Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Influence of nutrients addition and bioaugmentation on the hydrocarbon biodegradation of a chronically contaminated Antarctic soil

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Keywords
Antarctica, bacterial consortia, bioremediation, biostimulation, hydrocarbon-contaminated soil.

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2008/0249: received 13 February 2008, revised 21 July 2008 and accepted 10 September 2008

doi:10.1111/j.1365-2672.2008.04073.x

Abstract

Complexity involved in the transport of soils and the restrictive legislation for the area makes on-site bioremediation the strategy of choice to reduce hydrocarbons contamination in Antarctica. The effect of biostimulation (with N and P) and bioaugmentation (with two bacterial consortia and a mix of bacterial strains) was analysed by using microcosms set up on metal trays containing 2·5 kg of contaminated soil from Marambio Station. At the end of the assay (45 days), all biostimulated systems showed significant increases in total heterotrophic aerobic and hydrocarbon-degrading bacterial counts. However, no differences were detected between bioaugmented and nonbioaugmented systems, except for J13 system which seemed to exert a negative effect on the natural bacterial flora. Hydrocarbons removal efficiencies agreed with changes in bacterial counts reaching 86 and 81% in M10 (bioaugmented) and CC (biostimulated only) systems. Results confirmed the feasibility of the application of bioremediation strategies to reduce hydrocarbon contamination in Antarctic soils and showed that, when soils are chronically contaminated, biostimulation is the best option. Bioaugmentation with hydrocarbon-degrading bacteria at numbers comparable to the total heterotrophic aerobic counts showed by the natural microflora did not improve the process and showed that they would turn the procedure unnecessarily more complex.

Introduction

Contamination of soils with hydrocarbons has been reported in extremely cold areas like the Arctic (Whyte *et al.* 2001) and Antarctica (Aislabie *et al.* 2004). Because diesel fuels are required in large volumes for Antarctic activities, soils near stations are under an environmental risk that must not be ignored (Mac Cormack and Fraile 1997). In fact, chronically contaminated areas have been previously reported in several studies (Delille and Pelletier 2002; Aislabie *et al.* 2006). It is known that spillage of fuels into Antarctic soils induces an adaptive response of the indigenous microbial communities, leading to an enrichment in hydrocarbon-degrading bacteria (HDB; Aislabie *et al.* 2001; Ruberto *et al.* 2003). Bioremediation originated as a way to make profitable use of this natural response to the pollutants. It is an effective, low-cost and environmental-friendly tool to improve removal of contaminants from the environment (Gallego *et al.* 2001). At present, a common practice to deal with the problem posed by contaminated soils in Antarctica is the excavation of the affected soil, its storage and transport out of Antarctic area for treatment. This procedure is not only expensive but also considered as a potential source of environmental damage (Aislabie *et al.* 2004). Frequently, the contaminated soil remains stored as a hazardous waste for a long time because of lack of adequate treatment procedures on location and delays in transport. For these reasons, '*in situ*' remediation techniques should be

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considered the option for Antarctic soil restoration (Aislabie *et al.* 2006).

Depending on the time elapsed from the occurrence of the contamination event to the moment when the soil is treated, it can be considered as acutely contaminated (a previously pristine soil suddenly receiving a spill of fuels) or chronically contaminated (soil receiving repetitive quantities of hydrocarbons and hence remaining exposed to the pollutants during years or even decades). Bacteria inhabiting acute contaminated soils are not specialized in tolerating and metabolizing hydrocarbons, while those from a chronically contaminated soil have become adapted to the presence of the pollutants (Ruberto *et al.* 2008). The knowledge of this difference is crucial when the bioremediation strategy is to be chosen.

Bioremediation is a complex process and is affected by several environmental factors (Atlas and Bartha 2002). As low temperature represents one of the main limiting factors for biological processes in polar environments, bioremediation in Antarctica must be carried out with cold-adapted micro-organisms. The low concentrations of N and P available for bacterial growth and the imbalance in the C : N : P ratio are other relevant factors to take into account (Kerry 1993; Margesin and Schinner 1997a). Biostimulation provides adequate nutrient levels to increase degradation activity by the natural soil microflora. However, when HDB are scarce or absent, natural attenuation or biostimulation could prove to be not enough for an efficient removal of the pollutants (Snape *et al.* 2006). In these cases, inoculation with previously isolated HDB (bioaugmentation) could significantly shorten the bioremediation period. However, as was reviewed by Thompson *et al.* (2005), bioaugmentation is not just a simple procedure which only involves inoculating the soil with active HDB and waiting for the disappearance of hydrocarbons. Survival of the inoculum depends on factors such as microbial selection and inoculum size, which should be considered to avoid the frequently reported failure of bioaugmentation (Boon *et al.* 2000; Bouchez *et al.* 2000a). In this work, we present a report on an '*in situ*' bioremediation assay carried out in microcosms systems under Antarctic conditions where biostimulation and bioaugmentation strategies were tested on a soil chronically contaminated with diesel fuel. A mix of hydrocarbon-degrading strains and two different Antarctic hydrocarbon-degrading consortia were tested as inoculum in a 45-day-long assay.

Materials and methods

Study area

The assay was carried out during the Antarctic summer (February–March 2001) using the facilities of Jubany Scientific Station (62°14′S, 58°40′W) located on the coast of Potter Peninsula, King George Island (25 de Mayo Island), South Shetland Islands. During the assay, temperature ranged between −7·8°C and +7·3°C with a mean of +0·5°C, including 26 days in which temperature dropped below 0°C. Further information about climate conditions during the assay is provided in Table S1.

Soil preparation and analysis

Surface soil (0–30 cm depth) was collected from the surroundings of the fuel-storage tanks at Marambio Station (64°14′S, 56°37′W), an area chronically exposed to small spills occurring during the manipulation of fuels, mainly of which are diesel. The diesel fuel used in Antarctic areas is composed mainly of n-$C_{9–14}$ alkanes with minor levels of n-$C_{15–23}$ (Snape *et al.* 2006). The total hydrocarbon concentration of this soil was 11 972 ppm. Marambio Station is an important air facility that has been operating uninterruptedly since 1969 and has a higher fuel demand than any of the other Argentinean Antarctic stations. A quantity of approx. 45 kg of frozen soil was collected, stored at -20°C and shipped to Jubany station for use in the bioremediation assay. Large stones (>1 cm in diameter) were manually removed and the remaining soil fraction was homogenized by vigorously mixing it with a clean shovel. Homogenized soil was analysed for texture (Gee and Bauder 1986), total and soluble carbon (Richter and von Wistinghausen 1981), total nitrogen (Bremner and Mulvaney 1982) and phosphorus (Bray and Kurtz 1945). Water content was determined gravimetrically by heating subsamples at 105°C for 24 h. Soil pH was measured by duplicate in soil suspensions (10 g in 100 ml deionized water) after shaking (1 min, 250 rev min^{-1}).

Micro-organisms

Two hydrocarbon-degrading bacterial consortia and a mix of several hydrocarbon-degrading bacterial strains were used. Consortium J13 and the previously described strain *Rhodococcus* ADH AY220355 (Ruberto *et al.* 2005) were isolated from hydrocarbon-contaminated soils from Jubany station using polycyclic aromatic hydrocarbons (PAHs) and crude oil, respectively, as carbon source. Consortium M10 and strains M10dp and MP2–4 were isolated from hydrocarbon-contaminated soil from Marambio Station using PAHs as carbon source. DM1-41 strain was obtained from the same Antarctic station but using crude oil. Strains were identified on the basis of their morphological and metabolic characteristics (standard techniques and API 20 NE system). 16S rRNA gene sequencing was also performed. Amplification of the gene was made as described previously (Ruberto *et al.* 2003). PCR products

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were purified with the Wizard® PCR purification kit (Promega) and then sequenced. Partial 16S rRNA gene sequences were compared with known sequences in the Ribosomal Database Project (Cole *et al.* 2005).

Experimental design

Contaminated soil was distributed on steel trays (40 cm length, 32 cm wide, 5 cm height) coated with an inert enamel. Each tray was filled with 2·5 kg of soil, thus obtaining a 4 cm deep layer. The following systems were prepared in duplicates and exposed to the Antarctic climate conditions: AC (abiotic control, soil poisoned with 3% w/w $HgCl_2$ to evaluate hydrocarbon losses by nonbiological means); CC (community control system, to evaluate hydrocarbon removal by the indigenous microflora in soil without any manipulation); B (biostimulated system, soil amended with inorganic sources of N and P); S + B (soil bioaugmented with a mix of the strains); M10 + B (soil bioaugmented with consortium M10); J13 + B (soil bioaugmented with consortium J13). Inocula were prepared by culturing each strain and consortia (from stocks cryopreserved at −80°C) in 250 ml Erlenmeyer flasks with 50 ml of saline basal medium (SBM) and 2% v/v of diesel fuel, as was described by Espeche *et al.* (1994). Cultures were incubated at 15°C and 250 rev min^{-1} for 10 days. Consortia were harvested by centrifugation and resuspended in SBM (10 ml). Counts of total heterotrophic aerobic bacteria (THAB) in the suspensions used as inocula were $6{\cdot}4 \times 10^7$ and $1{\cdot}5 \times 10^8$ colony-forming units per ml (CFU ml^{-1}) for M10 and J13, respectively. Isolated strains ADH, M10dp, MP2-4 and DM1-41 were also harvested by centrifugation, separately resuspended in 2 ml SMB and mixed, resulting in a THAB value of $1{\cdot}6 \times 10^{10}$ CFU ml^{-1}. This mix, containing the four strains, was used as inoculum for the S + B system. All bioaugmented systems were also biostimulated using 13·4% w/v NH_4Cl and 3·13% w/v Na_2HPO_4 solutions resulting in a C : N : P ratio of 100 : 12·9 : 2·6. To maintain the initial water content in all systems, saline solution was added when necessary. Table 1 summarizes the conditions used in the microcosms.

Hydrocarbons analysis

Total hydrocarbons were quantified at 1, 10, 20, 30 and 45 days by the EPA 418·1 method (US Environmental Protection Agency). Approx. 1 g of soil was placed in a 20-ml glass vial with a metal-covered hermetic cap. HPLC grade CCl_4 (10 ml) and a spatula tipful of Na_2SO_4 were added to each flask. These flasks were sonicated overnight in an ultrasonic bath. Samples were subsequently transferred to a quartz cell and analysed in a Buck Model HC 404 Hydrocarbon analyser IR spectrometer (Buck Scientific, East Norwalk, CT). Chromatographic analysis was carried out with a Shimadzu GC-9A GC (Shimadzu, Corp., Japan) equipped with an FID (flame ionization detector) detector. Oven temperature was kept at 100°C for 1 min, then ramped at 10°C min^{-1} to 250°C and kept at that temperature for 5 min. The injector temperature was 280°C and separation was carried out with a 30 m × 0·25 mm i.d. (0·25 μm film thickness) fused-silica capillary column (cross-linked 5% PH ME siloxane). Carrier gas (He_2) flow was 31 cm s^{-1}. Data were collected using the PC-CHROM software (Buenos Aires University, Argentina).

Microbiological analysis

At 1, 10, 20, 30 and 45 days, serial dilutions (in 0·9% NaCl) were prepared and plated from 1 g of soil samples. THAB were determined (four replicates) on casein–peptone–starch agar, as was suggested by Wynn-Williams (1992) for Antarctic soil analysis. HDB were enumerated on solidified SBM with 2% filtered diesel fuel (filtered using 0·22 μm, 25 mm MSI nylon membranes provided by Micron Separations Inc., Westboro, MA) as sole carbon and energy source. Both, THAB and HDB plates were incubated for 30 days at 10°C and the results were expressed as CFU per gram of dry weight (CFU g^{-1}).

Statistical analyses

Bacterial counts from the different systems were analysed by repeated measured ANOVA and Tukey's multiple comparison test. Comparisons between hydrocarbon

Table 1 Details of the experimental design

System	Condition	$HgCl_2$ (3%) (ml)	N + P Solution (ml)	Inoculum size (UFC g^{-1})	Saline solution (ml)
AC	Abiotic control	380	–	–	–
CC	Indigenous microflora	–	–	–	380
B	Biostimulated indigenous microflora	–	100	–	280
S + B	Strains mixture bioaugmentation	–	100	$6{\cdot}4 \times 10^7$	270
M10 + B	Bioaugmentation with consortium M10	–	100	$2{\cdot}6 \times 10^5$	270
J13 + B	Bioaugmentation with consortium J13	–	100	$6{\cdot}0 \times 10^5$	270

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concentration at days 1 and 45 as well as comparisons among systems at day 45 were made using one-way ANOVA and Tukey's multiple comparison test.

Results

Analysis of the soil

The soil used in this study was sandy (64·4% sand, 24·5% silt, 11·1% clay). pH was 6·2, total C 0·74%, soluble C 0·024%, total N 0·072% and P $1{\cdot}09 \times 10^{-3}$%. Initial water content was 12% w/w. THAB counts in this soil were $5{\cdot}2 \times 10^6$ CFU g^{-1} and the HDB counts were $6{\cdot}0 \times 10^3$ CFU g^{-1}.

Characterization of the DM1-41, MP2-4 and M10dp strains

All three strains corresponded to psychrotolerant, aerobic, heterotrophic, Gram-negative rods. Morphological and biochemical characteristics of the strains are shown in Table S2. 16S rDNA partial sequence of DM1-41 strain (1493 bp) was closely related to *Stenotrophomonas rhizophila* (T) e-p10 AJ293463 (percentage of similarity: 99·7%). MP2-4 (1445 bp) proved to be related to *Pseudomonas migulae* (T) CIP 105470 AF074383 (percentage of similarity: 99·4%). Finally, 16S rDNA partial sequence from M10dp (830 bp) proved to be related to *Sphingobium xenophagum* (T) BN6 X94098 (percentage of similarity: 97·3%). According to the morphological, biochemical and molecular characteristics, strains were identified as *Stenotrophomonas* sp. (DM1-41), *Pseudomonas* sp. (MP2–4) and *Sphingobium* sp. (M10dp) and their GenBank accession numbers are DQ109991, EU195324 and EU195325, respectively.

Bacterial counts

In order to make evident the biological activity in the different systems, the changes in THAB and HDB counts were evaluated (Fig. 1). Although a higher THAB counts value was observed for S + B, no significant differences ($P > 0{\cdot}05$) were observed between all systems at day 1 (1 day after preparation of the systems). All systems increased in THAB, reaching similar values at the end of the assay. These values were not statistically different ($P > 0{\cdot}05$). No counts were detected in the abiotic control during the entire assay (data not shown).

When HDB counts were analysed, significant differences were found at day 1. Counts in the CC were lower and differed from B ($P < 0{\cdot}05$), S + B ($P < 0{\cdot}001$), M10 + B ($P < 0{\cdot}01$) and J13 + B ($P < 0{\cdot}01$). However, no differences were found between B, M10 + B and J13 + B. On the contrary, S + B showed significantly higher HDB counts.

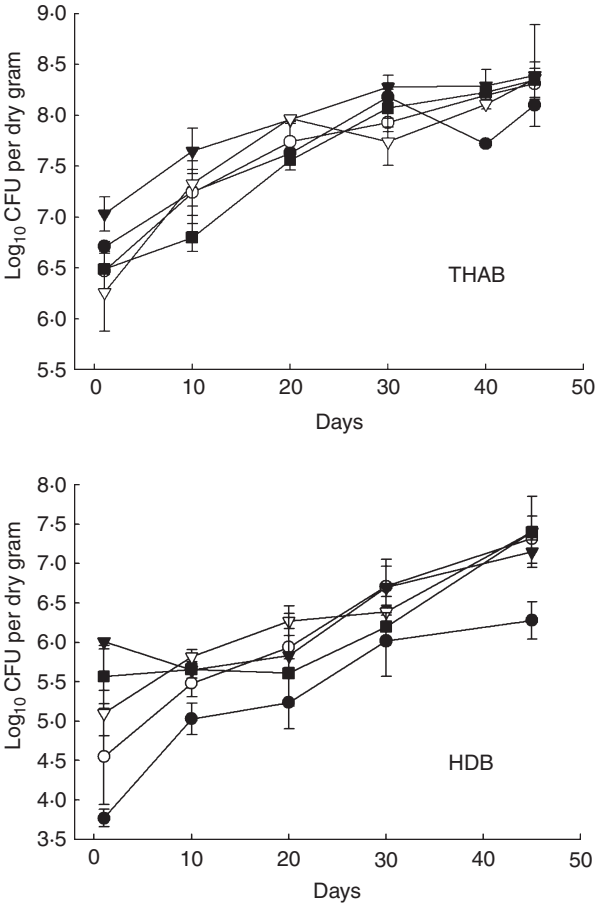

Figure 1 Influence of the different treatments on the number of total heterotrophic aerobic bacteria (THAB) and hydrocarbon-degrading bacteria (HDB) during the microcosms assay. Treatments: community control (CC); biostimulation of the indigenous microflora (B); biostimulation and bioaugmentation with a mix of strains (S + B); biostimulation and bioaugmentation with consortium J13 (J13 + B); biostimulation and bioaugmentation with the consortium M10 (M10 + B). (●, CC; ○, B; ▼, S+B; ▽, M10 + B; ■, J13 + B).

These results showed that 1 day after inoculation, the effect of bioaugmentation on the bacterial counts was only evident for S + B system. At day 45, all systems showed significant increases in HDB counts. At this time, as was observed at day 1, all biostimulated systems were different from CC ($P < 0{\cdot}05$). However, no differences were detected between them. These results indicated that an increase in the number of HDB occurs when either biostimulation or biostimulation/bioaugmentation strategies are applied to a chronically contaminated Antarctic soil.

Hydrocarbon concentration in microcosms

In all systems, including AC, a significant decrease ($P < 0{\cdot}01$) was observed between days 1 and 45 (Fig. 2). Main decreases in total petroleum hydrocarbon (TPH)

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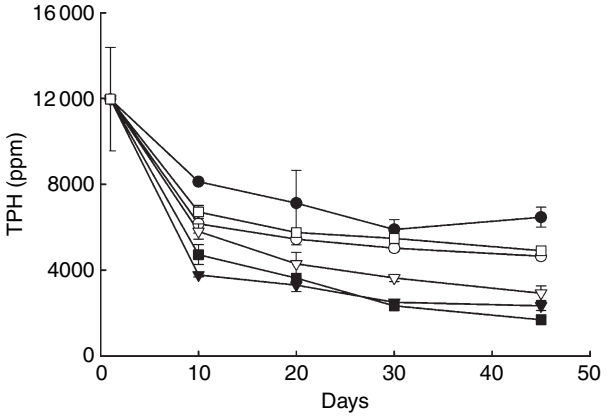

Figure 2 Changes in hydrocarbon concentration during the microcosms assay. Treatments: abiotic control (AC); community control (CC); biostimulation of the indigenous microflora (B); biostimulation and bioaugmentation with a mix of strains (S + B); biostimulation and bioaugmentation with consortium J13 (J13 + B); biostimulation and bioaugmentation with the consortium M10 (M10 + B).(–●–, AC; –○–, CC; –▼–, B; –▽–, S + B; –■–, M10 + B; –□–, J13 + B).

were observed between days 1 and 10 for all microcosms, system B showing the most significant one, followed by M10 + B and S + B. CC and J13 + B shared a very similar slope and the abiotic control showed the lowest one. From day 10, hydrocarbon removal rate was lower in all systems, evidencing the high abiotic removal derived from manipulation and farming of the soil during the first 10 days. Figure 3 shows hydrocarbon concentration at the end of the assay; comparison between TPH at days 1 and 45 and removal efficiencies (RE) calculated as indicated in eqn (1).

$$\mathrm{RE} = [\mathrm{TPH_{d1}} - \mathrm{TPHd_{45}})/\mathrm{TPH_{d1}}] \times 100 \qquad (1)$$

The highest removal efficiency (86%) occurred in M10 + B, where TPH concentration decreased from 11 972 ppm to 1687 ppm in 45 day (comparative GC chromatograms at day 45 for AC and M10 + B are shown in Fig. S1). System B (81% of removal) showed a TPH concentration at day 45 (2333 ppm), which did not differ from the one observed for M10 + B ($P > 0{\cdot}05$). Finally, S + B (76% of removal) reached a TPH concentration at day 45 (2924 ppm) that resulted significantly lesser ($P < 0{\cdot}05$) than that of M10 + B. In addition, the TPH concentration observed at day 45 in these three systems was significantly lower ($P < 0{\cdot}001$) compared with CC (4649 ppm, 61% of removal).

Although TPH concentration at day 45 was significantly lower in J13 + B compared with AC ($P < 0{\cdot}01$), it was not different from the TPH concentration observed for the CC, indicating a poor removal efficiency and suggesting some kind of negative effect on the biodegradation capacity compared with the indigenous biostimulated microflora.

In order to highlight the biological degradation activity, Fig. 4 shows the percentage of accumulated hydrocarbon removal in each system referred to the abiotic control at the same time and calculated as in eqn (2):

$$[\mathrm{TPH_{AC}} - \mathrm{TPH_Y}]/\mathrm{TPH_{AC}} \times 100 \qquad (2)$$

where AC is the abiotic control and Y represents any other system. These values do not indicate the total hydrocarbon disappearance, which would represent the absolute efficiency of a system. Such absolute efficiency is shown in Fig. 3. On the contrary, the percentage shown in Fig. 4 reflects the fraction of the hydrocarbons removed from each system once subtracted those fractions eliminated by abiotic factors and hence related to the biological activity.

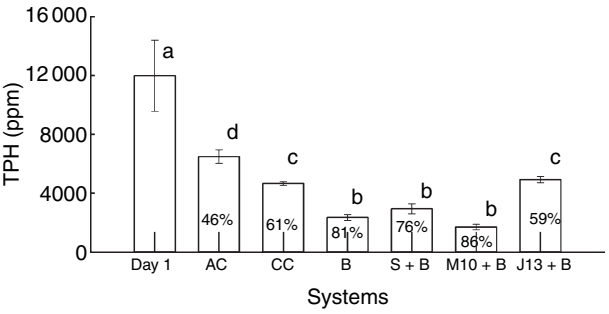

Figure 3 Removal efficiencies for each system and comparison between total petroleum hydrocarbon (TPH) concentration at days 1 and 45. Treatments: abiotic control (AC); community control (CC); biostimulation of the indigenous microflora (B); biostimulation and bioaugmentation with a mix of strains (S + B); biostimulation and bioaugmentation with consortium J13 (J13 + B); biostimulation and bioaugmentation with the consortium M10 (M10 + B).

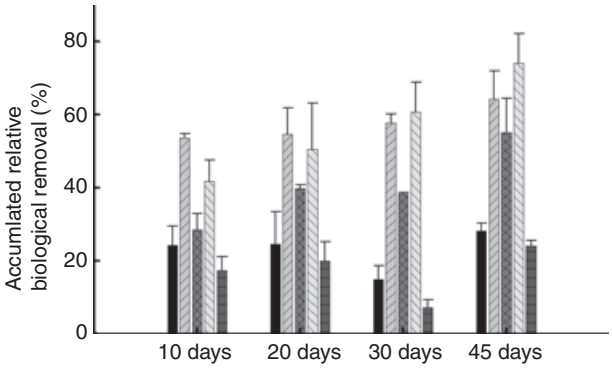

Figure 4 Percentage of hydrocarbon removal in each system referred to the abiotic control as a way to indicate biological removal. Treatments: community control (CC); biostimulation of the indigenous microflora (B); biostimulation and bioaugmentation with a mix of strains (S + B); biostimulation and bioaugmentation with consortium J13 (J13 + B); biostimulation and bioaugmentation with the consortium M10 (M10 + B). (■, CC; ▨, B; ▩, S + B; ▧, M10 + B; ▤, J13 + B).

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The system without biostimulation (CC) had a low degradation activity during the first 10 days (24·2%). This activity was negligible during the rest of the assay, determining the permanence of an approximately constant value of biological degradation from days 10 to 45. Biostimulated systems (except J13 + B) showed higher initial values of biological degradation (in the period 0–10 days). However, system B, which was the most efficient system during the first period (53·5%), showed no further significant increases in this value between days 10 and 45. On the contrary, M10 + B and S + B, which had lower initial values (41·9% and 28·6%, respectively) compared with B, showed low but significant values of biological degradation in the same period, proving that added micro-organisms maintained a certain degree of biodegradation until the end of the assay. Although the differences between inoculum sizes make it difficult to compare the performances of the S + B, M10 + B and J13 + B systems, this behaviour determined a similar ($P > 0·05$) biological hydrocarbon removal efficiency between B, S + B and M10 + B when considering the complete biodegradation processes. Finally, efficiency in J13 was always poor and showed similar levels compared with those obtained for the CC.

Discussion

Of the several biological systems used as bioaugmentation tools in this study, ADH strain was previously isolated and characterized as *Rhodococcus* sp. (Ruberto *et al.* 2005). The other three isolates used in the present assay proved to be a *Pseudomonas* sp. (MP2-4), a *Stenotrophomonas* sp. (DM1-41) and a *Sphingobium* sp. (M10dp). The metabolic versatility of the *Pseudomonas* is well known (Palleroni 1995). It has been reported that *Pseudomonas* can metabolize aliphatic hydrocarbons in low-temperature environments (Stallwood *et al.* 2005) and members of this genus also use PAHs as substrate (Aislabie *et al.* 2000). *Pseudomonas* became dominant in PAHs-contaminated sites. In this sense, a recent phylogenetic study performed by Ma *et al.* (2006) with 22 PAH-degrading bacterial strains isolated from Antarctic soils with naphthalene and phenanthrene as substrate resulted in the identification of 21 *Pseudomonas* sp. and one *Rahnella* sp., thus confirming the important role played by *Pseudomonas* in PAHs-contaminated cold environments. The isolation and selection of *Pseudomonas* MP2-4 strain from PAHs-enriched culture media are in accordance with the above-mentioned reports. In addition, *Sphingobium* M10dp were isolated from PAHs and selected by their high PAHs-degradation capacity. Other species included into the *Sphingomonadaceae* family were mentioned as PAHs degrading micro-organisms (Demanèche *et al.* 2004). In Antarctica, Saul *et al.* (2005) detected a low number of members of this genus in soils from Scott Base, but they became the dominant organisms in hydrocarbon-contaminated soils. A number of other authors have reported the presence of Sphingomonadaceae members in hydrocarbon-contaminated polar soils, as was reviewed by Aislabie *et al.* (2006). *Stenotrophomonas* DM1-41 was previously isolated from crude-oil-enriched cultures. Several *Stenotrophomonas* strains were isolated from different Antarctic natural environments (Vazquez *et al.* 2005). However, although some authors have reported the hydrocarbon biodegradation capacity of *Stenotrophomonas* strains in nonAntarctic soils (Boonchan *et al.* 1998; Juhasz *et al.* 2002); as far as we know, no reports about this degradation capacity of members of this genus have been published to date about Antarctic areas.

Diesel fuels are the main pollution source in Argentine Antarctic stations and a similar situation was reported for other Antarctic stations (Snape *et al.* 2001). Degradation of diesel and other fuels by bacteria was repeatedly demonstrated either on liquid cultures (Geerdink *et al.* 1996; Solano-Serena *et al.* 1999) or in diesel-contaminated soils (Gallego *et al.* 2001).

The significant influence of the harsh climatic factors, mainly the strong winds, on the abiotic elimination of volatile fractions of the hydrocarbons spilled on soils was mentioned in previous reports for diesel-contaminated Antarctic soils (Ruberto *et al.* 2005) as well as for other cold soils (Margesin and Schinner 1997a,b). However, this fact is basically to be expected in acutely contaminated soils because the light fractions of a recently-spilled fuel volatize faster, diminishing the TPH concentration without any significant biological contribution. In this respect, Kerry (1993) reported 50% of abiotic loss for a 1-year length assay in which a diesel spill was simulated in Antarctica. The extent of abiotic disappearance for chronically aged contaminated soil would not be expected to be as large as the one observed for a recently-spilled soil. However, our results show that an important fraction (46%) of hydrocarbons is eliminated by abiotic factors. Aeration and farming could be the cause of the observed losses, especially considering that soil from Marambio Station stays frozen and covered with snow for a long time. These environmental conditions could have limited the evaporation and partially protected the contaminated soil from a significant loss of the contaminant fuels despite the age of the spill, mainly of the *n*-alkanes with chain-lengths shorter than *n*-decane which is the fraction that suffers the fastest disappearance after a spill because of abiotic factors.

In this work, we demonstrate the feasibility of bioremediation of diesel-contaminated soils under Antarctic environmental conditions. The high TPH removal observed in

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the biostimulated systems (except J13) proved to be similar to those reported by Margesin and Schinner (1997b) for a 155-day-length diesel-oil removal study with Alpine subsoils (90·2%). Even in a very long-term assay performed during three summer seasons at Casey Station, Antarctica, Snape *et al.* (2003) reported 10·9% of residual hydrocarbons in biostimulated soils.

The high biodegradation rate evidenced by the biostimulated indigenous microflora during the first 10 days agrees with the concept that the bacterial flora long-term exposed to the contaminants are closely adapted to metabolize these compounds when other limiting environmental factors (C : N : P ratio, aeration, etc.) are improved. However, biodegradation activity of the indigenous microflora was almost negligible from days 10 to 45. One possible explanation is that all the really biodegradable compounds of the diesel fuel are utilized by the microflora during the first 10 days, the remaining fraction being refractory to the biodegradation by this microflora. Inocula, although selected for their high degradation activity *in vitro*, clearly had problems to adapt to a foreign environment. It was reported that inoculated micro-organisms are subjected to several biotic and abiotic sources of stress (Margesin and Schinner 1999) and for this reason, the ability to metabolize the pollutants is a necessary but no sufficient property to achieve the success of the inoculated micro-organisms. The reasons why inocula fail to work in nature as they work in the laboratory have been clearly enumerated by Goldstein *et al.* (1985) and also reviewed by El Fantroussi and Agathos (2005). Although in this assay no significant decreases in the total aerobic bacteria were detected, one of the causes for the lack of detectable improvement of biodegradation activity in the inoculated systems could be the phenomenon referred to as 'microbiostasis' by El Fantroussi and Agathos (2005) that consists in a rapid decline in number of the inoculated bacteria. In this work, the lack of a detectable decrease in cell numbers could be due to the fact that bacteria were inoculated to the same order of magnitude as that present in the soil, turning this kind of change undetectable by the culture-techniques applied. However, whereas several previous studies reported that inocula (or their biological activity) rapidly disappear from the experimental system and no further changes related to their presence can be detected (Margesin and Schinner 1997a; MacNaughton *et al.* 1999), in this work the strains mixture and M10 consortium seem to have overcome, to some extent, the limiting factors and preserved their degradation activity. In this sense, S + B and M10 + B showed a slow but increasing degradation activity, evidenced by the growing accumulated hydrocarbon-removal values observed in these systems. For the M10 consortium, this growing activity determined that, between days 20 and 30, system M10 + B overcomes the cumulative degradation activity of the natural microflora (system B). However, when the efficiency was analysed at the end of the assay (45 days), no differences were detected and B, M10 + B and S + B resulted in a similar hydrocarbon removal.

Another interesting point is that although it was expected that the hydrocarbon removal capacity in the bioaugmented systems should be higher or similar to that observed for the biostimulated natural microflora (because the same number of indigenous bacteria is present in all systems), there was a negative effect in these systems. This effect was evident during the first 10 days of the assay and correlated with the origin of the inocula. M10, which was isolated from the same place under bioremediation, exerted the lower perturbation. S + B, that included three bacterial strains from Marambio combined with another one isolated from Jubany, had a greater effect. Finally J13, which represents an absolutely foreign system, caused the highest negative impact. One possible explanation is the fact that massive inoculation could stimulate an increase in the bacterivorous population, which could induce a strong increase in the grazing pressure exerted on both, the inoculated and other bacterial species. This pressure could result in biological stress that affects the degradation capacity of the bacterial populations. Similar observations were made by Bouchez *et al.* (2000a,b). In these studies, massive inoculation with the aerobic denitrifying bacterium *Microvirgula aerodenitrificans* resulted in a breakdown of nitrification. These authors, working with FISH techniques, observed an increase in the number of bacteria with a positive signal in the digestive vacuoles of protozoa, and concluded that grazing is the main cause of the failure of bioaugmentation.

Introduction of hydrocarbons into a soil with low level of nutrients unbalances the C : N : P ratio. This fact limits the biodegradation rate because C cannot be assimilated by micro-organisms without adequate amounts of N and P. For this reason, amendment of contaminated soils with fertilizers is a common practice. However, it is important to select the most adequate source of nutrients and to avoid over-fertilization. As was reported previously (Ruberto *et al.* 2003), the addition of N and P at a C : N : P ratio of 100 : 12 : 3 in a diesel-oil-contaminated Antarctic soil inhibited bacterial growth during the first days of the experiment and resulted in a slower hydrocarbon-degradation activity. A similar observation was made using soils from Alaska by Braddock *et al.* (1997), who found an improvement of microbial hydrocarbon degradation after the application of N at levels of 50–100 mg kg^{-1} but an inhibitory effect after the application of 200 mg kg^{-1}. In the present work, biostimulation was

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applied at a C : N : P ratio of 100 : 12·9 : 2·6. The removal efficiency showed by the biostimulated indigenous microflora (B) suggests a high adaptation to the presence of hydrocarbons and a positive effect of the added level of nutrients.

Aislabie *et al.* (2006) pointed out that an appropriate inoculum for polar soils should contain a mixture of hydrocarbon degraders and that greater success would be expected using indigenous microbes adapted to cold condition rather than allochthonous strains. However, in this work, the absence of differences in TPH concentration at days 45 between systems B and M10 + B indicates that, for the studied soil, bioaugmentation with an indigenous bacterial consortium represents no advantage as compared with biostimulation of the indigenous microflora. It is important to highlight that the inoculum of M10 was to the same order of magnitude as was the culturable bacterial population present in the soil. Future works should investigate the success of bioaugmentation when the size of the inoculum is significantly higher when compared with the number of micro-organisms present in the contaminated soil. In this sense, we reported previously (Ruberto *et al.* 2006) a significant phenanthrene removal from soils inoculated with M10 consortium and amended with fish meal (as N and P source) and a surfactant (Brij®700; Sigma). In this case, the number of HDB inoculated was much higher than the number of HDB present in the assayed soil.

Based on the different efficiencies observed for consortia M10 (isolated from Marambio) and J13 (isolated from Jubany) when they were inoculated on Marambio-polluted soil, a site-specificity phenomenon could exist. Although this kind of site-specificity has been previously mentioned as a possibility, no previous reports on Antarctic soils bioremediation are known by us that make reference to the finding of this type of biological response.

Conclusion

Although under the assayed conditions an important removal because of abiotic factors was observed, these results show the feasibility of the application of bioremediation techniques for the clean-up of hydrocarbons-contaminated Antarctic soils under temperature conditions resulting adverse for nonadapted micro-organisms. If soils are chronically contaminated with diesel, biostimulation of the indigenous microflora should be the strategy of choice to reach an effective hydrocarbon removal, because bioaugmentation with a number of cells similar to those naturally present in the soil showed no positive effects. Inoculation with levels significantly higher than those applied in this study should be evaluated considering the resulted efficiency and the associated costs and efforts.

Acknowledgements

We thank Jubany and Marambio Station crews for the logistical support during the field studies and Cecilia Ferreiro for the correction of the English manuscript. This work was carried out under an agreement between the Instituto Antártico Argentino and the Facultad de Farmacia y Bioquímica of the Universidad de Buenos Aires and was supported in part by the Universidad de Buenos Aires (UBACyT U007) grant and the Agencia Nacional de Investigaciones Científicas y Técnicas (PICTO 11555).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 GC-MS chromatograms obtained for AC (A) and M10B (B) systems at day 45.

Table S1 Main climate conditions during the bioremediation assay.

Table S2 Main characteristics of three Antarctic hydrocarbon degrading strains.

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