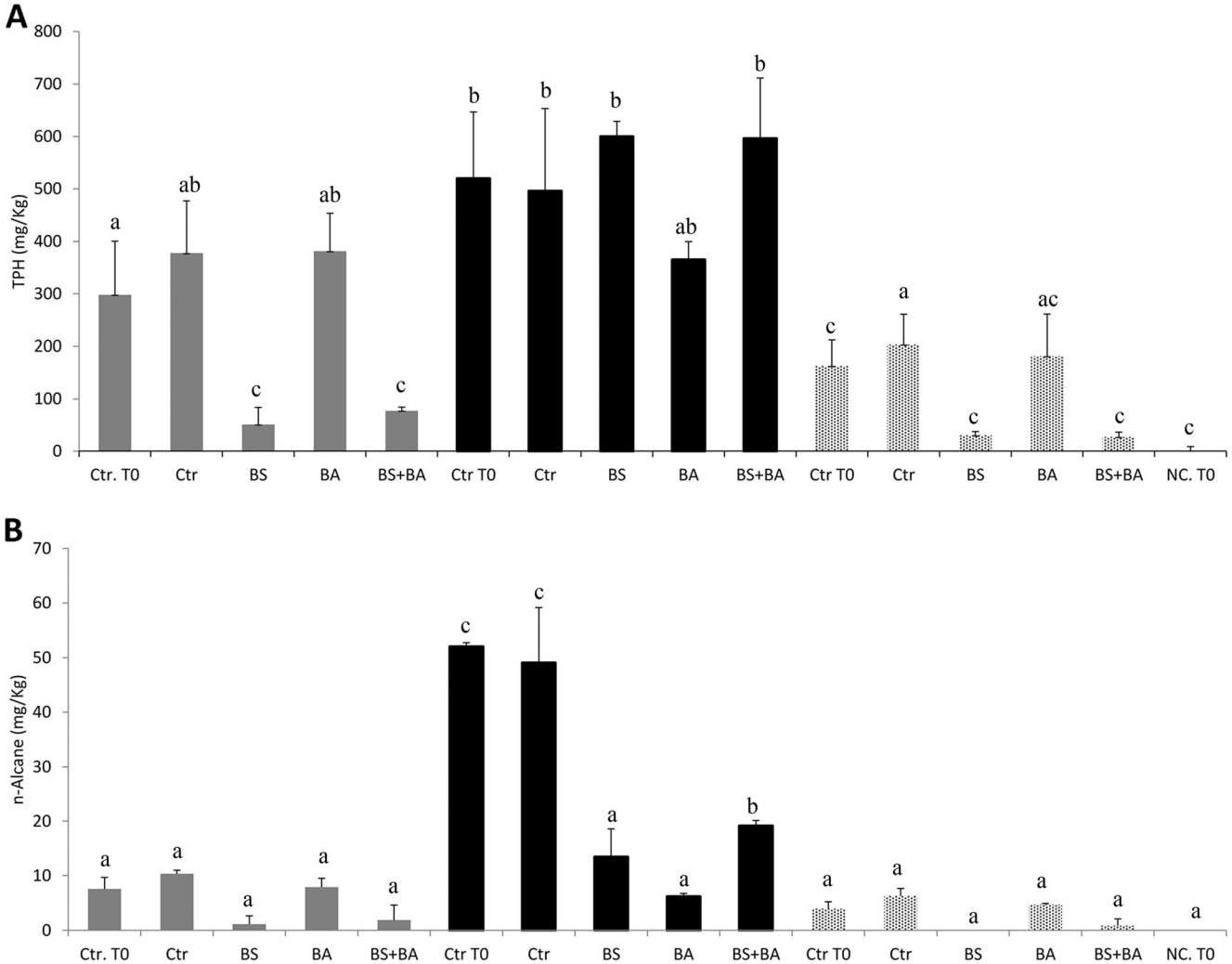

Fig. 1 Degradation of the TPH. *Gray bars*, chronically contaminated soil (C). *Black bars*, chronically contaminated soil added 2 % (*v*/*v*) of diesel oil. *Dashed bars*, non-contaminated soil added 2 % (*v*/*v*) of diesel oil. Values are the means from three replicates; *error bars* represent minimum and maximum values ($n=3$). Statistically significant differences were observed by ANOVA ($p<0.05$) and the variance between the treatments was observed by Tukey's test ($p<0.05$). *Different letters* (a, b, c) indicate statistically significant difference. Two letters appear when values are not significantly different from two groups

indicate differences in the communities of archaea when comparing different soils. However, data obtained for the *rrs* gene of archaea was not statistically significant once $p \geq 0.52$ values were obtained.

The DGGE gels generated *Alk*B gene clusters of the replicates of each treatment. Furthermore, the positioning of the samples in the graphic was also statistically significant with $p \leq 0.027$. However, the number of fragments observed was lower than was observed for the *rrs* gene, which was expected due to the difference in the gene copy numbers in the bacterial cells.

The graphic generated for the C soil shows a good separation. The replicas were well grouped, and axis 1 (40.2 %, $p=0.0167$) was the main influencer in the placement of the replicates, especially in the detachment of the BA samples (Fig. 3a), which reveal the strong effect, in the profile distribution for this gene, when bioaugmentation was applied.

With the C + O soil, the relationships between the treatments changed (Fig. 3b). In this situation, replicates of BS and BA ware positioned very closely, suggesting that the addition of fertilizer or the bacterial consortium generated a very similar profile for the *Alk*B gene.

The analysis of the N + O soil revealed that the distribution of the treatment groups changed. In this case, the BS group was further away from the other treatments, although it neared the Ctr treatment group in axis 1 (39.6 %, $p=0.0296$). Furthermore, the BA and BS + BA groups were nearest to each other on the same axis (Fig. 3c). In comparing the control replicates for each soil type analyzed in the graph, axis 1 (63.4 %, $p=0.0254$) emerged as the main influence on

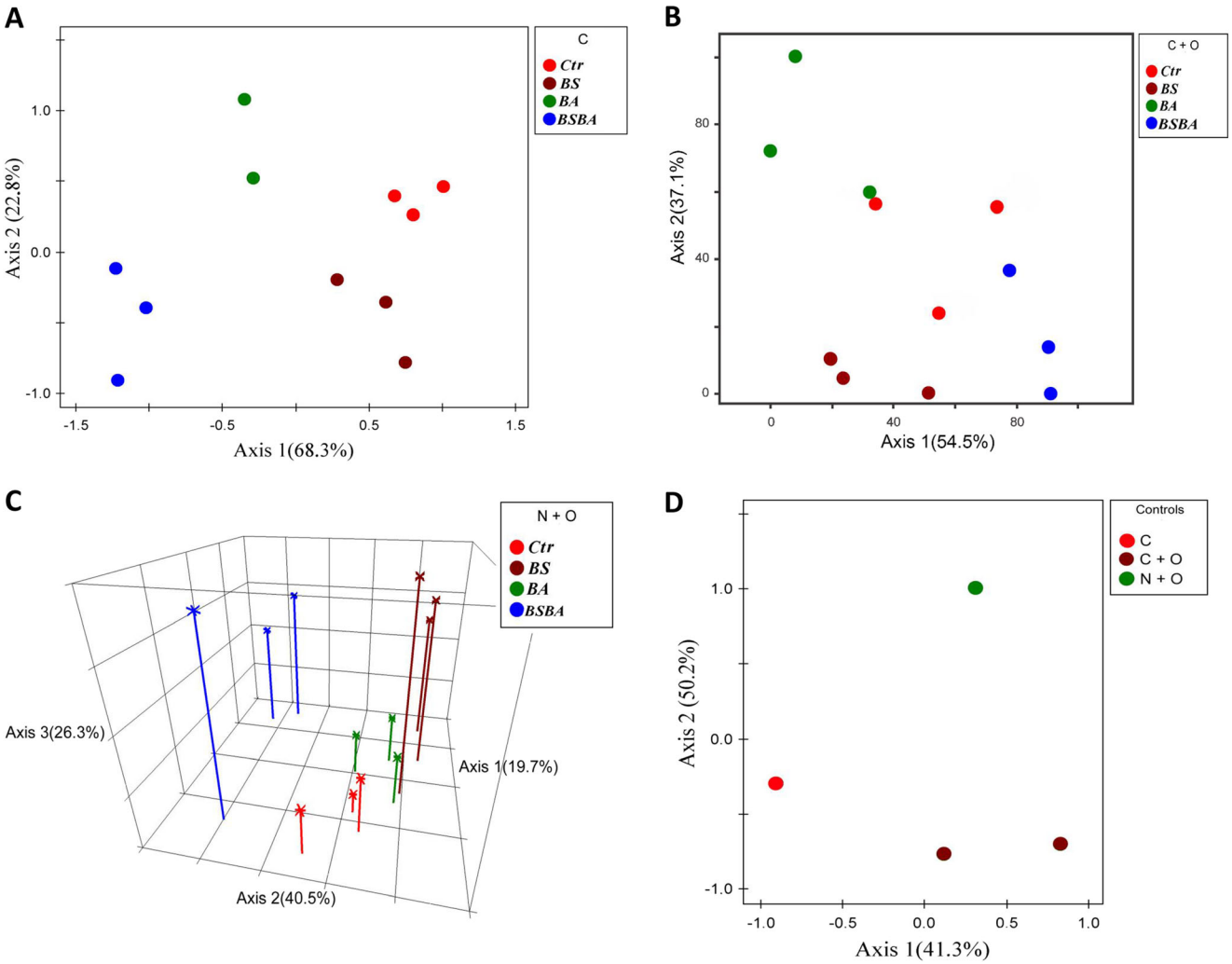

Fig. 2 Non-metric multidimensional ordination (NMS) of the DGGE profile for the *rrs* bacterial gene. **a** Soil with historical contamination by diesel oil (*C*). **b** Soil with historical contamination by diesel oil plus 2 % of diesel oil (*C* + *O*). **c** Uncontaminated soil plus 2 % diesel oil (*N* + *O*). **d** Control of the three models analyzed. The treatments used were as follows: control (*C*); biostimulation (*BS*); bioaugmentation (*BA*); biostimulation + bioaugmentation

positioning, and for this axis, we observed a similar result for the replicates from the C soil and the C + O soil (Fig. 3c).

Abundance of bacterial communities in the soils

The data obtained after performing the real-time PCR for the *rrs* gene revealed an average ranging from 10^{10} to 10^{12} copies per gram of soil. The analysis performed with chronically (C) and uncontaminated (N) soils, which were not subjected to experimentation, revealed the same number of copies for both (10^{12} copies). These values were higher before the experimentation (C + O and N + O) and the treatments (BS, BA, and BS + BA). The Ctr samples showed higher values than the treatments for all three models of soil studied. Among the treatments, the BA replicates (BA) generated higher values and the replicates that received fertilizer addition, BS and BS + BA, showed slightly lower values (ESM Fig. S5). These results suggest that the treatments did not lead to significant changes in the number of the gene copies.

Discussion

Here, we demonstrated changes in the bacterial community profiles of Antarctic soil contaminated by diesel oil in an in situ experiment. We compared the effects of adding nutrients (BS) to those generated by adding a preselected bacterial consortium (BA) and those of both treatments together (BA + BS), all compared to a control. The PVC tubes used were also previously used by Van Elsas et al. (1986) as well as by Fukuhara et al. (2013) to evaluate the colonization of wheat roots and in situ bioremediation, respectively. In general, the analytic data showed a small level of nitrogen in these soils, as we can see in the control and bioaugmentation treatment.

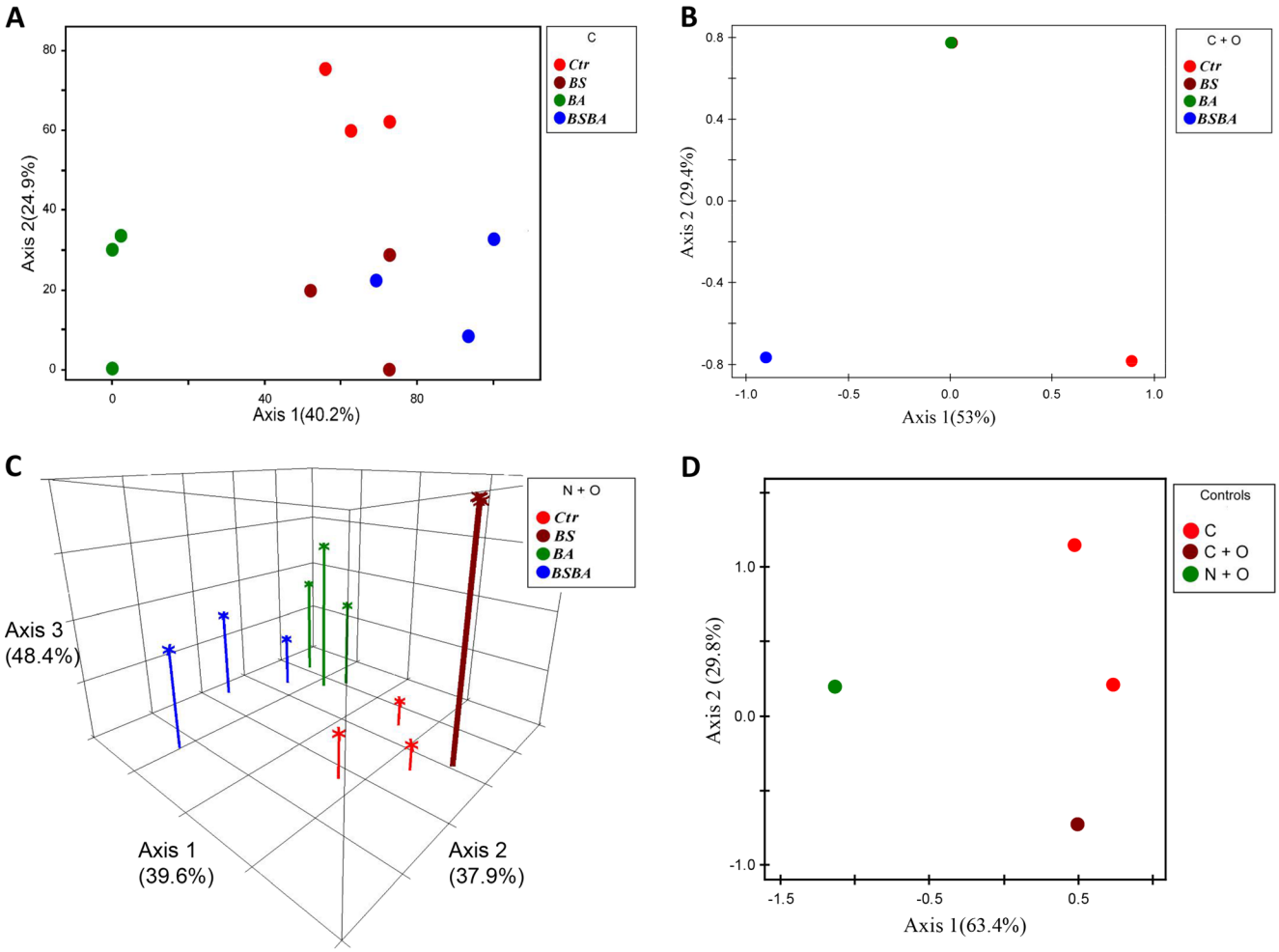

Fig. 3 Non-metric multidimensional ordination (NMS) of the DGGE profile for the *alk*B gene. **a** Soil with historical contamination by diesel oil (*SC*). **b** Soil with historical contamination by diesel oil plus 2 % of diesel oil. **c** Soil uncontaminated plus 2 % diesel oil. **d** Control of the three models analyzed. The used treatments were: control (*C*); biostimulation (*BS*); bioaugmentation (*BA*); biostimulation + bioaugmentation (*BS* + *BA*)

Where fertilizer was added, the level of nitrogen increased consistently, as did the phosphorus levels. Potassium seems not to be limiting, but for the BS + BA treated samples in the contaminated soil added 2 % of diesel oil (C + O), the low level of potassium seems have strongly affected the bacterial profile observed through DGGE analysis.

The analysis of TPH, comprising resolved and unresolved compounds, revealed degradation up to 87.5 % in the biostimulation treatment group. Several studies involving biostimulation have been carried out in many environments, and the addition of nutrients seems be the most effective treatment for removing the contamination. Jasmine and Mukherji (2014) observed increased microbial growth and microbial degradation after the addition of nutrients to sludge during 30 days of experiment. Furthermore, in an experiment carried out by Louati et al. (2014) in lagoon sediment, biostimulation was responsible for 98 % degradation at 15 °C. Despite the high levels of degradation we observed as the result of biostimulation, our study does not show an increase in the number of bacteria. In some cases, the changes caused by nutrient addition does not represent an increase in the number of microorganisms but instead increased dominance of specific groups, like for example groups known capable to degrade hydrocarbons compounds (e.g., *Rhodococcus*, *Sphingomonas*, *Pseudomonas*, and *Acinetobacter*).

Fukuhara et al. (2013) found numbers of bacteria ranging from 2.8×10^{9} to 5.4×10^{10} cells/g soil and numbers of bacteria carrying *alk*B ranging from 3.7×10^{7} to 5.0×10^{8} cells/g soil. Our work showed numbers of total bacteria exceeding 1.1×10^{8} cells/g soil in the contaminated and uncontaminated soils using MPN techniques. Hydrocarbon-degrading bacteria numbered 4.6×10^{6} to $>1.1\times10^{8}$ cells/g soil. The high bacterial numbers were remarkable as our soils did not carry vegetation and were nutritionally poor. Despite these high numbers, the bacterial numbers based on the 16S rRNA gene were slightly lower in the treatments (than in the control samples),

as verified by real-time PCR. This effect is likely related to the selection promoted by the treatments and certainly to the presence of the oil.

In cases where indigenous microorganisms do not possess a good potential to biodegrade oil residues or such degraders are present in low numbers, added microbial degrader communities can be used. Such consortia can be combined with nutrients, in attempts to enhance the degradation (Xu and Lu 2010). The consortium used in this study consisted of 10 bacterial strains, belonging to species of known hydrocarbon degraders, that were able to grow in culture medium containing a high level of diesel oil as a carbon source (2 % *w*/*w*) and seems to have been responsible for the most significant degradation in the bioaugmentation treatment of soil with major contamination (C + O, Fig. 1). These groups are often found in contaminated soils and are among those most responsible for promoting natural remediation (Baraniecki et al. 2002; Zanaroli et al. 2010; Gojgic-Cvijovic et al. 2011).

The archaeal community profiles were not strongly affected by the treatments. However, analyses including the controls showed some differentiation between the soil types studied. We observed that communities were influenced by the soil type but not by the treatments, showing that the changes introduced by the treatments did not change the profiles of the communities of archaea. This is consistent with data by Roling et al. (2004). These authors reported that archaea are unlikely to have a role in the degradation of oil. In addition, restoration of this group of microorganisms cannot be used as an indicator of the recovery of ecosystems impacted by oil after treatment for bioremediation.

Our PCR-DGGE (*alk*B and 16S rRNA gene) data revealed significant changes in the soils related to the treatments and type of soils used. For the 16S rRNA, we observed different grouping and spacing of the treatments used. Apparently, just an altered oil concentration was sufficient to change the profile, even with the same treatment. The analysis of the *alk*B gene showed a profile more specific for each treatment in each soil studied, which is related to the major specificity and preservation of this gene in comparison with 16S rRNA. Interestingly, the addition of two percent of diesel oil generated the same response to both biostimulation and bioaugmentation treatments. In this case, only the combination of these two treatments together was able to alter the contamination, showing a synergic effect. In the soil without historical contamination but with recent contaminant, the most different profile resulted from the biostimulation treatment. The *alk*B gene was studied by Guibert and colleagues (2012) using gene libraries and pyrosequencing with samples from sub-Antarctic coastal sediment; their study revealed a relevant ecological role for *alk*B, which was recommended as a biomarker for biodegradation in this environment.

In the Antarctic environment, Ruberto et al. (2009) also demonstrated the most efficient degradation as a result of BS treatment during 45 days of experimentation with a chronically contaminated Antarctic soil. Interestingly, in the same article, the author indicates that the most efficient degradation occurred in the first 10 days, for all systems, related to abiotic removal derived from manipulation of the soil in this period. However, the biostimulated system did not show a significant increase from 10 to 45 days of experimentation, whereas the systems using bioaugmentation maintained a degree of biodegradation until the end of the assay.

Our results showed excellent degradation of total TPH by BS treatment in both types of soil, C + O and N + O, followed by BA + BS and the control treatments. However, the results were surprisingly contrary in the soil with historical contamination that received more 2 % of diesel oil once bioaugmentation treatment presented the most efficient degradation. For this soil, no other treatment was able to degrade total TPH significantly. When we analyzed the *n*-alkane fractions, the BS and BS + BA treatments were able to degrade hydrocarbons but the BA treatment alone still showed the most efficient degradation.

These results indicate that the oil concentration in the C + O soil was very high, which, together with the addition of nutrients in the BS treatments, leads to a critical concentration and inability of the natural community to degrade the most complex fraction. Moreover, the degradation of *n*-alkane was hampered. On the other hand, BA treatment was more efficient in fostering the degradation at the level of TPH and also *n*-alkane. This is likely be related to the fact that the strains used in the consortium were isolated in medium with a high concentration of diesel oil (2 %).

Thus, the data obtained in this study show that if contaminating oil is present at a high level in soil, the most indicated treatment is the addition of a microbial degrader consortium isolated from the same environment with the same contaminant in a high concentration. This strategy appeared to be the most effective treatment, allowing us to obtain efficient degradation of resolved and unresolved compounds, beyond the degradation of *n*-alkane.

Acknowledgments This work integrates the National Institute of Science and Technology Antarctic Environmental Research (INCT-APA) that receives scientific and financial support from the National Council for Research and Development (CNPq process: no. 574018/2008-5) and Carlos Chagas Research Support Foundation of the State of Rio de Janeiro (FAPERJ). The authors also acknowledge the support of the Brazilian Ministries of Science, Technology and Innovation (MCTI), of Environment (MMA) and Inter-Ministry Commission for Sea Resources (CIRM).

Conflict of interest The authors declare that they have no conflict of interest.

References

Aislabie JM, Balks MR, Foght JM, Waterhouse EJ (2004) Hydrocarbon spills on Antarctic soils: effects and management. Environ Sci Technol 38:1265–1274. doi:10.1021/es0305149

Baraniecki CA, Aislabie J, Foght JM (2002) Characterization of *Sphingomonas* sp. Ant 17, an aromatic hydrocarbon-degrading bacterium isolated from Antarctic soil. Microb Ecol 43:44–54. doi:10.1007/s00248-001-1019-3

Bargagli R (2008) Environmental contamination in Antarctic ecosystems. Sci Total Environ 400:212–226. doi:10.1016/j.scitotenv.2008.06.062

Bargagli R, Nelli L, Ancora S, Focardi S (1996) Elevated cadmium accumulation in marine organisms from Terra Nova Bay (Antarctica). Polar Biol 16:513–520. doi:10.1007/BF02329071

Bargagli R, Monaci F, Cateni D (1998) Biomagnification of mercury in an Antarctic marine coastal food web. Mar Ecol Prog Ser 169:65–76. doi:10.3354/meps169065

Brown EJ, Resnick SM, Rebstock C, Luong HV, Lindstrom J (1991) UAF radiorespirometric protocol for assessing hydrocarbon mineralization potential in environmental samples. Biodegradation 2:121–127. doi:10.1007/BF00114602

Chénier MR, Beaumier D, Roy R, Driscoll BT, Lawrence JR, Greer CW (2003) Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl Environ Microbiol 69:5170–5177. doi:10.1128/AEM.69.9.5170

Clegg CD, Lovell RDL, Hobbs PJ (2003) The impact of grassland management regime on the community structure of selected bacterial groups in soils. FEMS Microbiol Ecol 43:263–270. doi:10.1111/j.1574-6941.2003.tb01066.x

Corsolini S (2009) Industrial contaminants in Antarctic biota. J Chromatogr A 1216:598–612. doi:10.1016/j.chroma.2008.08.012

Cury JC, Jurelevicius DA, Villela HDM, Jesus HE, Peixoto RS, Schaefer CEGR, Bícego MC, Seldin L, Rosado AS (2015) Microbial diversity and hydrocarbon depletion in low and high diesel-polluted soil samples from Keller Peninsula, South Shetland Islands. Antarct Sci 27:263–273. doi:10.1017/S0954102014000728

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685–5689. doi:10.1073/pnas.89.12.5685

Filler DM, Lindstrom JE, Braddock JF, Johnson RA, Nickalaski R (2001) Integral biopile components for successful bioremediation in the Arctic. Cold Reg Sci Technol 32:14. doi:10.1016/S0165-232X(01)00020-9

Fukuhara Y, Horii S, Matsuno T, Matsumiya Y, Mukai M, Kubo M (2013) Distribution of hydrocarbon-degrading bacteria in the soil environment and their contribution to bioremediation. Appl Biochem Biotechnol. doi:10.1007/s12010-013-0170-x

Gojgic-Cvijovic GD, Milic JS, Solevic TM, Beskoski VP, Ilic MV, Djokic LS, Narancic TM, Vrvic MM (2011) Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: a laboratory study. Biodegradation 23:1–14. doi:10.1007/s10532-011-9481-1

Guibert L, Loviso C (2012) Alkane biodegradation genes from chronically polluted subantarctic coastal sediments and their shifts in response to oil exposure. Microb Ecol 64:605–616. doi:10.1007/s00248-012-0051-9

Jacques RJS, Okeke BC, Bento FM, Teixeira AS, Peralba MCR, Camargo FAO (2008) Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. Bioresour Technol 99:2637–2643. doi:10.1016/j.biortech.2007.04.047

Jasmine J, Mukherji S (2014) Environmental science evaluation of bioaugmentation and biostimulation effects on the treatment of refinery oily sludge using 2 n full factorial design. Environ Sci Process Impacts 16:1889–1896. doi:10.1039/C4EM00116H

Lane DJ (1991) Nucleic acid techniques in bacterial systematics. In: Stackebrandt E, Goodfellow M (eds) Journal of basic microbiology. Wiley, Chichester, pp 115–175

Louati H, Said O, Ben SA, Got P, Cravo-Laureau C, Duran R, Aissa P, Pringault O, Mahmoudi E (2014) Biostimulation as an attractive technique to reduce phenanthrene toxicity for meiofauna and bacteria in lagoon sediment. Environ Sci Pollut Res Int 21:3670–3679. doi:10.1007/s11356-013-2330-5

Luna GM, Stumm K, Pusceddu A, Danovaro R (2009) Archaeal diversity in deep-sea sediments estimated by means of different terminal-restriction fragment length polymorphisms (T-RFLP) protocols. Curr Microbiol 59:356–361. doi:10.1007/s00284-009-9445-4

Martínez-Rosales C, Castro-Sowinski S (2011) Antarctic bacterial isolates that produce cold-active extracellular proteases at low temperature but are active and stable at high temperature. Polar Res. doi:10.3402/polar.v30i0.7123

McDonald SJ, Kennicutt MC, Brooks JM (1992) Evidence of polycyclic aromatic hydrocarbon (PAH) exposure in fish from the Antarctic Peninsula. Mar Pollut Bull 25:313–317. doi:10.1016/0025-326X(92)90688-3

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nübel U, Garcia-pichel F, Kühl M, Ku M, Muyzer G (1999) Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Appl Environ Microbiol 65:422–430

Peixoto R, Chaer GM, Carmo FL, Araujo FV, Paes JE, Volpon A, Santiago GA, Rosado AS (2011) Bacterial communities reflect the spatial variation in pollutant levels in Brazilian mangrove sediment. Antonie van Leeuwenhoek 99:341–354. doi:10.1007/s10482-010-9499-0

Robles-González IV, Fava F, Poggi-Varaldo HM (2008) A review on slurry bioreactors for bioremediation of soils and sediments. Microbiol Cell Fact 7:5. doi:10.1186/1475-2859-7-5

Roling WFM, de Brito IRC, Swannell RPJ, Head IM (2004) Response of archaeal communities in beach sediments to spilled oil and bioremediation. Appl Environ Microbiol 70:2614–2620. doi:10.1128/AEM.70.5.2614

Ruberto L, Vazquez SC, Cormack WPM (2003) Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int Biodeterior Biodegrad 52:115–125. doi:10.1016/S0964-8305(03)00048-9

Ruberto L, Vazquez S, Mac WP (2008) Bacteriology of extremely cold soils exposed to hydrocarbon pollution. In: Dion P, Nautiyal CS (eds) Microbiology of extreme soils, 13rd edn. Springer, Berlin, pp 247–274

Ruberto L, Dias R, Lo Balbo A, Vazquez SC, Hernandez EAM, Cormack WP (2009) Influence of nutrients addition and bioaugmentation on the hydrocarbon biodegradation of a chronically contaminated Antarctic soil. J Appl Microbiol 106:1101–1110

Santos HF, Carmo FL, Paes JES, Rosado AS, Peixoto RS (2010) Bioremediation of mangroves impacted by petroleum. Water Air Soil Pollut 216:329–350. doi:10.1007/s11270-010-0536-4

Tarnocai C, Campbell IB (2002) Soils of the polar regions. In: Dekker M (ed) Encyclopedia of soil science, 2nd edn. Lal R, New York, pp 1018–1021

Turner S, Pryer KM, Miao VP, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46:327–338. doi:10.1111/j.1550-7408.1999.tb04612.x

Van Dorst J, Siciliano S, Winsley T, Snape I, Ferrari B (2014) Bacterial targets as potential indicators of diesel fuel toxicity in subantarctic soils. Appl Environ Microbiol. doi:10.1128/AEM.03939-13

Van Elsas J, Dijkstra A, Govaert J, Vanveen J (1986) Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol Ecol 38:151–160. doi:10.1016/0378-1097(86)90046-7

Vázquez S, Nogales B, Ruberto L, Hernández E, Christie-Oleza J, Balbo A, Lo BR, Lalucat J, Cormack WM (2009) Bacterial community dynamics during bioremediation of diesel oil-contaminated Antarctic soil. Microb Ecol 57:598–610. doi:10.1007/s00248-008-9420-9

Versalovic J, Schneider M, De Bruijn F, Lupski J (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Xu Y, Lu M (2010) Bioremediation of crude oil-contaminated soil: comparison of different biostimulation and bioaugmentation treatments. J Hazard Mater 183:395–401. doi:10.1016/j.jhazmat.2010.07.038

Yang S-Z, Jin H-J, Wei Z, He R-X, Ji Y-J, Li X-M, Yu S-P (2009) Bioremediation of oil spills in cold environments: a review. Pedosphere 19:371–381. doi:10.1016/S1002-0160(09)60128-4

Yu Z, García-González R, Schanbacher FL, Morrison M (2008) Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-specific PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 74:889–893. doi:10.1128/AEM.00684-07

Zanaroli G, Di Toro S, Todaro D, Varese GC, Bertolotto A, Fava F (2010) Characterization of two diesel fuel degrading microbial consortia enriched from a non acclimated, complex source of microorganisms. Microbiol Cell Fact 9:10. doi:10.1186/1475-2859-9-10