International Biodeterioration & Biodegradation 75 (2012) 96–103

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Bioremediation of an aged diesel oil-contaminated Antarctic soil: Evaluation of the "*on site*" biostimulation strategy using different nutrient sources

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ARTICLE INFO

Article history:
Received 21 March 2012
Received in revised form
12 June 2012
Accepted 24 July 2012
Available online 17 October 2012

Keywords:
Antarctica
Hydrocarbons contamination
Biostimulation
Soil remediation
Oil Spill Eater (OSEII)

ABSTRACT

Under Antarctic conditions, bioremediation processes are highly limited. Although chronically contaminated soils seem to require no bioaugmentation, biostimulation proved to be beneficial although diverse results have been reported in relation to the type of nutrient source and the best experimental design. In this work we evaluated, in "*on site*" land plots, the effect that on the hydrocarbon removal and bacterial community structure of a fuel contaminated soil have an inorganic salts mixture, a complex organic matrix (fish meal) and a commercial product listed by the EPA. Also the effect of a surface active compound (Brij700) on biodegradation process was studied. Brij700 did not improve biodegradation in any of the studied conditions but induced relevant changes on bacterial community of soil amended with fish meal. Although fish meal significantly enhanced bacterial counts, this effect was unspecific, drastically changed the bacterial community structure and did not improve hydrocarbon removal. Salts amended systems evidenced a non significant decrease in contaminant concentration. Commercial product caused the higher reduction (49.4%, $p < 0.05$) of hydrocarbons compared with the control system after 45 d of treatment and cause the minor changes in bacterial community, constituting a promising alternative for some hydrocarbon-contaminated Antarctic soil restoration.

1. Introduction

Oil-derivative fuels (diesel oil, gasoline, etc) are essential for most human activities in Polar Regions (continental Antarctica and the Arctic). Power supply, heating and vehicles (cars, ships and aircrafts) are fuels dependent. For that reason small contamination events frequently occur during transport and refuelling procedures (Aislabie et al., 2006; Rayner et al., 2007). This situation makes it necessary the development of remediation strategies in order to keep Antarctic soils and waters under adequate environmental conditions (Wardell, 1995; Snape et al., 2001). Up to date, several physical and chemical methods have been used worldwide for removal of oil and its derivatives from soils (Khan et al., 2004), but many of them are very expensive to be applied on a full scale and generally only move the contaminant from one site to another. For these reasons, the processes that harness the natural degradative capacity are, at the present days, the tool of choice for *on site* remediation of hydrocarbon contaminated soils (Rayner et al., 2007; Yang et al., 2009). Scientific Antarctic Stations are located in areas where temperature seldom exceeds 10 °C and the soil remains frozen most of the year, determining a very low biodegradation activity (Brakstad, 2008). This low microbial growth rate increases during the Antarctic summer, favouring a significant rise in the indigenous bacterial number and activity. To take advantage of this increased bacterial activity during the short summer period could be the basis of an efficient, environmentally-friendly, versatile and economic tool to eliminate organic pollutants from Antarctic soils.

Many Antarctic soils have low levels of phosphorous and nitrogen and, when a significant amount of hydrocarbon is spilled on these soils, the C:N:P ratio becomes unbalanced, reaching values

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http://dx.doi.org/10.1016/j.ibiod.2012.07.020

far from the 100:10:1, which has been largely considered as the most adequate ratio to promote microbial growth (Ron and Rosenberg, 2010). The adjustment of the C:N:P ratio by fertilization (biostimulation strategy) have been reported as a powerful tool to enhance the removal of different organic contaminants in diverse environments (Margesin and Schinner, 2001; Röling et al., 2002). However, other authors have found a negative effect of nutrient addition; probably occurring when the concentration exceeds a certain threshold that mostly depends on the characteristics of the local natural microbiota (Braddock et al., 1997). These results evidenced that both, the sources of nutrients and the fertilization strategy are relevant factors that should be taken into account to achieve a successful bioremediation process (Walworth et al., 2007; Liu et al., 2010).

Some authors have reported that surfactants contribute to enhance microbial hydrocarbon degradation activity (Zhou et al., 2008). However, other studies mentioned no positive or even negative effects of the surface-active compounds on degradation activity (Chen et al., 2001; Mosche and Meyer, 2002). These previous studies evidenced that the effect caused by the addition of a surfactant in a contaminated soil depends on several factors, including how microorganisms interact with hydrocarbons, the chemical structure, hydrophobicity and bioavailability of the pollutants and the physicochemical properties of the soil, among others. For this reason, and taking into account that the addition of surface-active compounds increases the cost of the processes, it is relevant to evaluate the effect of these compounds on the biodegradation of pollutants for each particular pollution case using *on site* experimental approaches.

Considering the above mentioned antecedents, the main objective of this study was to evaluate the effect of different biostimulation strategies on the diesel fuel removal efficiency and to analyse the changes suffered by the structure of the soil bacterial community under bioremediation treatment. For this purpose we amended, using organic and inorganic nutrient sources, a chronically contaminated Antarctic soil in the presence or absence of a surfactant agent. The study was performed in Carlini (Jubany) Station, located in the coast of Potter Cove, close to the Antarctic Specially Protected Area (ASPA) N°132. Because of the permanent human presence and the great volume of fuels required, soils near the station are under continuous risk of pollution. Our aim is that these results can contribute in the near future to the design of a simple and easy-to-apply protocol for its use after contamination events of Antarctic soils.

2. Materials and methods

2.1. Experimental design

Experiment was carried out at Carlini (Jubany) Station (62° 14′ S, 58° 40′ W), located on Potter Cove, 25 de Mayo Island (King George Island), South Shetland Islands. Chronically hydrocarbon-contaminated soil used in this work had been collected from the concrete pools containing the fuel-storage tanks and was kept for more than two years in metal containers. After manually removal of the large stones the soil was sieved (2 mm mesh) in order to obtain a homogeneous material.

Eighteen land-plot microcosms (15 cm depth, 50 cm length and 50 cm width) were set in January 2007. Plots were delimitated with a wooden frame and caved to the desired depth. The caves were filled with the contaminated soil previously mixed with different components, according to the five designed treatments: NSS (soil amended with inorganic salts), NSSB (soil amended with inorganic salts and surfactant Brij700), FM (soil amended with fish meal), FMB (soil amended with fish meal and surfactant Brij700) and CP (soil amended with a commercial product). An additional system CC (non amended soil) was performed as control (see Table 1 for a more detailed description of the systems). Among the nutrient sources used in the experimental systems, inorganic salts represent a water-soluble component. Fish meal, although not considered strictly as a slow-release nutrient, represents a rich N and P source that reduces the washing in the natural environment. The commercial product OSEII® (Oil Spill Eater International, Corp.) is reported as an oleophilic material containing N and P that could be also delaying the washing processes, more likely suffered by the hydrophilic nutrients.

Blocks of three plots were run per treatment, distributed at random on the ground and sampled at 0, 6, 10, 15, 25, 35 and 45 d. Each microcosm sample was prepared by combination of three subsamples and used for chemical, microbiological and molecular analyses. The surface active compound selected for this study was Brij700®, a high molecular weight polyoxyethylene stearyl ether (MW 4670) having a hydrophilic/lipophilic balance of 18.8 and representing a potent solubilizer agent. The commercial bioremediation agent OSEII® was also used. This bioremediation agent is listed on the US Environmental Protection Agency's National Contingency Plan for Oil Spills and is reported as a nutrient/enzyme additive consisting of "nitrogen, phosphorus, readily available carbon, and vitamins for quick colonization of naturally occurring bacteria" (Zhu et al., 2004).

2.2. Soil analysis and total hydrocarbons quantification

The soil was analysed for texture by the pipette method (Gee and Bauder, 1986), organic carbon (Walkley and Black, 1934), extractable phosphorous (Bray and Kurt, 1945) and total Kjeldhal nitrogen. Water content was determined gravimetrically by heating samples at 105 °C during 24 h. For pH measurements, 10 ml of sterile saline solution (NaCl 8.9 g l^{-1}) were added to 1 g of soil and vortexed for 1 min. Total hydrocarbon concentration (THC) in soils was measured based on the EPA 418.1 method (USEPA, 1983). Briefly, 1 g of soil was accurately weighed and placed into 20 ml glass-flasks. HPLC grade CCl_4 (10 ml) and a spatula-tip of anhydrous Na_2SO_4 were added to each flask. Flasks were sonicated overnight in an ultrasound bath. Samples were then filtered, transferred to a quartz-cell and analysed (Buck Model HC 404 Hydrocarbon analyzer IR spectrometer). Results were expressed as μg g^{-1} of dry soil (dw).

Table 1
Conditions applied to the different microcosms containing fuel-contaminated Antarctic soil.

Microcosm	Treatment	Description
CC	Untreated soil	Chronically contaminated soil placed in the plot without any treatment.
NSS	Biostimulation with inorganic salts	NH_4NO_3, Na_2HPO_4 and KH_2PO_4 added to chronically contaminated to reach a C:N:P ratio of 100:10:1 based on total organic carbon
NSSB	Biostimulation with inorganic salts + surfactant	Idem NSS but added with 0.3% w w^{-1} of Brij700®
FM	Biostimulation with a complex organic matrix	Chronically contaminated soil amended with 3% w w^{-1} of fish meal.
FMB	Biostimulation with a complex organic matrix + surfactant	Chronically contaminated soil amended with 3% w w^{-1} of a mix of fish meal and surfactant Brij700® (9:1).
CP	Biostimulation with a commercial product	Chronically contaminated soil added with a commercial bioremediation agent (OSEII®).

2.3. Culturable bacterial counts

One gram of recently sampled soil was suspended in 10 ml of sterile saline solution with 1% v v^{-1} Tween80 and vigorously shaken for 5 min. Serial dilutions of soil suspension were plated on casein–peptone–starch (CPS) agar (Wynn-Williams, 1992) for the evaluation of total heterotrophic aerobic bacteria (HAB) and on solidified saline basal medium (SBM) supplemented with 2% v v^{-1} diesel oil for enumeration of hydrocarbon degrading bacteria (HDB). All plates were incubated 30 d at 15 °C and the results were expressed as colony forming units per gram of dry weight (CFU g^{-1} dw).

2.4. DNA extraction

Total community DNA was extracted from samples using a modification of the protocol described by Kuske et al. (1997). A mix (solution A) containing 2.25 ml of tris–EDTA (TE) buffer, 0.25 ml of sodium dodecyl sulphate (SDS) solution (10% w v^{-1}), 35 μl of lysozyme (300 mg ml^{-1}) and 30 μl of proteinase K (10 mg ml^{-1}) were added to 2 g of soil, in sterile tubes. The samples were incubated during 1 h at 37 °C followed by another 30 min at 70 °C, mixing the tubes every 15 min. After that, 1.5 g of glass beads (0.1 mm, Sigma) was added to the tubes and they were manually shaken for 4 min and then centrifuged for 10 min at 6000 rpm. The obtained supernatants were placed on ice and the soil pellets were added with fresh A solution but containing no lysozyme or proteinase K. Tubes were incubated for 30 min at 70 °C and processed as was described above. The two supernatants were pooled in a new sterile tube and extracted twice with 1 volume of a mix of chloroform:isoamyl alcohol (1:1). After centrifugation at 8000 rpm during 5 min, the aqueous phase was separated and placed in a new sterile tube and 0.1 volume of 5 M NaCl solution and 0.6 volume of isopropyl alcohol were added. Tubes were incubated for 10 min at room temperature and centrifuged for 20 min at 14,000 rpm. DNA pellets were washed with 2.5 volumes of 70% v v^{-1} of ethanol, centrifuged at 14,000 rpm for 15 min and the supernatant was discarded. DNA pellets were air-dried and resuspended in 50 μl of sterile TE buffer, pre-heated at 60–80 °C. An additional purification step with a commercial kit (Illustra Blood GenomicPrep Mini Spin Kit, GE) was employed to avoid PCR inhibition with humic acids. DNA quality was confirmed by agarose gel electrophoresis stained with Ethidium Bromide.

2.5. PCR amplification and DGGE of bacterial community

Two independent amplifications by polymerase chain reaction (PCR) were made for each sample. In each one, the V3–V5 variable regions of the 16S rRNA gene were amplified using GC-341F (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTCCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) primers (Muyzer et al., 1998). The PCR reaction mixture contained 1 μl of the extracted genomic DNA, 5 μM of each primer, 2.5 μM of each deoxyribonucleotide triphosphate, 10 mg ml^{-1} of bovine serum albumin, 1 U of GoTaq (Promega) and 1× GoTaq buffer, in a final volume of 30 μl. The PCR program included an initial denaturation step for 4 min at 95 °C; ten cycles consisting of 94 °C for 30 s; 62 °C for 45 s; and 1 min at 72 °C followed by a step down of 30 s at 94 °C, 45 s at 57 °C, and 72 °C for 1 min (25 cycles) and a final extension at 72 °C for 10 min. The PCR products were analysed by agarose gel electrophoresis. The purified PCR amplicons were applied onto 6% w v^{-1} polyacrylamide gels (acrylamide:N,N′-methylenebisacrylamide, 37.5:1) in a denaturing gradient gel electrophoresis (DGGE) that was performed on Bio-Rad D GENE System (Bio-Rad, Munich, Germany). The denaturing gradient used ranged from 45 to 70 % denaturant (100% denaturant corresponds to 7M urea and 40% v v^{-1} formamide). DGGE was performed in 1× TAE buffer containing 10 mM of sodium acetate, 0.5 mM of Na_2-EDTA and 20 mM of Tris base, pH 7.4, at 100 V and 60 °C for 16 h. The gels were stained for 1 h with SybrGold (Invitrogen) in 1× tris-acetate–EDTA (TAE) buffer, viewed under UV light and analysed using GelComparII software package (Applied Maths, Kortrijk, Belgium). In order to normalize the gel and band positions, sample FMB was used as a standard in the first and last lanes of the gel (not shown in Fig. 3).

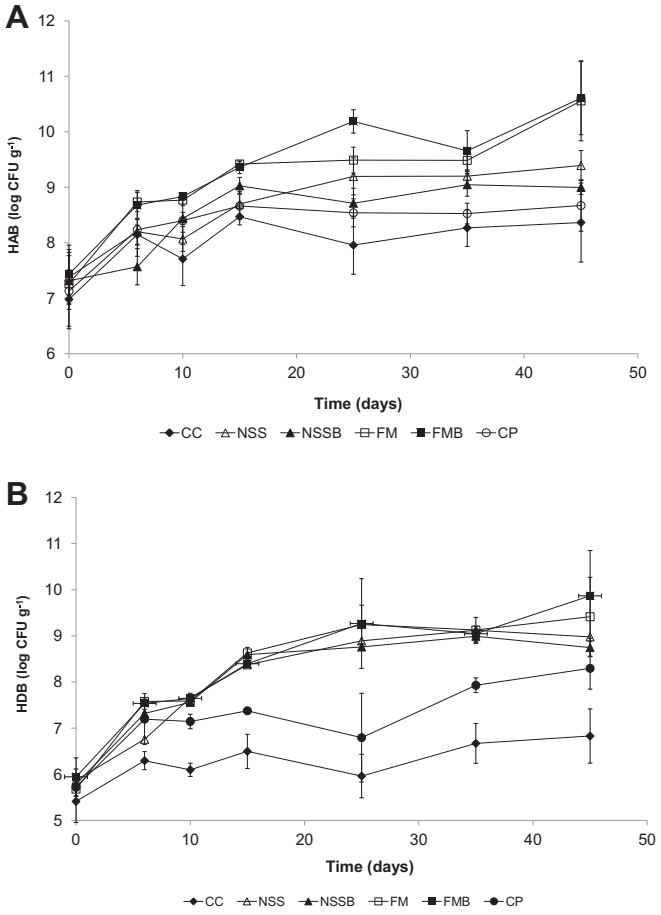

Fig. 1. Changes in heterotrophic aerobic bacterial counts (A) and hydrocarbon degrading bacterial counts (B) during the bioremediation assay in Antarctic soil microcosms with different biostimulation strategies. CC: control system, FM: system amended with fish meal, FMB: system amended with fish meal and Brij700, NSS: system amended with inorganic salts, NSSB: system amended with inorganic salts and Brij700, CP: system amended with the commercial product OSEII®. Bars represent SD of three independent replicates. For description of each microcosm see Table 1.

Similarity matrixes of the banding patterns were made using Pearson's product correlations (Pearson, 1926). The dendrogram was calculated by the unweighted pair group method with arithmetic mean (UPGMA) (Sokal and Michener, 1958). Principal components analysis (PCA) was made using InfoStat statistic software (Di Rienzo et al., 2001). Richness (number of bands, S), dominance (Berger–Parker index, D) and diversity (Shannon–Wiener index, H) values were calculated using PAST data analysis package (Hammer et al., 2001). DGGE banding data were used to estimate the diversity indexes treating each band as an individual OTU and using the number of bands as an indicator of richness (S). The H and D indexes were calculated from the number of bands present and their relative intensities.

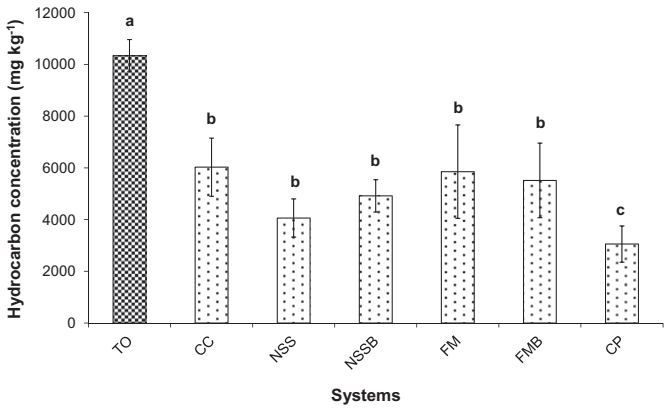

Fig. 2. Total hydrocarbon concentration (THC) in soil at day 1 and at the end of the assay (45 d) in the different microcosms systems. T0: control system at day 1, CC: control system at day 45, NSS: system amended with inorganic salts at day 45, NSSB: system amended with inorganic salts and Brij700 at day 45, FM: system amended with fish meal at day 45, FMB: system amended with fish meal and Brij700 at day 45. Bars represent SD of three independent replicates. Different letters represent significant differences between systems. For description of each microcosm see Table 1.

2.6. Statistical analysis

Hydrocarbon concentration and bacterial counts data obtained from the different systems were compared by One-way Analysis of Variance (ANOVA) and Dunnett or Tukey Multiple Comparisons Tests.

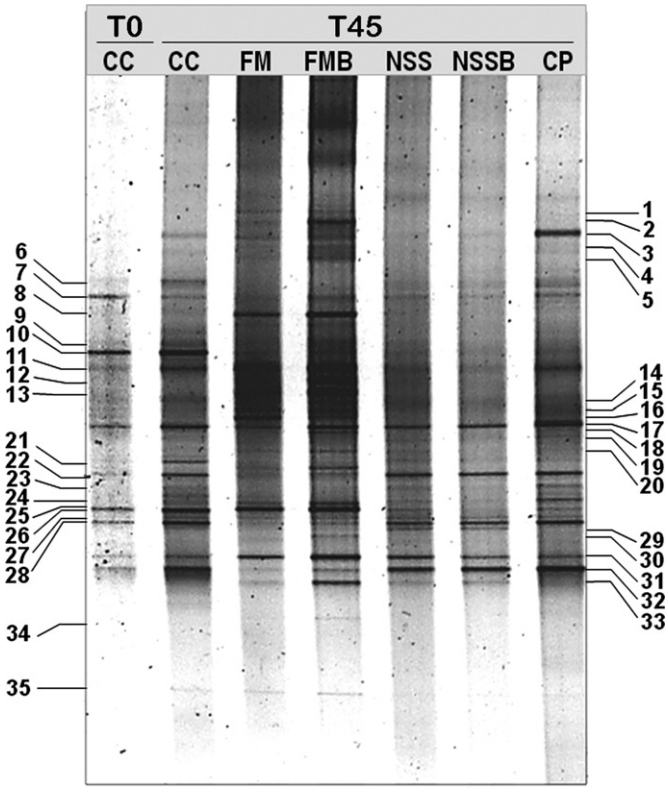

Fig. 3. Fingerprinting from 16S rRNA gene fragments for samples of the six soil microcosms. Thirty-five bands were identified. T0: initial sample (day 1), T45: end of the assay (day 45), CC: control system, FM: system amended with fish meal, FMB: system amended with fish meal and Brij700, NSS: system amended with inorganic salts, NSSB: system amended with inorganic salts and Brij700, CP: system amended with the commercial product OSEII®. For description of each microcosm see Table 1.

3. Results

3.1. Soil properties

The soil had a sandy texture, containing 1.8% clay, 3.8% silt, and 94.4% sand. Organic carbon, total Kjeldhal nitrogen and extractable phosphorous levels were 10.21 g kg^{-1}, 0.32 g kg^{-1} and 5.0 mg kg^{-1} respectively. The pH was 6.8 and the water content 10%. This chronically contaminated soil had an initial total hydrocarbon concentration of 10,336 mg kg^{-1} dw. Whereas mean air temperature during the experiment was 2.6 C° (range −2 C° to +7.8 °C), soil temperature showed a mean value of 4.8 °C and a range of −1.4 to +15.1 °C.

3.2. Heterotrophic bacterial counts

The effect of the different treatments on heterotrophic aerobic bacterial counts (HAB) is shown in Fig. 1A. All systems evidenced an early stimulation of the counts during the first 15 d. After that, all systems tended to stabilize the HAB levels until the end of the study. The exception were systems containing fish meal (FM and FMB) where heterotrophic bacteria increases until day 45 significantly differentiating from all the others systems ($p < 0.05$) and evidencing that the presence of this complex organic source of nutrients greatly enhance the heterotrophic bacterial growth despite the presence or absence of the surfactant.

When only the final HAB counts values (at d 45) were compared, the fish meal amended systems showed to be significantly different ($p < 0.05$, one way Anova and Tukey post test) compared to all the rest of the amended systems and also compared to CC ($p < 0.01$ one way Anova and both, Dunnet and Tukey post tests). The other treatments were not different from the control.

3.3. Diesel-degrading bacterial counts

The level of hydrocarbon degrading bacteria (HDB) in the control system (Fig. 1B) raised only one order of magnitude along the study determining HAB/HDB ratios exhibiting minor changes with time (range 0.75—082). Evolution of hydrocarbon degrading bacteria in all the other systems significantly differed ($p < 0.01$) from the control (CC). System containing OSEII® evidenced a similar behaviour compared to CC but at higher HDB values ($p < 0.01$). Control system also differed from those containing fish meal but not from the salts amended systems (although both, NSS and NSSB were at the limit of significance). At the end of the assay (45 d) HDB values in all treated systems were significantly higher than the control ($p < 0.01$) but no significantly differences were evidenced between them. At this time HAB/HDB ratios of the treated systems increased, rising to 0.89 in FM, 0.93 in FMB, and 0.96 in CP, NSS and NSSB.

3.4. Total hydrocarbon biodegradation

From the initial level of hydrocarbon measured in soil, CC system exhibited a reduction of 42% as result of the abiotic processes and the biological removal by the already adapted microbiota of the soil (Fig. 2). Despite the great increase in bacterial counts observed in fish meal amended systems, they showed a not significant reduction in hydrocarbons level compared with CC at the end of the assay. Although salts amended systems showed higher hydrocarbon removal compared to FM systems, no significant differences were detected between salts (NSS and NSSB) and fish meal (FM and FMB) fertilized systems. However, the OSE amended system showed a significantly higher ($p < 0.05$) hydrocarbon removal compared with all the other treated systems. In this

sense, at d 45, CP exhibited 49.4% of hydrocarbon removal compared with CC system. CP also showed to be more efficient compared with FM, FMB, NSSB and NSS (47.8%, 44.6%, 37.9% and 24.8% respectively). Considering that the studied soil contained a high initial level of contaminants and that it was chronically contaminated, a removed fraction of 70.5% of the initial level in only 45 d under Antarctic conditions can be considered as promissory. The presence of the surfactant did not evidence any positive effect on biodegradation efficiencies as was evidenced by the similar values exhibited by systems with and without Brij despite the source of nutrient used in biostimulation (fish meal or salts).

3.5. Effect of the treatments on the dynamic of soil bacterial community

DGGE pattern of the soil bacterial communities from the different microcosms at the end of the assay and the control system at day 1 are shown in Fig. 3. The dendrogram obtained from the cluster analysis, after the densitometric study of the gel and construction of the similarity matrix is shown in Fig. 4. Dendrogram evidenced that the fish meal amended systems (with or without Brij) suffered notorious changes in bacterial community composition at d 45 not only in comparison with the CC at d 1 but also compared with all the other systems at the end of the assay. This fact suggests that fish meal promote the growth and predominance of bacterial populations different to those favoured under the environmental conditions present in the rest of the microcosms.

Bacterial community structure in CC at d 45 evidenced changes compared with CC at d 1. However, several bands in both microcosm pattern were present during all the trial and, at the end of the assay (t45) exhibited a higher intensity than at the beginning of the experiment (t0). This could be reflecting a positive effect of the aeration and mixing of this contaminated soil on some aerobic populations of the natural bacterial community, which passed to predominate at the end of the assay. The salt amended microcosms NSS and NSSB exhibited similar DGGE profiles and, although included in the same subcluster, they showing notorious changes compared with the CC and CP systems. Changes induced in NSS by the presence of Brij seem to be minor, as occurred also with FM systems. It is interesting to note that bacterial community structure in OSEII® amended system showed a similar evolution (percent similarity of 74%) that those showed by CC, suggesting that among treated systems this commercial product was those causing the minor changes compared with the control.

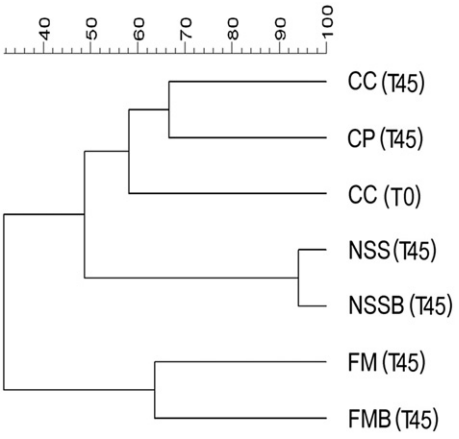

Fig. 4. UPGMA dendrogram resulting from cluster analysis of the data reporting the presence/absence and abundance of DGGE bands in the six microcosms systems at the end of the assay (T45) and the control system at day (T0). CC: control system, FM: system amended with fish meal, FMB: system amended with fish meal and Brij700, NSS: system amended with inorganic salts, NSSB: system amended with inorganic salts and Brij700, CP: system amended with the commercial product OSEII®. For description of each microcosm see Table 1.

Analysis of the diversity parameters in the different treatments (Table 2) showed that all systems, including CC, increased their richness at the end of the assay. In accordance with the high increases in bacterial numbers and with differences observed in the DGGE banding patterns fish meal amended systems exhibited the higher increases in richness, probably by favouring several populations able to the growth on easily degradable carbon sources. Changes in CC system were significant, exhibiting an increase in the H index caused by an increase in richness (S index) accompanied by a decrease in dominance (D index). In comparison with control system at day 1, CP system at d 45 showed a moderate increase in The H index, caused mainly by the increase in richness seeing as the dominance value did not evidence changes.

PCA analysis (Fig. 5) clearly confirms differentiation of the FM systems from the rest of treatments, with the two main components explaining 60.6% of the variance. A remarkable point is that the FM systems clearly favoured a group of DGGE bands which was different to those that were present in the CC, CP and NSS systems. Another groups of systems defined with this analysis comprised the salt amended systems (NSS and NSSB) whose showed minor differences between them. The third group was represented by the control soil (CC) at the initial and final time and the CP. Changes suffered by CC from d 1 to d 45 were probably caused by the aeration and manipulation of soil and CP system evidenced a similar evolution, being no differentiated from CC at d 45 by the PC 1.

4. Discussion

Antarctic continent, as a whole, do not represent a site exhibiting serious problems of hydrocarbons contamination. Although when such problems occurs it could be considered as restricted to a small area, the isolation and the strict permanent environmental conditions turn any contamination event of significant environmental concern and hard to remediate. For this reason, the development of *in situ* or *on site* bioremediation systems is crucial to confront this problem and to comply with the proposals of the international Antarctic community (ATCM XXXIV, 2011).

The microcosm-based study presented in this paper has revealed a number of relevant facts. One of these facts is represented by the magnitude of the detected natural attenuation, which reduced 42% of the initial hydrocarbon concentration. Although soils suddenly contaminated with diesel fuel in temperate areas (where both, abiotic factors and degradation activity of the natural microbiota, exhibit rates higher than those present in cold areas) have shown very high levels (90%) of natural attenuation (Sarkar et al., 2005), it is known that low temperatures and ageing of contaminated soils, which increase interaction between soil particles and hydrocarbons reducing bioavailability (Alexander, 2000),

Table 2
Values of richness, dominance and diversity calculated from DDGE band patterns obtained from hydrocarbon contaminated soil at the start of the experiment and from all the microcosms at d 45. For description of each microcosm see Table 1.

Microcosm	Richness (S)	Dominance (D)	Diversity (H)
CC (t0)	10	0.143	2.030
CC (t45)	16	0.092	2.566
NSS	9	0.133	2.063
NSSB	8	0.168	1.940
FM	17	0.096	2.485
FMB	27	0.071	2.915
CP	14	0.124	2.292

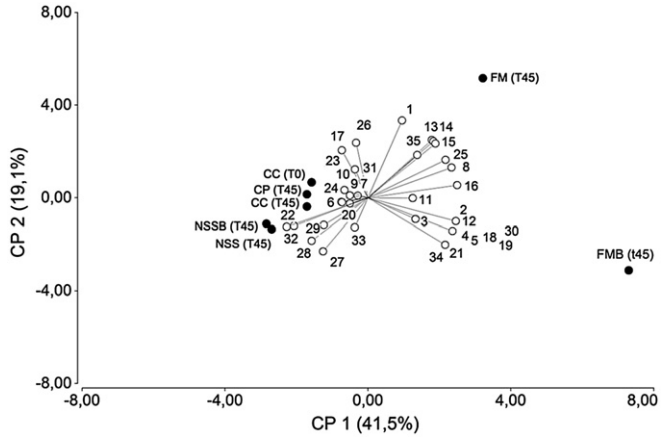

Fig. 5. Biplot corresponding to the principal component analysis (PCA) showing the differentiation of the FM systems from the rest of treatments. Letters in the vectors represents the bands as were labelled in the DGGE gel from Fig. 3. CC (T0): control system at day 1. CC (T45): control system at day 45, FM (T45): system amended with fish meal at day 45, FMB (t45): system amended with fish meal and Brij700 at day 45, NSS (T45): system amended with inorganic salts at day 45, NSSB (45): system amended with inorganic salts and Brij700 at day 45, CP: system amended with the commercial product OSEII® at day 45. For description of each microcosm see Table 1.

reduce hydrocarbon removal from cold soils. For this reason, chronically contaminated Antarctic soils, as the studied in this work, not only usually exhibit natural attenuation levels lower than the corresponding acutely polluted Antarctic soils but also showed levels notoriously lower than the reported for chronically contaminated soils from temperate areas. In this sense, an own previous study using land plots as experimental model showed only 10% of hydrocarbon removal attributable to natural attenuation (Vázquez et al., 2009). It's important to highlight that in the mentioned study the assay was performed using the 50 cm depth surface soil layer, without any intermediate storage. For this reason, the soil had the natural water content and a low degree of compression that could have favoured a significant level of removal of hydrocarbons previously to its use as experimental system (mainly by stripping and evaporation of the lighter fraction by abiotic factors). On the contrary, the present study was made with a chronically polluted soil that has been removed from their original site and maintained highly compressed in metal containers during two years. These could have avoided significant loss of hydrocarbons either by abiotic loss of the volatile fraction or by microbial activity, factors that only could have turned significant when soil was prepared for the assay, resulting in the high level of natural attenuation observed.

This remaining of a large fraction of the fuel components in the soil was observed by other authors working in Antarctica (Aislabie et al., 2006) as well as in several other cold-climate environments, as Patagonia (Acuña et al., 2008) and the Arctic (Mohn et al., 2001). The permanence of this great fraction of the hydrocarbons initially spilled on Antarctic soils has been registered even several years after contamination event (Coulon and Delille, 2006).

Because natural attenuation in Antarctica do not reach the desired soil restoration at the required levels and times, is that biostimulation and bioaugmentation are considered as relevant tools to shorten the bioremediation processes in Antarctica, making it compatible with the short summer period in which microbial activity is significant, at least in sites located in the Antarctic Peninsula, as Carlini station is.

Bacterial counts values also highlight the effect of manipulation and preparation. All experimental systems, including control, evidenced a rapid increase in HAB during the first 15 d. This positive effect of the soil manipulation that caused a one order of magnitude increase in counts had been previously observed by us in Antarctic soil bioremediation systems in microcosms (Ruberto et al., 2006; Vázquez et al., 2009). It was evident that the presence of a complex organic nutrient source, as the fish meal, represented a strong additional stimulus for the soil bacterial growth and permitted a continuous increase in HABC, even during the last 20 d, when the rest of the experimental systems showed no significant increases in HAB counts. This observation suggests that fish meal not only represents a long-lasting source of inorganic nutrients but also provides soil microorganisms with a long-term source of easily degradable organic molecules. However, the fact that FM systems exhibited significant differences in HAB counts but no differences in HDB compared with all the other systems at d 45, shows that the growth enhancing induced by the fish meal in this contaminated soils was unspecific, favouring any population able to metabolize the easily degradable substrates but failing in promote a differential growth and activity of the hydrocarbon degrading populations. This fact could explain the low hydrocarbon removal efficiency showed by the fish meal amended systems, whose values were similar to the non fertilized control system. Also agree with the molecular analysis of the bacterial community composition, where FM systems were clearly separated from all the other systems by both the cluster and PC analysis. It is possible that fish meal allowed the growth of some populations of the community, initially present in very low and non detectable levels in this Antarctic soil that proved to have low levels of organic carbon sources. The fast growth response in the presence of the meal substrates could have increased their abundance, reaching detectable levels by the used DNA fingerprint methods. Although as far as we know no studies have been conducted in order to analyse the changes caused by fish meal on the community structure of a soil, Delille and Coulon (2008) reported a positive effect of fish meal addition on bioremediation of a sub Antarctic soil contaminated with diesel fuel. Although this positive effect was observed after a period of time as long as one year and using a different experimental model (biopiles and acute contamination on a pristine soil), the different response of the bacterial community to the same source of nutrients reinforce the assessment of a close case-dependency of the bioremediation efficiency in Antarctic soils. In relation to the inorganic salts amended systems, the level of BDH at d 45 were similar to those observed for the FM systems. However, although remnant hydrocarbon concentration values tended to be consistently lower than in the fish meal amended systems, such differences were not statistically significant. This represented an unexpected result, because even though it has been reported that high initial levels of salts added as nutrients to the Antarctic and Arctic soils could result in an inhibition of the biodegradation activity (Braddock et al., 1997; Ruberto et al., 2003), at the level used in this work, several own previous studies (Ruberto et al., 2008, 2010; Vázquez et al., 2009) as well as from other authors (Margesin and Schinner, 2001; Powell et al., 2006; Delille and Coulon, 2008) have found that the adjustment of N and P concentration produce a significant increase in biodegradation activity. Evidently, also the response of the Antarctic soil bacteria to biostimulation is highly influenced by the soil properties, as was clearly indicated by Delille and Coulon (2008) from mesocosms studies with two different soils at sub Antarctic Kerguelen Island. It is also possible that, under the strict natural conditions and starting with a highly compacted soil that remained two years in containers, 45 d of treatment were not enough to reveal significant changes between these systems. It is possible that longer time period of process could evidence such differences.

The commercial product OSEII® was the only one exhibiting a significant improvement in hydrocarbon removal compared with

CC. Although this product does not contain living bacteria in their composition, the combination of the enzymes, nutrients and surfactant improved biodegradation under the Antarctic conditions. The continuous increase in HDB observed until the end of the assay in this experimental system also suggests that OSEII® maintained the adequate releasing of the inorganic nutrients required by the natural microbiota to grow all along the study. Evidently, this commercial product does not provide the microorganisms with organic sources of nutrients, favouring only those populations able to use hydrocarbons as carbon and energy source. Interestingly, CP system also caused the minor changes in the bacterial community structure, as estimated by DGGE patterns. These results reinforce the above commented effect of OSE, because suggest that this commercial product stimulated mainly some bacterial populations previously present at detectable levels in the soil, which would proliferate using hydrocarbons as carbon source. These favoured populations could be the responsible of active hydrocarbon degradation, resulting in the best degradation removal with the minor changes in bacterial community structure. This assumption is also supported by the diversity index showed in Table 2. Increase in H index evidenced by the control system (from d 1 to d 45) was caused mainly by the increase in richness and, in a minor extent, by a decrease in dominance. Compared with the evolution of the CC at d 45, CP system showed a lower diversity index, caused by a slight decrease in richness and a slight increase in dominance. The PCA reinforce the evidence that CC and CP evolved in a similar way and differed from salt and fish meal amended systems.

It is well known that DGGE method has a number of limitations to describe microbial diversity (Pedrós-Alió, 2006). This technique allows amplification of only the dominant members of the soil bacterial community and, due the use of universal primers and the high similarity between 16S rRNA gene sequences, it is possible that more than one species is represented in each band and also that bands on the same level in a gel correspond to different species. In this sense, the number of bands obtained from each community allows estimating only the minimum number of dominant OTUs present in a sample. Although the true diversity of the studied bacterial communities cannot be inferred from the band patterns, the classical richness and diversity indexes can be calculated from the matrices obtained from the DGGE gels and used to compare the structure of communities from different samples and the changes suffered under different conditions or treatments.

Although both, increases (Zucchi et al., 2003; Kaplan and Kitts, 2004) and reductions (Ogino et al., 2001) of bacterial diversity were reported during bioremediation processes, in our study we observed lower H index values compared with the control system at the same time. The exception was FMB system. No previous studies about the effect of different biostimulation strategies on the bacterial community structure of hydrocarbon contaminated Antarctic soils were reported. However, Jimenez et al. (2007), who studied the bacterial community dynamics during a field biostimulation assay in land plots defined on a beach affected by the *Prestige* heavy fuel-oil spill, found that the addition of the oleophilic fertilizer S200 caused an important change of the microbial community at 30 d. In contrast, in our study, OSEII fertilizer caused minor changes in the community structure at d 45 contrasting with the notorious changes induced by fish meal amendment.

The absence of a positive effect when Brij was added represents other relevant point. Positive (by increasing solubility and dissolution of hydrocarbons or enhanced mass transport) and negative effects (attributed at a great number of factors) of the addition of surfactants to soil have been previously reported by several authors (Li and Chen, 2009). Even into the Brij family of surfactants, effect caused on hydrocarbon biodegradation has been contradictory. So, The use of non-ionic Brij's surfactants was reported as positive on naphthalene contaminated aqueous systems (Boonchan et al., 1998), phenanthrene containing solutions (Zhao et al., 2005), soil slurry (Kim et al., 2001) and PAHs contaminated soils (Bueno-Montes et al., 2011). However, Brij's surfactants did not enhanced or even evidenced negative effect on hydrocarbons biodegradation in several other studies (Laha and Luthy, 1992; Yuan et al., 2000). Although the reasons why the addition of Brij did not enhance biodegradation in this experiment were not investigated, it is possible that the main components of the remaining fuels in the studied soil (mainly linear and ramified aliphatic between C10 and C20) have been not significantly affected in their diffusion rates and bioavailability in the presence of the surfactant as have been reported that the aromatic hydrocarbons are (Volkering et al., 1995; Garon et al., 2002).

In summary, based in these results and those previously obtained with contaminated Antarctic soils we can conclude that the success of bioremediation strategies for Antarctic soils is highly site-specific and depends on contaminant properties (and history), the nature of the nutrient products and the characteristics of the environments. Despite that several own previous works proved the efficiency of salts mediated biostimulation, the present work failed to evidence this fact. Although in general, commercial oleophilic nutrient products have not shown to be more effective than the agricultural fertilizers or inorganic salts in stimulating oil degradation, in this particular situation, the OSEII product evidenced the better performance with the minor perturbation of the natural bacterial community structure, constituting a promising alternative for some hydrocarbon contaminated Antarctic soil restoration.

Acknowledgements

We acknowledge the financial support of this research by the Agencia Nacional de Promoción Científica y Tecnológica (PICTO N° 35778) and the Universidad de Buenos Aires (UBACyT 001).

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