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ORIGINAL PAPER

Hydrocarbon removal and bacterial community structure in on-site biostimulated biopile systems designed for bioremediation of diesel-contaminated Antarctic soil

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Abstract Several studies have shown that biostimulation can promote hydrocarbon bioremediation processes in Antarctic soils. However, the effect of the different nutrient sources on hydrocarbon removal heavily depends on the nutrients used and the soil characteristics. In this work, using a sample of chronically contaminated Antarctic soil that was exposed to a fresh hydrocarbon contamination, we analyzed how a complex organic nutrient source such as fish meal (FM) and a commercial fertilizer (OSEII) can affect hydrocarbon biodegradation and bacterial community composition. Both amended and unamended (control) biopiles were constructed and controlled at Carlini Station and sampled at days 0, 5, 16, 30 and 50 for microbiological, chemical and molecular analyses. FM caused a fast increase in both total heterotrophic and hydrocarbon degrading bacterial counts. These high values were maintained until the end of the assay, when statistically significant total hydrocarbon removal (71 %) was detected when compared with a control system. The FM biopile evidenced the dominance of members of the phylum Proteobacteria and a clear shift in bacterial structure at the final stage of the assay, when an increase of Actinobacteria was observed. The biopile containing the commercial fertilizer evidenced a hydrocarbon removal activity that was not statistically significant when compared with the untreated system and exhibited a bacterial community that differed from those observed in the unamended and FM-amended biopiles. In summary, biostimulation using FM in biopiles significantly enhanced the natural hydrocarbon-degradation activity of the Carlini station soils in biopile systems and caused significant changes in the bacterial community structure. The results will be considered for the future design of soil bioremediation protocols for Carlini Station and could also be taken into account to deal with diesel-contaminated soils from other cold-climate areas.

Keywords Antarctic soils · Hydrocarbons · Biopiles · Biostimulation · Fish meal · Commercial fertilizer

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Introduction

In the last years, there has been increased interest in the Antarctica and its economic, political and scientific importance. As a consequence, human activity in this region (setting up scientific stations, increased tourism, aircraft transportation and establishment of fisheries) has increased, with the concomitant rise of the need for petroleum transportation, storage and consumption. These activities represent an important risk of hydrocarbon pollution in soil and water, which was a major concern in the Antarctic Treaty Consultative Meetings (ATCM 2012, 2013). In a remote and isolated area such as Antarctica, on-

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site bioremediation is the only feasible clean-up option for hydrocarbon spills (Aislabie et al. 2004; Delille et al. 2009) because transportation of contaminated soil does not represent a practical option, and also once in the soil, the rate of natural attenuation is not fast enough to reduce the hydrocarbon levels in an adequate time period (Snape et al. 2006). The success of bioremediation technology in Antarctica is largely conditioned by several local environmental factors such as the low temperatures and low availability of nutrients, mostly N and P (Greer 2009).

The hydrocarbon spills generally produce an imbalance in the C:N:P ratio. Under this condition, microbial biomass formation in soil could be limited by the low N and P levels. This problem is particularly relevant in many Antarctic soils, because they normally exhibit a low availability of nutrients (Ferguson et al. 2003; Ruberto et al. 2009). In order to balance the C:N:P ratio in fuel-contaminated soils, nutrient supplementation (biostimulation) has been reported to be an effective tool for enhancing biodegradation activity and hence for reducing the ecological impact of oil derivative pollution in the Arctic and Antarctic areas (Braddock et al. 1997; Aislabie et al. 2006). Although it is not generally known which indigenous microorganisms should be favored to optimize the rate and extent of bioremediation in a particular contaminated environment, it was reported that added nutrients are not incorporated equally by bacterial taxa in hydrocarbon-contaminated soils (Bell et al. 2011). This fact could cause a change in the composition and metabolic activity of the autochthonous microbial community.

For this reason, in this work we aimed to study hydrocarbon removal and bacterial community shifts occurring when a chronically contaminated Antarctic soil is biostimulated with different nutrient sources. Although chronically contaminated, the soil had also been impacted by a recent diesel spill. This represents a relatively frequent situation occurring near the fuel storage tank area of the Antarctic station. While several papers have reported studies on hydrocarbon bioremediation in polar soils, either freshly (Delille 2000; Ruberto et al. 2003) or chronically (Stallwood et al. 2005; Kauppi et al. 2011) contaminated, to our knowledge no studies analyzing the biostimulation effect were conducted using chronically contaminated soil that had been exposed to a fresh hydrocarbon contamination. Although the accidental fresh contamination could provide growth-supporting energy and carbon to the previously selected microbial community, a negative impact could also be expected because of the toxic effects (Kang and Park 2010). In order to cover different nutrient requirements of the authoctonus microbial community surviving the fresh contamination event, we decided to test two different nutrient sources. One of these nutrients sources, fish meal (FM), is a low-cost and easily available material in the south of Argentina that proved to be efficient to stimulate microbial growth and hydrocarbon biodegradation (Lee et al. 1995; Delille et al. 2008). This complex matrix also acts by increasing soil porosity, hence favoring the aerobic metabolism of the pollutants (Kauppi et al. 2011). The other nutrient source was the commercial fertilizer OSE II® (Oil Spill Eater International Corp.), which is listed on the US Environmental Protection Agency's National Contingency Plan for Oil Spills and is reported to be a multienzyme liquid concentrate containing nutrients, which was reported to produce a significant hydrocarbon reduction after 45 days in a chronically contaminated antarctic soil (Dias et al. 2012). The amended soil was arranged in biopile systems. Biopiles have proved to be effective for reducing the hydrocarbon concentration in contaminated soils of different origins (Jørgensen et al. 2000; Delille et al. 2008; Sanscartier et al. 2009). An advantage of this design is the possibility to treat a large soil volume in a smaller area, compared with other designs such as landfarming (Sanscartier et al. 2009). Studies performed by Yergeau et al. (2009, 2012) using ex situ biopiles (where soils were excavated, aerated and fertilized to specifically stimulate growth of aerobic bacteria) for the treatment of hydrocarbons contaminated high Arctic soils showed that biopile treatment causes major shifts in microbial communities, favoring the presence of aerobic hydrocarbon-degrading components. To investigate the changes in microbial community of the amended soil in the biopiles, in this work we applied denaturing gradient gel electrophoresis (DGGE), one of the most frequently used techniques for this purpose (Ahn et al. 2006; Chemlal et al. 2012; Adetutu et al. 2014). Knowledge about these changes is relevant to understand the effect of a disturbance in the natural microbial communities and is required to achieve an efficient bioremediation process management. (Popp et al. 2006; Baek et al. 2007, Jin et al. 2013).

In this context, the aim of the study was to evaluate biodegradation efficiency and changes in bacterial community structure caused by both a product specially designed to enhance the soil hydrocarbon degrading capability and a waste organic matter, such as FM. We used small biopiles as the experimental design, containing chronically contaminated and freshly diesel-impacted soil from Carlini Station.

Materials and methods

Study site and experimental design

A 50-day (4 January–23 February) experiment using soil biopiles was conducted during the 2010 Antarctic summer at the Carlini Argentinean Scientific Station (62°14′S,

58°40′W), which is located on Potter Peninsula, 25 de Mayo Island (King George Island) in the South Shetland Islands (Fig. 1). During the study period (50 days), the total precipitation was 35.5 mm, while the average of the daily mean temperature was 0.9 °C with a maximum of 7.7 °C and a minimum of −5.5 °C.

The chronically contaminated soil used in this study was collected just under an aerial diesel transport pipe, near the fuel storage tanks. This soil had suffered several previous small spills of diesel as a result of fuel manipulation and transportation, but there had also been a spill of fresh diesel a few weeks before the assay. Hence, this soil had a combination of old and recent hydrocarbon contamination.

The contaminated soil was sieved (2 mm mesh) in order to obtain a homogeneous material. Biopiles consisted of 30 kg of sieved soil placed in plastic boxes (22.5 cm depth, 49.5 cm length and 35.5 cm width), which were located in an open area, partially protected from rain and snow in order to avoid the waterlogging of the systems. Three biopiles were prepared: FM (containing soil biostimulated with 3 % w/w of dry and previously sterilized FM), CP (containing soil amended with 15 ml/kg of the commercial product OSE II®, as suggested by the manufacturer for surface spills on land) and CC (control system containing unamended soil). The FM chemical composition was 9 % (w/w) of total N and 4.27 % (w/w) of P content and provided proteins, lipids, fibers, vitamins and calcium. OSE II® is listed in the US Environmental Protection Agency's National Contingency Plan as useful for oil spills, and it was applied following the manufacturer's instructions. The N and P content of the OSE II® was estimated as 0.055 and 0.0065 %, respectively, based on the information of the original patent of the product (Stillman 1992).

Biopiles were sampled in triplicate during summer at days 0, 5, 16, 30 and 50. Before sampling, biopiles were gently mixed, and three subsamples were taken and used independently for chemical and microbiological analysis.

Soils and chemical analysis

The soil was analyzed for texture using the pipette method (Gee and Bauder 1986). Organic carbon (Walkley and Black 1934), extractable phosphorous (Bray and Kurtz 1945) and total Kjeldhal nitrogen were also evaluated. Water content was determined gravimetrically by heating samples at 105 °C for 24 h. For pH measurements, 10 ml of sterile saline solution (NaCl 8.9 g/l) was added to 1 g of soil and vortexed for 1 min. The total hydrocarbon content

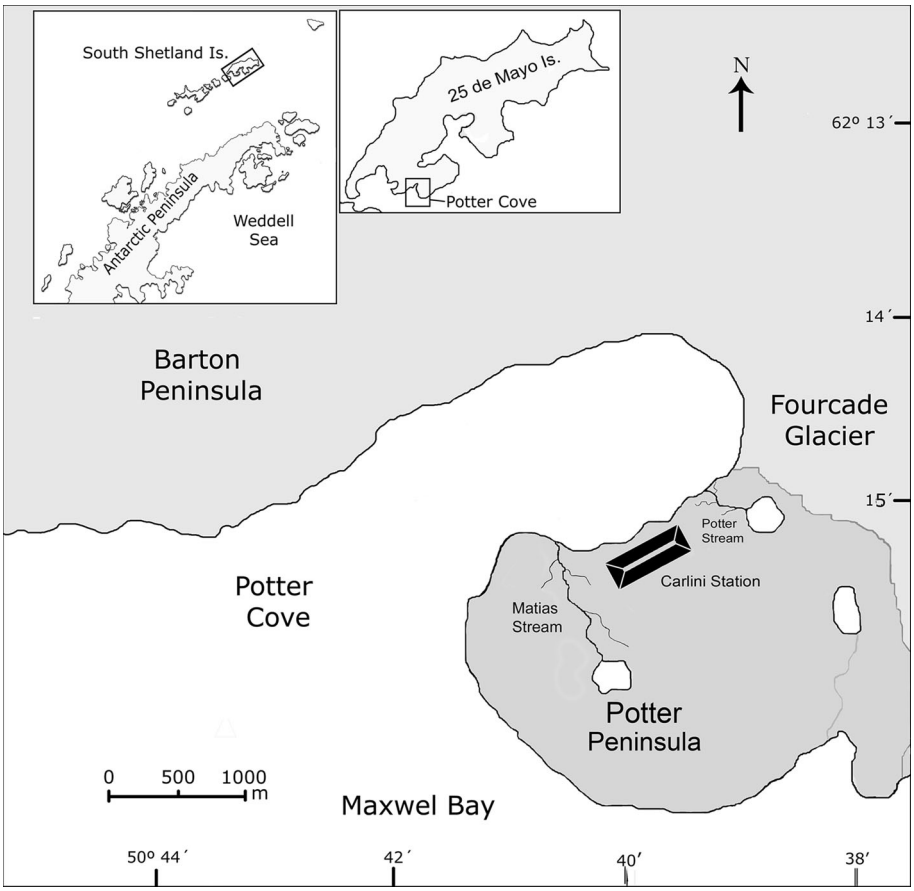

Fig. 1 Geographic location of Potter Cove and Potter Peninsula showing Carlini station, where the biopile systems were located

was determined by GC using a Shimadzu GC-9A equipped with a flame ionization detector (FID) and a capillary column (30 m × 0.25 mm i.d) with a coating of 5 % PH-ME siloxane (0.25 µm inner layer). Ten milliliters of CH_2Cl_2 was added to 5 g of wet soil in a flask. Then, a spatula tip of anhydrous Na_2SO_4 was added to each flask. Samples were sonicated for 1 h and centrifuged at 1,000*g* for 15 min, and the supernatant was carefully separated to analyze the total hydrocarbon concentration. GC operating conditions were set up as follows: the oven temperature was 100 °C for 1 min and ramped to 250 °C (10 °C for 1 min), keeping this temperature for 5 min. The injector temperature was 280 °C. The carrier gas (He_2) was used at a flow rate of 31 cm/s. Results were obtained and analyzed with PC-Chrom software.

Bacteriological counts

The amounts of total heterotrophic aerobic bacteria (HAB) and hydrocarbon-degrading bacteria (HDB) in each soil sample were estimated by viable cell counts. One gram of recently sampled soil was suspended in 10 ml of sterile saline solution with 1 % (v/v) of Tween 80 (to favor detachment of cells from the soil particles) (Vester et al. 2014) 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 HAB and on solidified saline basal medium (SBM) supplemented with 2 % (v/v) diesel oil for enumeration of HDB. SBM was prepared as described by Espeche et al. (1994).

Plates were incubated for 30 days at 15 °C and results expressed as colony-forming units per gram of dry weight soil (CFU/g dw). Differences among mean values for bacterial counts (and also for hydrocarbon content) were determined using one-way ANOVA and Tukey's post test.

Isolation of DNA and PCR amplification

For molecular analysis, three soil subsamples from each biopile were pooled, and total DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. DNA was purified using the Genomic Blood DNA Purification Kit (GE), spectrophotometrically quantified with QUBIT (Invitrogen, Brazil) and also evaluated by electrophoresis in 1.5 % (w/v) agarose gels stained with ethidium bromide.

A fragment of the variable region V3–V5 of the 16S rDNA was amplified using the eubacterial primers 341-F-GC and 907-R (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 of bovine serum albumin, 1 U of GoTaq (Promega, USA) and 1X GoTaq buffer. PCR was performed in a total volume of 30 µl. Thermocycling was performed under the following conditions: an initial denaturation step for 4 min at 95 °C; ten cycles consisting of 94 °C for 30 s; 62 °C for 45 s; 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 quality of the PCR products was analyzed by agarose gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE)

PCR products were separated by denaturing gradient gel electrophoresis, using DGGE (Bio-Rad D GENE System, Munich, Germany), on a 6 % (w/v) polyacrylamide gel (acrylamide:*N*,*N*′-methylenbisacrylamide, 37.5:1) and 1× TAE buffer (10 mM sodium acetate, 0.5 mM Na_2-EDTA and 20 mM Tris base, pH 7.4). The denatured gradient of the gel was 45–70 % (100 % denaturant corresponds to 7 M urea and 40 % v/v formamide). Gels were run at 60 °C for 16 h at 100 V, stained for 1 h with SybrGold (Invitrogen) in 1× TAE buffer, viewed under UV light and analyzed using the GelCompar II® software package (Applied Maths, Kortrijk, Belgium). Densitograms were obtained from DGGE lanes and used for calculating a similarity matrix using Pearson's product correlations (Pearson 1926). Only bands representing more than 2 % of total relative intensity in the DGGE banding pattern were considered. The dendrogram was calculated by the unweighed pair group method with the arithmetic mean (UPGMA) (Del Panno et al. 2005).

Clone library sequencing and phylogenetic analysis of DGGE bands

In this part of the work, we focused our analysis on the identification of the dominant taxonomic groups present in the sole system exhibiting significant hydrocarbon removal. For this purpose, PCR products (at the initial and final time) from the FM biopile were analyzed and compared with those from the CC biopile. PCR products were purified with the Genomic Blood DNA Purification Kit (GE), cloned into the pGEM-t easy vector (Promega, Madison, WI) and transformed into competent *Escherichia coli* DH5α, as described by the manufacturer's protocol. Blue-white screening was used, and 30 white colonies were randomly picked.

In order to verify whether the cloned sequences were present in the original bacterial community patterns revealed by DGGE, a colony PCR was performed. For this purpose, each clone was picked up with a tip and resuspended with 30 µl of distilled water in a 0.2-ml PCR tube. The bacterial suspension was kept for 10 min at 95 °C to disrupt the cells. After that, the PCR master mix was

added, and the amplification was performed as described above. Colony PCR products and PCR products from the original bacterial community were separated by DGGE in order to verify the clone position and, consequently, the presence of each sequence in the original community. Cloned sequences correlating with bands present in the original bacterial community were sequenced (Macrogen® Korea) compared with the GenBank database using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) and classified using the Ribosomal Database Project (RDP) 16S rDNA database (release 8.1, Cole et al. 2009).

Bands located in the same position in different lanes of the DGGE gel were identified with the same number in the DGGE pattern.

Results

Soil characterization

The analyzed soil had a sandy texture with 2.7 % clay, 4 % silt and 93.3 % sand. Organic carbon, total nitrogen and extractable phosphorous levels were 0.98, 0.045 and 0.0033 %, respectively. The C/N relation was 21.7. The water content was 11 %, and the pH was 7.1. The initial hydrocarbon concentration in the soil was 21,909 mg/kg dw.

Bacterial counts and total hydrocarbon removal

Evolution of the heterotrophic aerobic bacterial (HAB) counts is shown in Fig. 2a. An initial increase (days 0–5) was observed in the FM biopile. After day 5, the counts mantained a constant level that was significantly higher than CC at the end of the assay ($p < 0.01$). The FM system also showed an increase in the HDB during the first 5 days (Fig. 2b). After a slow decrease, the counts reached approximately 10^8 CFU/g until the end of the assay.

The control system showed a similar initial increase in HAB counts, but exhibited a decrease between days 5 and 16, with values dropping to levels near 10^7 CFU/g until day 50 and determining the above-mentioned significant differences with FM. HDB counts in CC decreased the first 5 days and subsequently maintained a level of 10^6 CFU/g, which was significantly lower than for FM ($p < 0.01$).

The CP system exhibited an initial decrease in HAB counts of one order of magnitude. After that, values showed oscillations but ranged near 10^7 CFU/g. On the other hand, after 5 days the HDB counts increased up to 15 days and were higher than those from CC ($p < 0.01$). This system showed an HDB/HAB ratio close to 1 from days 16 to 50.

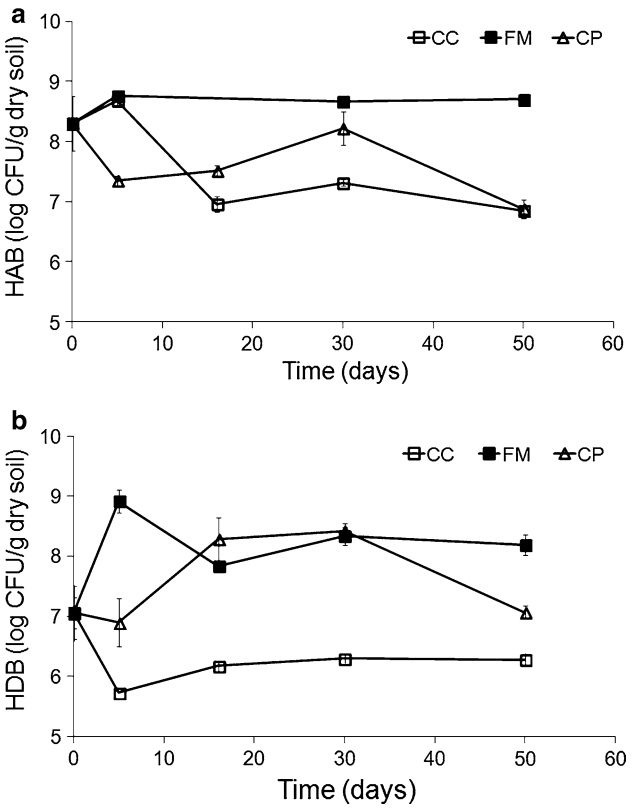

Fig. 2 Changes in **a** heterotrophic aerobic bacterial counts (HAB) and **b** hydrocarbon degrading bacterial counts (HDB) during the 50-day bioremediation assay in Antarctic soil biopiles with FM and the commercial product OSE II® (CP). *CC* control system. *Bars* represent SD from triplicates

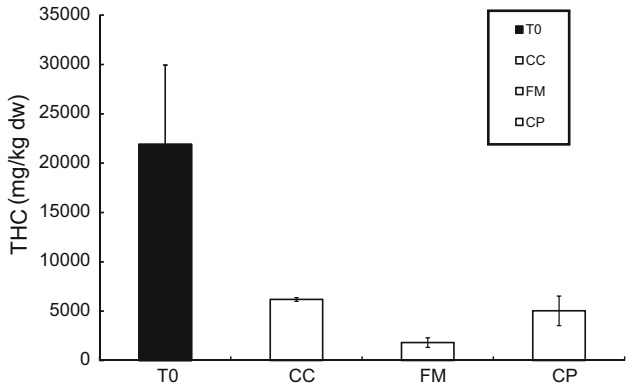

Fig. 3 Total hydrocarbon concentration (THC) in soil at day 0 (*black column*) and biopiles at day 50 (*white columns*). *CC* control system, *FM* fish meal, *CP* commercial product OSE II®. *Bars* represent SD from triplicates

Figure 3 shows the total hydrocarbon concentration (THC) in the contaminated soil at day 0 and in the three biopiles at day 50. From the initial concentration of hydrocarbon measured in soil (21,909 mg/kg dw), 6,190 mg/kg dw remained in the CC biopile at day 50, accounting for 72 % of hydrocarbon loss. At the end of the

assay, the THC value from FM biopile was 1,815 mg/kg dw, which represented a significant reduction ($p < 0.01$) of 71 % compared to CC at the same time. Conversely, the THC value from CP biopile was 5,037 mg/kg dw, resulting in a nonsignificant THC decrease (19 %) compared to CC at day 50.

Effect of nutrient amendment on bacterial community structure and dynamics

The effect of nutrients on the bacterial community structure was investigated using 16S rDNA-based PCR-DGGE. The DGGE banding pattern in the different treatments at days 0, 5 and 50 is shown in Fig. 4.

The dendrogram obtained from the densitometric analysis of DGGE (Fig. 5) evidenced that all samples from CC systems clustered together exhibiting a high percentage of similarity (86 %) and evidenced minor changes in bacterial community structure along the time in the unamended soil. Soil samples from FM systems at days 0 and 5 shared the same community structure as evaluated by DGGE. Although grouped in a different cluster, samples from days 0 and 5 of FM and the CC samples showed a high percentage of similarity (82 %).

FM at day 50 exhibited a dramatic shift in bacterial community structure, sharing only 32 % of similarity with the cluster comprising all the rest of samples.

All samples from the CP system formed a subcluster with an 82 % similarity. This subcluster shared a 68 % similarity with all the CC and the early stages of FM. This suggests that the main change in CP community structure seems to occur just after the nutrient addition. After that, only minor changes occurred in the CP system.

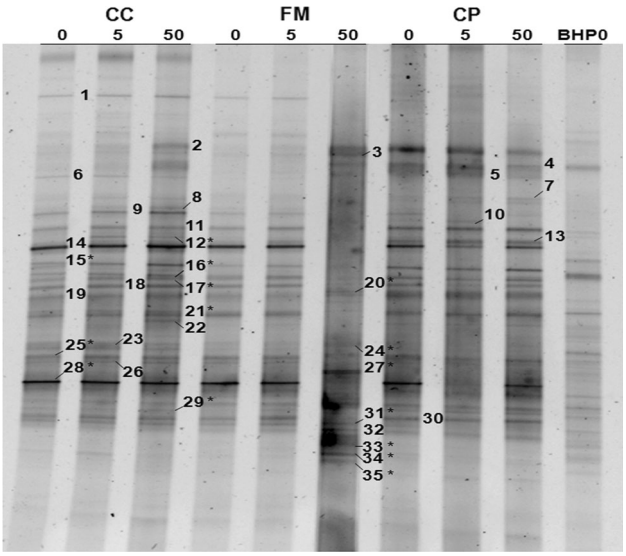

Fig. 4 Denaturing gradient gel electrophoresis (DGGE) profile of the bacterial community from the soil biopiles at days 0, 5 and 50 of the assay. FM and CP represent biopiles amended with FM and the commercial product OSE II®, respectively. *CC* control system. BHP0 represents the marker lane. *Numbers 1–35* indicate the DGGE bands included in the PCA. *Asterisks* indicate the sequenced DGGE bands

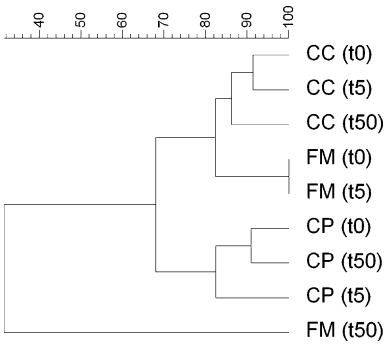

Fig. 5 UPGMA dendrogram resulting from cluster analysis of the data reporting the presence/absence and abundance of DGGE bands in the three microcosms systems at days 0, 5 and 50 of assay. *CC* control system, *FM* fish meal, *CP* commercial product OSE II®

Identification of the dominant bacterial DGGE bands

The characteristics of the sequenced bands are described in Table 1. It is interesting to note that many of the closest organisms were previously isolated from soil and water samples from cold environments. Fifty-three percent of the total sequenced bands were ascribed to the phylum Proteobacteria. As mentioned above, most of these bands were present in the CC biopile, for example, bands ascribed to the families Caulobacteraceae (genus *Brevundimonas*), Acetobacteraceae and Comamonadaceae (genus *Polaromonas*). These bands were not detected in the FM biopile at day 50. Sequences of the families Carnobacteriaceae (genus *Carnobacterium*) and Micrococcaceae (genera *Arthrobacter* and *Cryobacterium*), corresponding to the phylum Actinobacteria, represented 20 % of the total sequenced bands and were only identified in the FM biopile at day 50. Finally, members from the families Planococcaceae (genus *Planococcus,* phylum Firmicutes) and Sphingomonadaceae were found in both biopiles during the entire assay.

Discussion

The results from the bioremediation assay presented in this work evidenced a positive effect of the biostimulation with FM on the bioremediation of an Antarctic soil from Carlini station.

One of the observations worthy of comment is the 72 % of reduction in the initial hydrocarbon content observed in the CC biopile at day 50 that corresponds to both abiotic loss

Table 1 Phylogenetic affiliation of cloned sequences from DGGE gels

Band	Band detection	Closest organism[a]	% Similarity	Isolation origin
12	All except FM t50 (**CC t50**)	*Brevundimonas* sp. TP-Snow-C32 (HQ327141.1)	99	Snow from the Tibetean Plateau
15	All except FM t50 (**CC t0**)	Uncultured bacterium clone (GU183588.1)	97	Arsenic-contaminated aquifer at 85 m depth
16	All except FM t50 (**CC t50**)	*Sphingomonas* sp. FG03 (EU784670.1)	99	Phenol-contaminated soil in Northwest of China
17	CC t0, t5 and **t50**	Uncultured Sphingomonadaceae bacterium (GU300207.1)	98	Soil from Canada
20	**FM t50**	Uncultured Alpha-proteobacterium (DQ316817.1)	99	Uranium-contaminated sediment in Oak Ridge, TN, USA
21	All except FM t50 (**CC t50**)	*Caulobacter* sp. ECN-2008 (AM940947.1)	99	Contaminated *Paulinella cromatofora* culture
24	CC (t0, 5 and 50) and **FM t50**	*Carnobacterium* sp. G4a-1 (FN397989.1)	100	Gas hydrate containing sediment. (1,049 m water depth; 77 m below seafloor)
25	All except FM t50 (**CC t0**)	Uncultured bacterium clone (GU453471.1)	99	Hydrocarbon-contaminated aquifer
27	**FM t50**	Uncultured bacterium (JF357615.1)	100	Straw compost from Yongji City, China
28	All except FM t50 (**CC t0**)	Uncultured *Rhodoferax* sp. (GQ366604.1)	99	Soil near Roopkund glacier, Himalayan mountain ranges, India
29	All except FM t0 (**CC t50**)	*Bacillus* sp. G2 (2006) (DQ667102.1)	91	Ross Sea, Antarctica
31	All except CP (t5 and 50) (**FM t50**)	*Planococcus* sp. ZD22 (DQ177334.1)	99	Soil contaminated by petroleum effluents from Da Qing oil field, China
33	**FM t50**	*Arthrobacter* sp. 4–20 (GU213306.1)	99	Freshly deposited granite sand, Central Alps
34	**FM t50**	*Arthrobacter* sp. KOPRI 25422 (GU062491.1)	100	Arctic soil from Svalbard, Norway
35	**FM t50**	*Cryobacterium* sp. Hh31 (JF267311.1)	100	Soil of a glacier from Xinjiang Uyghur Autonomous Region, China

Samples in bold indicate the system from which the band was obtained, cloned and sequenced

[a] Sequences were matched with the closest relative from the GenBank database

and natural biodegradation. Although chronically contaminated, the soil had also suffered a recent spill of fresh diesel; hence, it contained a high proportion of light hydrocarbons that could undergo volatilization and stripping processes. Also, as was reported by Delille et al. (2008), working with pilot biopiles containing diesel-contaminated subAntarctic soils, freshly sieved and aerated soil prior to biopile processing seems to be an important factor determining the high hydrocarbon removal efficiency. Therefore, sieving and mixing could be another factor favoring the increase of hydrocarbon loss in all biopiles, including the controls. Values as high as 75 % of hydrocarbon removal in a newly diesel-contaminated Antarctic soil were observed by Ruberto et al. (2003) after 51 days in microcosms performed in 1-l flasks. It could be possible that in the work of Ruberto et al. (2003) the small size and high surface/volume ratio of the flask microcosms greatly contributed to the high hydrocarbon removal in the control treatment.

The fast stimulation on the HAB and HDB counts caused by FM evidenced that this organic nutrient represents an excellent substrate for enhancing growth of the soil hydrocarbon-degrading bacteria.

Although there is no consensus on the optimum C to N ratio for enhancing biodegradation of hydrocarbons in soil, some investigations have recommended that the maximal N application can be calculated as the mass of N per mass of soil water rather than to C concentrations (Ferguson et al. 2003). As polar soils often have low water-holding capacities, it may be difficult in practice to maintain optimal nutrient concentrations while precluding osmotic stress caused by overfertilization with inorganic fertilizers. Thus, the use of slow-release fertilizers, such as FM, was tested in cold soils (Aislabie et al. 2006) and sediments (Pelletier et al. 2004) and was also used as a nutrient source and carrier material in a recently patented biorremediaton method for treatment of ice-covered polar regions (Helmke et al. 2013). In our treatment, after the 3 % FM addition, the N and P content was increased up to 0.32 and 0.13 %, respectively, in the FM biopile, providing a readily biodegradable form of organic matter and a spongy texture to

the soil that could improve oxygen availability. This positive effect of FM on soil biodegradation has previously been proved for other bulking agents (Hamzah et al. 2012) and certainly could have contributed to the observed stimulation of bacterial counts and the consequent high elimination efficiency. High hydrocarbon elimination efficiency using biopiles with subAntarctic soils amended with FM was also observed by Delille et al. (2008), working with pristine soils artificially contaminated with fresh diesel. As was mentioned in the introduction section, revision of the literature evidenced that the biopile technique was successfully applied in a variety of contaminated polar soils amended with N and P. The FM had also been previously used by us in a bioremediation treatment of chronically contaminated Antarctic soil performed in land plots. However, nonsignificant hydrocarbon removal was observed (Dias et al. 2012). In that case, it was reported that growth enhancement caused by FM seemed to be unspecific, favoring the activity of the total bacterial community but not the specific hydrocarbon-degrading fraction. In contrast, it is possible that in the present work, in the presence of a fresh fraction of hydrocarbons, the FM resulted in a cometabolic source, which promoted the degradation of the available hydrocarbon coming from the fresh spill.

The biopile amendment with the commercial product OSE II® showed a different result. HDB counts were increased in CP, but no differences in hydrocarbon elimination were observed when comparing this system with the unamended control.

This commercial product is listed as a nutrient/enzyme additive and consists of nitrogen, phosphorus, readily available carbon and vitamins for quick colonization of naturally occurring bacteria. There is very little information available in the scientific literature reporting OSE II® application in hydrocarbon-contaminated soils. A field demonstration was carried out by Zwick et al. 1997 at a bioventing site at a Marine Corps Air Ground Combat Center (MCAGCC) in California to investigate the efficacy of OSE II in a fuel-contaminated vadose zone. The selection of OSE II was based on the recommendation of its use by EPA and also on evidence from a previous microcosm study in which various amendments were evaluated by monitoring microbial respiration using soils collected from oil-contaminated estuarine environments (Zhu et al. 2004). Also in our own previous study carried out in land plots, the presence of OSE II® resulted in significant hydrocarbon removal from chronically contaminated soil in the Carlini base (Dias et al. 2012). Considering the fact that our both bioremediation treatments were applied to similar soils from the same station and using the same fertilization protocol, we principally attribute this divergence in the efficiency of OSE II® in hydrocarbon elimination to the different soil contamination histories. The chronically polluted soil used in our previous study (10.336 mg/kg dw of total hydrocarbon concentration) had been stored for years in metal containers until it was decided to test the efficiency of OSE II in a bioremediation treatment. The observed stimulating effect could probably be related to the fact that OSE II® supplies a variety of growth factors that promote the elimination of aged hydrocarbons (Zhu et al. 2004) and also surface active compounds enhancing the bioavailability of recalcitrant hydrocarbons (Stillman 1992). Instead, the soil of the present study, despite having a long history of hydrocarbon contamination, was also under acute contamination caused by a recent fresh diesel spill, which increased the overall hydrocarbon concentration to 21,909 mg/kg dw. Under this hydrocarbon excess, the concentration of OSE II could prove to be low for maintaining an actively degrading bacterial population during the whole experiment. One of the reasons for this result could be the level of N and P provided by OSE II®, which could be insufficient for a good biodegradation rate under this higher level of fresh hydrocarbons present in this assay.

DGGE analysis showed similar band patterns at 0, 5 and 50 days from the unamended biopile, suggesting that minor changes were induced in the soil bacterial community structure by manipulation. Although grouped in a different cluster, the profiles at days 0 and 5 from FM biopile evidenced a high similarity percentage (82 %) with those from CC biopile, suggesting that although the FM addition had a great effect on the bacterial density, it only caused minor changes in the bacterial community structure at the first stages of the process. The treatment progress could be inferred by the obvious structural change observed at the last sampling time in the FM biopile, which correlated with the high hydrocarbon elimination. In correspondence with the observed bacterial counts, the OSE II® addition produced only comparatively minor temporal changes in the bacterial community structure for all of the treatment (68 % similarity). This observation was accompanied by a minor hydrocarbon removal activity.

Identification of the taxonomic groups from the most efficient treatment (FM biopile), although limited to the dominant groups, revealed the dominance of members of the phylum Proteobacteria (genera *Caulobacter*, *Sphingomonas*, *Brevundimonas*, *Rhodoferax*). Members of this phylum were reported to be predominant in chronically hydrocarbon-contaminated soils from Antarctica (Greer et al. 2010; Mac Cormack et al. 2011) and also from the Arctic (Bell et al. 2013). The presence of available hydrocarbon and FM could promote the selection of an active and fast hydrocarbon degrading population (r-strategist). Despite the evidence of an inhibitory effect of the fresh spill on the bacterial population density in the CC

biopile, the surviving population was also mainly represented by members of the phylum Proteobacteria. Toward the end of the treatment, with lower levels of available hydrocarbons and probably also low nutrient concentrations, better adapted bacteria as members of Actinobacteria were selected in the FM biopile. Actinobacteria were frequently reported in hydrocarbon-contaminated Antarctic soils (Saul et al. 2005; Aislabie et al. 2014) and also seem to be involved in aerobic and microaerophilic hydrocarbon degradation in soils from different cold environments (Whyte et al. 2002; Margesin et al. 2003; Björklöf et al. 2008; Ruberto et al. 2008). This group was considered to be k-strategist microorganisms and has been referred to as a major component in the later stages of successions of soils (Shrestha et al. 2007). Its absence in the first stages of the treatment could be related to the fact that the soil, despite its long previous history of hydrocarbon exposure, had received a significant amount of fresh diesel, resulting in an unfavorable environmental condition for Actinobacteria.

In summary, the results evidenced that biostimulation using the complex organic matrix FM is effective to enhance hydrocarbon bioremediation in biopile systems of frequently impacted soil as a consequence of the various activities conducted at the station. Comparison of the results with those previously obtained evidenced that bioremediation success is closely dependent on the previous history of pollution and mode of soil storage, even applying the same amendment procedure. Finally, the increase in hydrocarbon removal mediated by FM was accompanied by a clear change in the bacterial community structure and an increase in the proportion of the k-strategist Actinobacteria at the final stages of the process.

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References

Adetutu EM, Bird C, Kadali KK, Bueti A, Shahsavari E, Taha M, Patil S, Sheppard PJ, Makadia T, Simons KL, Ball AS (2014) Exploiting the intrinsic hydrocarbon-degrading microbial capacities in oil tank bottom sludge and waste soil for sludge bioremediation. Int J Environ Sci Technol 1–10. doi:10.1007/s13762-014-0534-y

Ahn JH, Kim MS, Kim MC, Lim JS, Lee GT, Yun JK, Kim T, Ka JO (2006) Analysis of bacterial diversity and community structure in forest soils contaminated with fuel hydrocarbon. J Microbiol Biotechnol 16:704–715

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

Aislabie J, Saul D, Foght J (2006) Bioremediation of hydrocarbon-contaminated polar soils. Extremophiles 10:171–179

Aislabie JM, Novis PM, Ferrari B (2014) Microbiology of eutrophic (ornithogenic and hydrocarbon-contaminated) soil. In: Cowan DA (ed) Antarctic terrestrial microbiology. Springer, Berlin, pp 91–113

ATCM XXXV (2012) Environmental issues related to the practicality of repair or remediation of environmental damage. Working paper 026 presented to ATCM XXXV, Hobart, New Zealand

ATCM XXXVI (2013) Repair or remediation of environmental damage: report of the CEP intercessional contact group. Working paper 027 presented to ATCM XXXVI, Bruseles, Belgium

Baek K-H, Byung-Dae Y, Byung-Hyuk K, Dae-Hyun C, In-Sook L, Hee-Mock O, Hee-Sik K (2007) Monitoring of microbial diversity and activity during bioremediation of crude oil-contaminated soil with different treatments. J Microbiol Biotechnol 17:67–73

Bell TH, Yergeau E, Martineau C, Juck D, Whyte LG, Greer CW (2011) Identification of nitrogen-incorporating bacteria in petroleum-contaminated Arctic soils by using [^{15}N]DNA-based stable isotope probing and pyrosequencing. Appl Environ Microbiol 77:4163–4171

Bell TH, Yergeau E, Maynard C, Juck D, Whyte LG, Greer CW (2013) Predictable bacterial composition and hydrocarbon degradation in Arctic soils following diesel and nutrient disturbance. ISME J 7:1200–1210

Björklöf K, Salminen J, Sainio P, Jørgensen K (2008) Degradation rates of aged petroleum hydrocarbons are likely to be mass transfer dependent in the field. Environ Geochem Health 30:101–107

Braddock JF, Ruth ML, Catterall PH, Walworth JL, McCarthynd KA (1997) Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: implications for nutrient-amended bioremediation. Environ Sci Technol 31:2078–2084

Bray RH, Kurtz LK (1945) Determination of total organic and available forms of phosphorus in soil. Soil Sci 591:39–45

Chemlal R, Tassist A, Drouiche M, Lounici H, Drouiche N, Mameri N (2012) Microbiological aspects study of bioremediation of diesel-contaminated soils by biopile technique. Intl Biodeterior Biodegradation 75:201–206

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:141–145

Del Panno MT, Morelli I, Engelen B, Berthe-Corti L (2005) Effect of petrochemical sludge concentrations on microbial communities during a soil bioremediation process. FEMS Microbiol Ecol 53:305–316

Delille D (2000) Response of Antarctic soil bacterial assemblages to contamination by diesel fuel and crude oil. Microb Ecol 40:159–168

Delille D, Duval A, Pelletier E (2008) Highly efficient pilot biopiles for on-site fertilization treatment of diesel oil-contaminated sub-Antarctic soil. Cold Reg Sci Technol 54:7–18

Delille D, Pelletier E, Rodriguez-Blanco A, Ghiglione J-F (2009) Effects of nutrient and temperature on degradation of petroleum hydrocarbons in sub-Antarctic coastal seawater. Polar Biol 32:1521–1528

Dias RL, Ruberto LAM, Vázquez SC, Hernández E, Lo Balbo A, Del Panno MT, Mac Cormack WP (2012) Bioremediation of an aged diesel oil-contaminated Antarctic soil: evaluation of the "on site" biostimulation strategy using different nutrient sources. Int Biodeterior Biodegradation 75:96–103

Espeche ME, Mac Cormack WP, Fraile ER (1994) Factors affecting growth of an *n*-hexadecane degrader Acinetobacter species isolated from a highly polluted urban river. Int Biodeterior Biodegradation 33:187–196

Ferguson SH, Franzmann PD, Revill AT, Snape I, Rayner JL (2003) The effects of nitrogen and water on mineralisation of hydrocarbons in diesel-contaminated terrestrial Antarctic soils. Cold Reg Sci Technol 37:197–212

Gee GW, Bauder JW (1986) Particle-size analysis. In: Klute A, Madison WI (eds) Methods of soil analysis, Part 1. Physical and mineralogical methods. Agronomy Monograph No. 9, 2nd edn. Soil Science Society of America, Madison, pp 383–411

Greer CW (2009) Bioremediation of contaminated sites in the Canadian Arctic: monitoring performance and the effects of biostimulation using molecular methods. In: Bej A, Aislabie J, Atlas RM (eds) Polar microbiology: the ecology, diversity and bioremediation potential of microorganisms in extremely cold environments. Taylor and Francis CRC Press, Boca Raton, pp 321–340

Greer CW, Whyte L, Neiderberger TD (2010) Microbial communities in hydrocarbon-contaminated temperate, arid, tropical, alpine, and polar soils. In: Timmis KN (ed) Polar microbiology: the ecology, diversity and bioremediation potential of microorgansims in extremely cold environments. Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 2313–2328

Hamzah A, Salleh SNMd, Lee SL, Sarmani S (2012) Bioaugmentation of microbial consortia and supplementation of bulking agents in removal of crude oil from soil. In: Mendez-Vilas A (ed) Microbes in applied research. Current advances and challenges. World Scientific Publishing Co, London, pp 39–43

Helmke E, Gerdes B, Juergens J, Reuter K (2013) Bioremediation method for accelerated biological decomposition of petroleum hydrocarbons in sea ice-covered polar regions, and bacteria and enzyme mixtures as agents for carrying out said method. US patent 2013/0210112 A1, issued Aug 15, 2013

Jin HM, Choi EJ, Jeon CO (2013) Isolation of a BTEX-degrading bacterium, *Janibacter* sp. SB2, from a sea-tidal flat and optimization of biodegradation conditions. Bioresour Technol 145:57–64

Jørgensen KS, Puustinen J, Suortt AM (2000) Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 107:245–254

Kang YS, Park W (2010) Protection against diesel oil toxicity by sodium chloride-induced exopolysaccharides in *Acinetobacter* sp. strain DR1. J Biosci Bioeng 109:118–123

Kauppi S, Sinkkonen A, Romantschuk M (2011) Enhancing bioremediation of diesel-fuel-contaminated soil in a boreal climate: comparison of biostimulation and bioaugmentation. Int Biodeterior Biodegradation 65:359–368

Lee K, Tremblay GH, Cobanli SE (1995) Bioremediation of oiled beach sediments. Assessment of inorganic and organic fertilizers, evolving technologies. In: Proceedings 1995 int oil spill conference, American Petroleum Institute, Washington DC, pp 107–112

Mac Cormack WP, Ruberto LAM, Curtosi A, Vodopivez C, Pelletier E (2011) Human impacts in the Antarctic coastal zones: the case study of hydrocarbons contamination at Potter Cove, South Shetland Islands. In: Coffen-Smout S (ed) Ocean year book, vol 25. Brill/Martinus Nijhoff, Dalhousie University, Nova Scotia, pp 141–170

Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. Appl Environ Microbiol 69:3085–3092

Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Waver C (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) Molecular microbial ecology manual. Kluwer Academic, Dordrecht, pp 1–27

Pearson K (1926) On the coefficient of ratial likeness. Biometrika 18:105–117

Pelletier E, Delille D, Delille B (2004) Crude oil bioremediation in sub-Antarctic intertidal sediments: chemistry and toxicity of oiled residues. Mar Environ Res 57:311–327

Popp M, Schlömann M, Mau M (2006) Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiol 152:3291–3304

Ruberto LAM, Vázquez SC, Mac Cormack WP (2003) Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. Int Biodeterior Biodegradation 52:115–125

Ruberto LAM, Vázquez SC, Mac Cormack WP (2008) Bacteriology of extremely cold soils exposed to hydrocarbon pollution. In: Dion P, Nautiyal CS (eds) Microbiology of extreme soils. Soil biology series 13, chapter 12. Springer, Berlin, pp 247–274

Ruberto LAM, Dias R, Lobalbo A, Vázquez SC, Hernández EA, Mac 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

Sanscartier D, Zeeb B, Koch I, Reimer K (2009) Bioremediation of diesel-contaminated soil by heated and humidified biopile system in cold climates. Cold Reg Sci Technol 55:167–173

Saul DJ, Aislabie J, Brown CE, Harris L, Foght JM (2005) Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. FEMS Microbiol Ecol 53:141–155

Shrestha PM, Noll M, Liesack W (2007) Phylogenetic identity, growth–response time and rRNA operon copy number of soil bacteria indicate different stages of community succession. Environ Microbiol 9:2464–2474

Snape I, Ferguson SH, Harvey PMA, Riddle MJ (2006) Investigation of evaporation and biodegradation of fuel spills in Antarctica: II. Extent of natural attenuation at Casey Station. Chemosphere 63:89–98

Stallwood B, Shears J, Williams PA, Hughes KA (2005) Low temperature bioremediation of oil-contaminated soil using biostimulation and bioaugmentation with a *Pseudomonas* sp. from maritime Antarctica. J Appl Microbiol 99:794–802

Stillman NW (1992) Bioremediation yeast and surfactant composition. U.S. patent 5160488 A. Filed September 14, 1990, and issued Nov 3, 1992

Vester J, Glaring M, Stougaard P (2014) Discovery of novel enzymes with industrial potential from a cold and alkaline environment by a combination of functional metagenomics and culturing. Microb Cell Fact 13:72

Walkley A, Black IA (1934) An examination of the Degtjareff method for determining organic carbon in soils: effect of variations in digestion conditions and of inorganic soil constituents. Soil Sci 63:251–263

Whyte LG, Schultz A, Van Beilen JB (2002) Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol Ecol 41:141–150

Wynn-Williams DD (1992) Counting bacterial colony-forming units in soil. In: Wynn-Williams DD (ed) BIOTAS manual of methods for Antarctic terrestrial and freshwater research, section II. Scientific Committee on Antarctic Research, Cambridge, p 3

Yergeau E, Arbour M, Brousseau R, Juck D, Lawrence JR, Masson L, Whyte LG, Greer CW (2009) Microarray and real-time PCR analyses of the responses of high Arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. Appl Environ Microbiol 75:6258–6267

Yergeau E, Sanschagrin S, Beaumier D, Greer CW (2012) Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high Arctic soils. PLoS One 7:1–10

Zhu X, Venosa AD, Suidan MT (2004) Literature review on the use of commercial bioremediation agents for cleanup of oil-contaminated estuarine environments. EPA/600/R-04/075 July 2004

Zwick TC, Foote EA, Pollack AJ, Boone JL, Alleman BC, Hoeppel RE, Bowling L (1997) Effects of nutrient addition during bioventing of fuel contaminated soils in an arid environment. In: Alleman BC, Leeson A (eds) In-situ and on-site bioremediation, vol 1. Battelle Press, Columbus, pp 403–409