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Bioremediation of hydrocarbon-contaminated soils in cold regions: Development of a pre-optimized biostimulation biopile-scale field assay in Antarctica

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HIGHLIGHTS

- Geomembrane-covered 0.4 ton biopiles were chosen as experimental systems.
- RSM-optimized levels of N and P significantly enhanced diesel fuel biodegradation.
- Average temperature was higher in covered systems than in the non-covered ones.
- In situ bioremediation of Antarctic soils is a useful tool for hydrocarbon removal.
- High removal (75%) of fuels from amended Antarctic soils was achieved in 40 days.

GRAPHICAL ABSTRACT

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ABSTRACT

Bioremediation proved to be an effective approach to deal with soil contamination, especially in isolated, cold environments such as Antarctica. Biostimulation, involving the addition of macronutrients -mainly nitrogen and phosphorous- is considered the simplest and cheapest bioremediation process. Optimizing the levels of these nutrients is a key step prior to the application of a biostimulation strategy. In this work, N and P levels, optimized by Response Surface Methodology (RSM) at lab-scale, were applied to an Antarctic hydrocarbon contaminated soil. The process was performed *on-site*, using high density polyethylene geomembranes (800 µm) to isolate treated soil from the surroundings and under environmental conditions at Carlini station (Antarctica) during 50 days. Two 0.5 ton biopiles were used as experimental units; a control biopile (CC), and a biostimulated system (BS), amended with N and P. At the end of the assay, hydrocarbon removal was significantly higher in BS system compared to CC (75.79% and 49.54% respectively), showing that the applied strategy was effective enough to perform a field-assay in Antarctica that significantly reduce soil contamination levels; and proving that RSM represents a fundamental tool for the optimization of nutrient levels to apply during bioremediation of fuel contaminated cold soils.

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1. Introduction

Contamination with organic compounds is an issue of general concern around the world. A myriad of hydrocarbon polluted sites can be found worldwide (Sharma et al., 2000; Konečný et al., 2003; Shi et al., 2008; Panagos et al., 2013). This environmental problem occurs even in cold isolated regions such as the Arctic and Antarctica (Aislabie et al., 2006; Gomez and Sartaj, 2013) due to the hydrocarbon-based fuels which are used as energy source in these locations (Aislabie et al., 2004). In Antarctic stations, fuel management has caused both minor and major spills, deeply affecting the surrounding soils, waters and sediments. Given the remoteness from suitable infrastructure for remediation of the Antarctic affected environment, bioremediation is considered the most adequate approach to recover these soils from contamination (Ruberto et al., 2003; de Jesus et al., 2015). In this frozen continent, environmental liabilities dating back to times when fuel management was environmentally inattentive, as well as new contamination events require the development of simple bioremediation protocols, able to be applied in locations where the availability of machinery and facilities is scarce.

From a biological point of view, soil is a substantially complex matrix, particularly those contaminated with organic compounds. In these soils, natural carbon and energy sources coexist with several kinds of xenobiotic compounds, each one with its own chemical structure, individual concentrations and toxic effects on the soil biota. It is estimated that 1 g of soil could contain as much as 10^{10}–10^{11} bacteria (Horner-Devine et al., 2004), comprising 6000 to 40,000 different species (Curtis et al., 2002). These huge biomass values refer also to fungi, which could reach up to 200 m of hyphae per gram of soil (Leake, 2004). Although Antarctic soils present lower biomass and microbial diversity values than tropical ones, these numbers give an idea of the biological potential of a soil and reflect a wide catabolic capability to support a bioremediation process. In this regard, even the Antarctic soils have proved to contain a diverse microbiota capable of reaching satisfactory levels of contaminant removal under biostimulation processes (Coulon and Delille, 2003; Ruberto et al., 2008).

Due to its biological nature, bioremediation is dependent on several physicochemical factors (Boopathy, 2000). Thus, the development of bioremediation processes should consider the features of the treatment site. These include soil texture, air temperature and other climate variables, water availability, existing vegetation and terrain topology (Ruberto et al., 2013). Concentration of nutrients (such as bioavailable Nitrogen and Phosphorus, total organic matter and O_2 levels) and the thermal amplitude to which each soil is exposed to, can also be considerably diverse in different soils. All these physicochemical variables should be taken into consideration when a biostimulation approach is intended.

Different C:N:P ratios were considered as reference for nutrient addition in biostimulation approaches over the years (Waksman, 1927, Redfield et al., 1963, Brown et al., 1983, Morgan and Watkinson, 1989, Zhou and Crawford, 1995, Dong et al., 2015). Considering the stoichiometry of microbial growth, a C:N:P ratio of 100:10:1 has been reported as the optimum choice (Cheng and Mulla, 1999; Dibble and Bartha, 1979). However, in order to get optimal results, this established ratio should be tested whenever a bioremediation process is considered, since each soil has its own biological diversity and requirements. These considerations are clearly visible when an excessive addition of nutrients is provided, resulting in the inhibition of biological activity (Liu et al., 2011). For this reason, the optimization of this strategy is a key step prior to field application.

Landfarming is not suitable for a large scale process with Antarctic soils, because water content is considerably altered by snow and wind. In addition, wind can spread contaminated soil particles to a larger area or even to the sea. On the other hand, indoor processes present the advantage of enabling temperature regulation and avoiding sharper modifications in water content. However, these kinds of processes require adequate facilities and entail costs associated to heating. For these reasons, geomembrane covered biopiles are a suitable alternative, combining smaller surface requirements than landfarming and the protective effect of the membrane, avoiding abrupt water content changes as well as wind drag and lixiviation. Coverage of soil with coatings has also been reported as a way to enhance soil average temperature and to reduce thermal amplitude inside biopiles, benefiting the degrading potential of the microbial community and achieving a higher biological hydrocarbon removal (Mohn, 2001; Delille et al., 2004, 2008; Gomez and Sartaj, 2014).

As information about "in situ" bioremediation in Antarctica is scarce, and based on the priority that environmental restoration has for our country and the rest of the Antarctic Treaty members, in this work we report the results obtained from a field assay consisting on biostimulation of a biopile arranged with a diesel fuel contaminated soil from Carlini Station (25 de Mayo Island, South Shetlands, Antarctica) with levels of N and P previously optimized using Response Surface Methodology (RSM) (Martinez Alvarez et al., 2015). As far as we know, this experiment constitutes the first report of a rational bioremediation design based on such an optimization for Antarctic gasoil contaminated soil.

2. Materials and methods

2.1. Soil analysis and characterization

Soil for this field assay was gathered in December 2013, during 2013–2014 Argentinian Antarctic expedition from the diesel fuel storage tanks surrounding area at Carlini scientific station, Isla 25 de Mayo (King George Island), South Shetlands, Antarctica (62°14′18″S 58°40′00″W). Contaminated soil containing an average hydrocarbon concentration of 2180 mg kg^{-1} was sieved (10 mm mesh) to remove stones, concrete, large paint residues and any other rough material. Sieved soil was divided in two fractions. One of them was used for the field assay and the other one stored at −20 °C for further studies. The soil was also analyzed for texture by the pipette method (Gee and Bauder, 1986), organic carbon (Walkley and Black, 1934), extractable phosphorous (Bray and Kurtz, 1945) and total Kjeldhal nitrogen. Water content was determined gravimetrically by drying samples at 105 °C during 24 h. For pH measurements, 10 ml of sterile saline solution (NaCl 8.9 g/L) were added to 1 g of soil and vortexed for 1 min. The pH of the resulting suspension was measured using a Docu pH+ meter probe (Sartorius). All soil samplings were randomly gathered from the biopile after proper homogenization and mixture in order to assure representative samples.

2.2. Biopiles design

For the field assay, approximately 830 kg of sieved contaminated soil were homogenized using a rotating drum, and then divided into two equivalent fractions in order to set up the community control biopile (CC) and the biostimulated biopile (BS). CC experimental unit consisted in contaminated soil with periodical mixing and water addition to maintain moisture target level (15% w/w). The BS experimental unit was similar to CC, but with the addition of nutrients to reach the previously optimized C:N:P ratio of 100:17.6:1.73 (Martinez Alvarez et al., 2015). Water content and mixing frequency were the same in both units. Both systems were arranged to define a truncated pyramid or pyramidal frustum (210 cm × 140 cm, 200 cm × 130 cm, h = 20 cm), holding 0.53 m^3 of soil (Fig. 1). This arrangement provides a trade-off between surface and volume, allowing the treatment of a large soil volume using a moderate surface size of terrain, minimizing the contaminant volatilization as well as the geomembrane requirements. In addition, it represents an adequate configuration to withstand strong winds.

Soil biopiles were isolated from the surroundings using a 3 × 5 m high-density polyethylene geomembrane (800 μm). This conformation

provides not only isolation from the contiguous soil, avoiding lixiviation, but also protection against wind, snow and rain, being completely inert towards petroleum hydrocarbon compounds. As summertime in polar regions is characterized by long days and short nights resulting in extended periods of solar irradiation; and as dark materials tend to absorb solar radiation more efficiently than clear ones, a potential warming effect on the biopiles was evaluated. Soil temperature, water content and conductivity were measured at 2hs-interval using a Decagon Data logger equipped with four 5TE sensors, one inside each biopile, and two sensors (controls 1 and 2) buried in the surrounding soil. Biopiles were placed adjacent to the diesel storage tanks, near the site where the contaminated soil was taken from. Biopiles were opened and manually mixed using shovels every two days. Water content was adjusted, when necessary, by adding filtered (0.22 μm) distilled water.

2.3. Nutrients addition

Biostimulation treatment was performed in accordance with soil initial hydrocarbon concentration, and applying the optimized nutrient levels obtained from a response-surface methodology (RSM) experiment previously reported (Martinez Alvarez et al., 2015). Nitrogen and Phosphorous were added as ammonium nitrate and monosodium phosphate respectively (Analytical grade, Anedra). A sterile solution (autoclaved at 121 °C for 15 min) containing both salts was prepared dissolving 372.5 g of NH_4NO_3 and 56.3 g of KH_2PO_4 in 5 L of distilled water. After sterilization, the solution was diluted with deionized water to a volume of 30 L and sprayed onto each system. In this manner, nutrients were better distributed in the bulk soil and, at the same time, water content was adjusted to the target level of 15% w/w. This water content allows proper biological activity without flooding the studied soil (US EPA, 1994).

2.4. Hydrocarbon analysis

For hydrocarbon quantification, soil samples (5 g) were extracted with 20 mL of tetrachloroethylene (IR quality, Sintorgan) in 40 ml Teflon tubes (Nalgene). A spoon tip of sodium sulfate was added to each tube in order to remove any remaining aqueous fraction in the organic phase. After that, 0.5 g of Silica Gel 60 (0.063–0.200 mm, Merck) was added to each tube to remove polar compounds from the organic phase. Flasks were shaken (200 rpm) for 3 h at 25 °C. Afterwards, samples were centrifuged (15 min, 7600 g), filtered to avoid the presence of soil particles and transferred to a quartz-cell for analysis. Five replicates were extracted and measured at each sampling time. The results were expressed as the average of those five determinations.

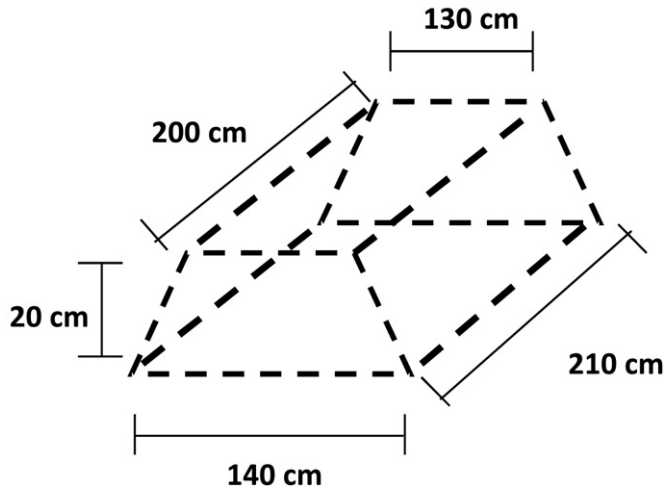

Fig. 1. Biopiles shape and dimensions. Volume can be calculated as $V = \frac{h}{3} \times (A + A' + \sqrt{A + A'})$, where h stands for height, A represents the surface of the larger base and A′ the surface of the smaller base.

Hydrocarbon quantification was performed at Carlini's scientific station laboratory with a Fourier Transform Infrared spectrophotometer (IR Affinity 1, Shimadzu, Corp., Japan), employing a modification of ASTM D7066 method. Calibration was performed with diesel fuel standards prepared in HPLC-grade tetrachloroethylene (blank, 500 and 1000 mg kg^{-1}), and peak areas in the range of 2850 to 3000 cm^{-1} were considered. The Infrared spectra determination was set for 45 scans per sample and resolution was set at 2.0. Samples were measured following the same protocol, and final values were obtained extrapolating each sample peak area in the respective calibration curve.

For gas Chromatography analysis (GC) soil samples (5 g) were extracted with 40 mL of hexane:acetone solution (1:1) in 40 mL Teflon tubes (Nalgene). Sample conditioning included the same steps as for FTIR measurement. Chromatographic analysis was carried out in a Shimadzu GC-9A Gas Chromatograph (Shimadzu, Corp., Japan) equipped with a flame ionization detector (FID) and a 30-m-long 0.25 mm i.d. (0.25 μm film thickness) fused silica capillary column (cross-linked 5% PH ME siloxane). Oven temperature was kept at 100 °C for 1 min, then ramped at 10 °C/min to 250 °C and kept at this temperature for 5 min. Injector temperature was 280 °C. Carrier gas (He_2) flow was 31 cm/s. Samples from day 50 were not evaluated due to problems during storage and transportation. Data were collected using the PC Chrome software (Universidad de Buenos Aires, Argentina). The total area of all peaks ranging from C9 to C28 in samples was compared to those from suitable standards (Supelco Diesel Organic Range Calibration Mix).

2.5. Biological activity

Biological activity was inferred by two different methods: Fluorescein diacetate (FDA) hydrolysis and bacterial counts. Total microbial activity was followed by the fluorescein diacetate method (Adam and Duncan, 2001). Briefly, 2 g of soil were incubated (20 °C and 200 rpm) with 15 ml of a phosphate solution (8.7 g/L K_2HPO_4, 1.3 g/L KH_2PO_4, pH 7.6) and 200 μL of a 2000 μg/L solution of Fluorescein Diacetate. After 30 min, the reaction was stopped by adding 15 ml of Chloroform: Methanol (2:1) and the solutions were centrifuged for 3 min at 480g. Absorbance of the aqueous phase was spectrophotometrically measured at 490 nm.

On the other hand, the number of total heterotrophic aerobic bacteria (THAB) was determined by plating serial dilutions of soil samples on half strength R2A agar (Oxoid), in order to provide a more oligotrophic media -in accordance with the low organic nutrient level present in Antarctic soils. Dilutions were prepared by mixing 1.0 g of sieved soil with 10 ml of saline solution (0.9% NaCl) containing 0.01% Tween 80. Hydrocarbon degrading bacteria (HDB) were enumerated by plating 0.1 ml of the serial dilutions on agarized SBM containing 1% diesel fuel as sole carbon and energy source. All samples were vigorously vortexed (15 min) to allow an efficient detachment of microbial cells from soil particles. Both, THAB and HDB plates were incubated 14 d at 15 °C and the results expressed as CFU per gram of dry weight (CFU g^{-1} dw). For both, FDA hydrolysis and bacterial counts, the results shown represents the average obtained for five replicates. R2A halved medium contained (in g/L): Yeast extract, 0.25; Proteose peptone, 0.25; Casein hydrolysate, 0.25; Glucose, 0.25; Starch, 0.25; K_2HPO_4, 0.15; $MgSO_4$, 0.0012; Sodium pyruvate, 0.15; Agar, 15.0.

3. Results

3.1. Biological activity

With the exception of t_0, biological activity (as FDA levels) showed significantly higher values in the biostimulated system in comparison to those observed in the control ($p > 0.05$, Fig. 2). In BS treatment, the

biological activity significantly increased over time (more than 9-fold increment during the first 30 days), reaching its highest point at day 40. The ratio between biological activities in both systems (BS/CC) increased almost linearly up to day 40, reaching a 7:1 ratio at the end of the experiment (day 50). This fact, and the scarce increase in biological activity showed by CC, where no N and P were added, highlights the positive effect of nutrient addition (biostimulation) on the biological activity of the microbial community present in the treated soil. This result also implies that the soil used in this experiment could not provide enough amounts of these nutrients to allow an optimum -not limited- hydrocarbon catabolic rate.

3.2. Bacterial counts

THAB and HDB exhibited a considerable increase in counts during the first 10 days, reached its maximum at day 30 and then decreased in both CC and BS systems (Fig. 3a, b). The initial rise was observed in both CC and BS systems, suggesting that it is related, in part, to aeration and manipulation -in addition to the biostimulation effect. After that, THAB and HDB continued increasing at a lower rate under both conditions (CC and BS), although HDB grew proportionally more than THAB in the BS system, as evidenced by the BDH/THAB ratio (Fig. 3c). THAB and HDB counts reached values over 1×10^8 total CFU/ml and over 1×10^6 CFU/ml respectively in BS system. This result is in accordance with the percentage of degrading bacteria, which also showed its maximum at day 30 (Fig. 3b), indicating that the observed increase and subsequent decrease in total CFU's is triggered by the rise and fall of the number of hydrocarbon degrading bacteria. In any case, nutrients addition proved to be indispensable for the development of bacterial flora. This fact was supported by the observation that in all cases, bacterial counts were substantially higher in the biostimulated system. After day 40, counts tended to decrease, suggesting the appearance of some kind of limiting factor for bacterial growth, possibly due to the shortage of easily-degradable organic carbon.

3.3. Physical properties follow-up

3.3.1. Soil analysis

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} (1.02%), 0.32 g kg^{-1} and 5.0 mg kg^{-1} respectively; while pH was 6.8 and the water content 10%. Total hydrocarbons concentration was 2180 ± 165 mg kg^{-1}.

3.3.2. Conductivity

A clear difference was observed in the conductivity values for each biopile even at t_0 (Fig. 4). These differences can be attributed to nutrients addition. The use of NH_4NO_3 and KH_2PO_4 in aqueous solution as N and P source for biostimulation in BS biopile, provided the soil with a larger amount of ions compared to the CC biopile, where only filtered (0.22 μm) distilled water was added. Also, CC biopile showed almost no change in conductivity values, consistent with the low levels of ions present or available in this Antarctic soil. In BS system, the obtained conductivity patterns presented peaks every two days, in accordance with soil mixing days. This observation suggests that soil mixture is vital for solubilization and, consequently, availability of nutrients for the microbial flora. The intensity of these peaks decreased throughout the experimental period. However, average conductivity remained almost unchanged during the entire experiment, as is reflected by the tendency line slopes in both systems (−0.0003 for BS and 0.0001 for the CC).

3.3.3. Temperature

Mean temperature measured for both membrane-covered systems (6.5 °C for BS and 6.7 °C for CC) showed to be significantly different (ANOVA, $p < 0.001$) than those registered for the controls (5.2 °C and 5.3 °C for soil-buried sensors, controls 1 and 2 respectively). As it can be seen in Fig. 5, most of the points are over the zero line, meaning that temperature was higher in covered systems than in the non-covered ones –only 12% below zero line. The difference reached its highest point on an unclouded day, with high solar radiation, at 4:00 am (ΔT 4.8 °C), whereas the lowest value (ΔT −6.7 °C) was recorded at 16:00 pm the same day. This pattern of positive differences during night and negative differences during the day was consistent during the entire experiment and suggests that the membrane kept soil warm during the non-solar radiation periods and made covered soil inside it less sensitive to solar heating. In other words, it reduced the thermal amplitude inside the biopiles, while keeping them warmer than uncovered soil.

3.4. Hydrocarbon content

Hydrocarbon concentration decreased from an initial 2180 (±165) mg kg^{-1} to a final value of 527.79 (±27) mg kg^{-1} in the biostimulated

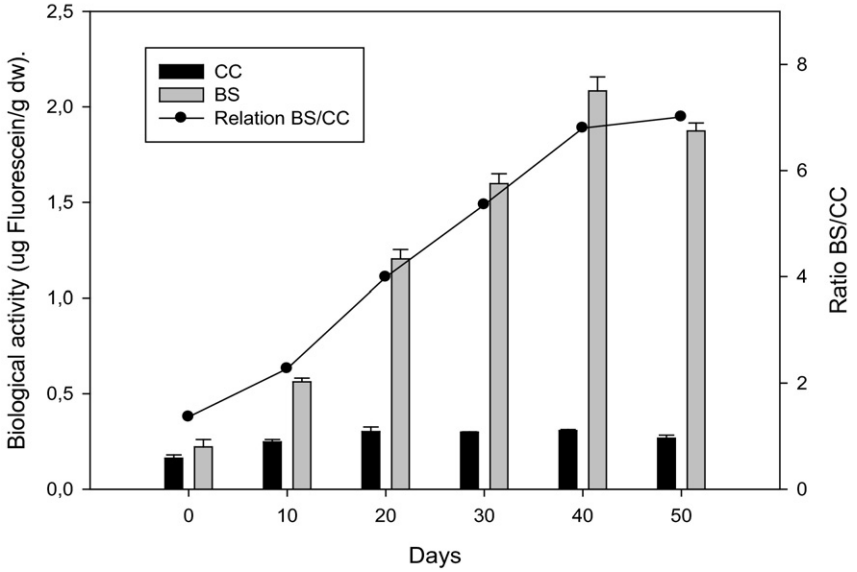

Fig. 2. Biological activity levels, measured as released fluorescein, for Control (CC) and Biostimulated system (BS).

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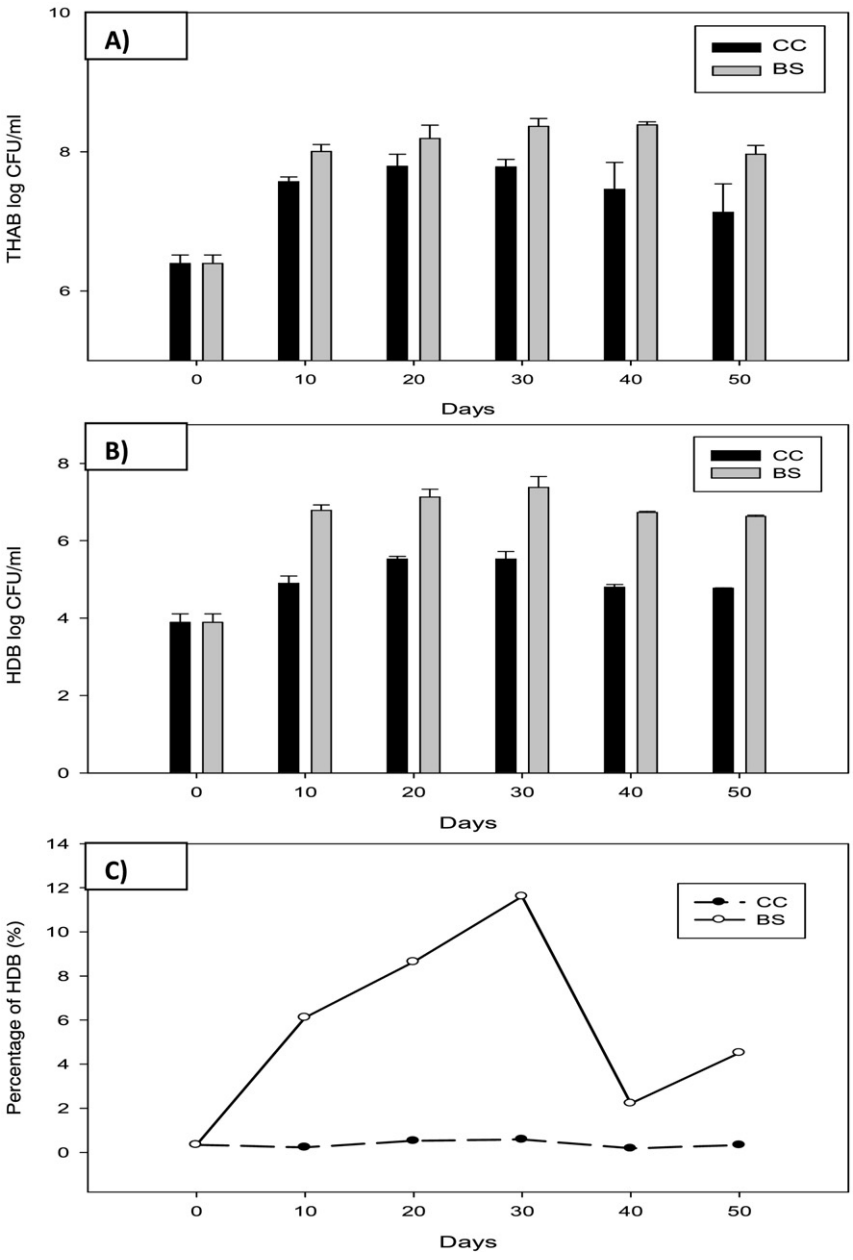

Fig. 3. Bacterial counts for control (CC) and biostimulated (BS) systems. A) Total heterotrophic aerobic bacteria (THAB) expressed as colony forming units per ml. B) Hydrocarbon degrading bacteria (HDB) expressed as colony forming units per ml. C) Relative percentage of HDB/THAB (%).

system (75.79% removal), while 1110.1 ($\pm$88) mg kg^{-1} was the final hydrocarbon content in the control biopile (49.54% removal). The highest removal rates were found up to day 30 for both systems. After this time, hydrocarbon concentration values tended to become stable (Fig. 6). Although Infrared spectra measurement includes some uncertainty when working in time-series analysis, due to the fact that hydrocarbon composition –and not only concentration- changes through time, it is important to highlight that it represents an appropriate method for hydrocarbon quantitation.

The analysis of some isoprenoids hydrocarbons by GC-FID (Fig. 7) was performed in order to assess evaporation losses due to their resistance to biodegradation and their relatively low volatility (Snape et al., 2005; Martinez Alvarez et al., 2015). It is considered that isoprenoids are resistant to biodegradation. The percentage removal of iC_{14}, iC_{15} and iC_{16} in the control (CC) and the biostimulated treatment (BS) are shown in Table 1.

The results obtained for CC biopile evidenced that, in spite of the use of a closed system, hydrocarbon removal due to evaporation was considerable during the process, ranging between 18 and 43%, depending on the isoprenoid. Nonetheless, the difference in the elimination percentage between CC and BS systems for all the considered isoprenoid compounds suggests that isoprenoids are being mainly biodegraded, or at least selectively eliminated in the system where the biological activity was stimulated (BS).

4. Discussion

The assay here presented demonstrate that a biopile full-scale bioremediation of hydrocarbon contaminated soil is feasible in Antarctic regions, reaching up to 75.9% of removal in treated systems.

This field assay arose from a previous laboratory analysis, in which C:N:P ratio for Carlini Station contaminated soil was optimized

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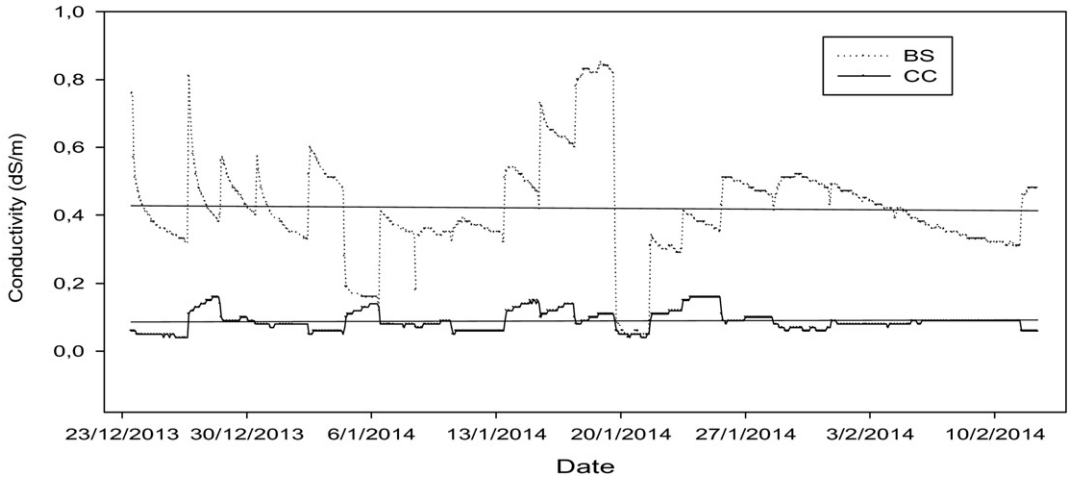

Fig. 4. Conductivity values for the entire experimental period, for BS and CC systems.

(Martinez Alvarez et al., 2015). That optimum ratio was 100:17.6:1.73, and was obtained for a soil containing near 1200 mg kg^{-1} of hydrocarbons. The central aim of this work was to test if lab-optimized C:N:P ratio also leads to high bioremediation efficiency in the field, where several environmental variables –such as temperature, winds, precipitations- are not controlled. For this purpose, geomembrane-covered 0.4 ton biopiles were chosen as experimental systems. High density polyethylene geomembranes (HDPEG) had been recently tested as proper materials for isolation and covering of hydrocarbon-contaminated Antarctic soil biopiles (McWatters et al., 2016). Due to its resistance and robustness (it can maintain its physicochemical properties for at least three years of exposure to harsh environmental Antarctic conditions) HDPEG proved to be an adequate tool to contain and treat contaminated soil.

The experiment showed that, when biostimulated, up to 80% of the hydrocarbons could be removed from soil in a single step treatment of 50 days, when the initial level of contamination is around 2000 mg kg^{-1}. One important discovery refers to the levels of removal showed by the control system (CC), where no biostimulation treatment was applied. Values near 50% of the initial level of contaminants were

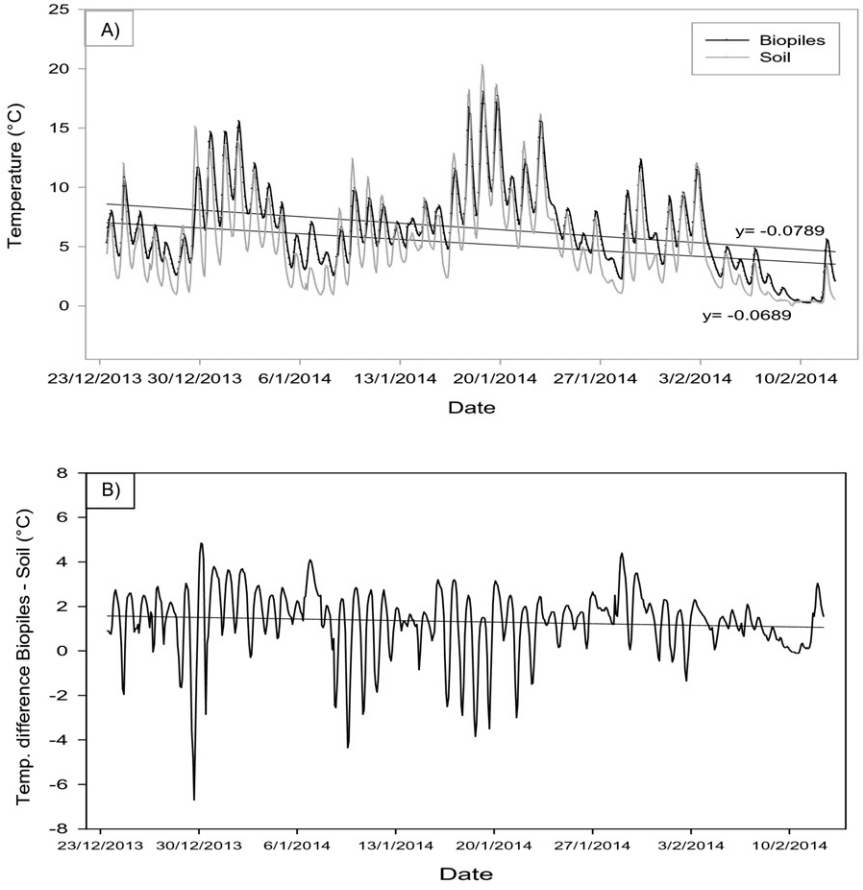

Fig. 5. A) Temperature plots throughout the entire experimental period for Biopiles and uncovered soil. B) Plot showing temperature differences between biopiles and uncovered soil for the entire experimental period.

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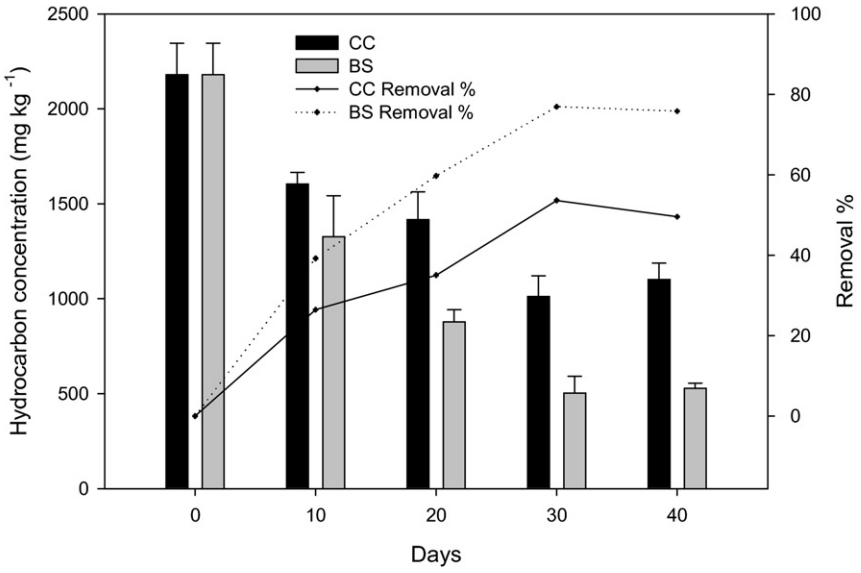

Fig. 6. Hydrocarbon concentration (mg kg^{-1}), and cumulative removal percentage (%) throughout experimental period. Error bars indicate standard deviation.

removed as the result of abiotic losses and intrinsic biodegradation capacity of the biota. Additionally, it is possible to assume that there was a significant abiotic loss due to volatilization at initial stages of the process. This hypothesis is supported by the facts that: 1) there was only a slight increase in biological activity for CC system; 2) HDB/THAB ratio did not increase during the entire assay for CC; and 3) the main hydrocarbon removal in this system occurred during the first ten days.

Significant losses of hydrocarbons due to volatilization showed to be highly relevant for the lightest aliphatic fractions of diesel fuels in biopiles containing contaminated Antarctic soils, as reported by (Whelan et al., 2015). These authors found -testing a dynamic multimedia model for bioremediation prediction- that although biodegradation was predicted to be the dominant loss mechanism for several hydrocarbon fractions, volatilization showed to be highly relevant for the lightest aliphatic fractions of diesel fuels.

In relation to the volatilization of the lightest compounds, isoprenoids hydrocarbons are used as evaporation reference molecules in contaminated soils (Snape et al., 2005; Snape et al., 2006). Consistently, in our assay, the analysis of the isoprenoid hydrocarbon fraction (iC_{14}–iC_{16}) evidenced that hydrocarbon evaporation was significant during the experiment, even though in an aged soil the volatile fraction is supposed to be lessened (Alexander, 2000). It is possible that mixing and laboring the soil exposed pockets of fresh fuel, allowing its contact with air and favoring evaporation.

It is interesting to highlight the increased elimination of isoprenoids hydrocarbons in the system where biostimulation was applied. Considering that CC and BS systems were identically designed, it is expected that both have similar evaporation rates, and therefore the increased elimination could not be attributed to evaporation, but to other processes related with nutrient addition. *Pseudomonas* sp. was shown to break down isoprenoid compounds (Cantwell et al., 1978), and a similar behavior was previously reported for Carlini station hydrocarbon contaminated soils (Martinez Alvarez et al., 2015), where members of the *Pseudomonadaceae* family are abundant inhabitants (Vazquez et al., 2013). Studies focusing on the degradation of this isoprenoid fraction are needed to gather more information of such biodegradation processes.

Also, it is likely that the thermal protection caused by the geomembrane cover may be partially isolating the microbial flora from environmental rough conditions, and therefore favoring natural attenuation. This natural attenuation may also have been enhanced by the thermal versatility that non-isothermal incubation provides to the microorganisms in the field assays in Antarctica. The increase of both, THAB and HDB values in response to soil aeration and humidity adjustment, as well as the difference in the hydrocarbon removal between CC and BS systems support previous observation that the microbial flora in Antarctic chronically-contaminated soils is adapted to tolerate and to metabolize fuels derived contaminants (Ruberto et al., 2009, Dias et al. 2012), and is responsible in part of the observed levels of natural attenuation.

The present experiment designed was based on mathematical and statistical predictions obtained from a response surface assay, which was planned and conducted for a previously known hydrocarbon concentration (Martinez Alvarez et al., 2015). From the stoichiometric point of view, this optimized ratio between C, N and P is theoretically lineal and therefore, an increase in C amount must be followed by an increase in the N and P levels needed to be added to that soil in order to allow a proper contaminant removal. However, other phenomena like modification in the bioavailability of any of the elements in this ratio could break the linearity. In addition, the fact that petroleum hydrocarbons are intrinsically toxic should not be ignored. Higher levels of hydrocarbons may represent a lethal concentration for microbial species that are tolerant to this contaminant, causing a decrease in biological activity of the treated soil, and therefore a decrease in the overall process efficiency. For these reasons, more and deeper investigations are necessary to explore the pollutant concentration range in which the prediction is fulfilled.

Hydrocarbons removal rate is crucial when time is a limiting factor. This is particularly true for the Antarctic continent, where any large-scale bioremediation scheme is limited to the summer months, a season in which soil is thawed and snow-free. In this regard, Bardi et al. (2000) compared the degradation rate of some model hydrocarbons in liquid media at 28 °C using β-cyclodextrine as surfactant. They reported the complete degradation of 40.000 mg kg^{-1} of dodecane in 4 days for the system containing the surfactant and in 9 days in its absence. These values resulted in removal rates of 10.000 and 4.444 mg kg^{-1} day^{-1} for the system with and without surfactant addition respectively. Despite having been obtained in liquid media and at 28 °C using a simple aliphatic hydrocarbon as a target molecule, these values could be considered as reference for a very high removal rate. In this same way, Liu et al. (2011), working with soil contaminated with 14.000 mg kg^{-1} of diesel fuel, reported a removal efficiency (140 days) of 79.7% for a biostimulated system having a C:N:P ratio of 100:11:3.7 by the addition of NH_4NO_3 and K_2HPO_4. The authors referred

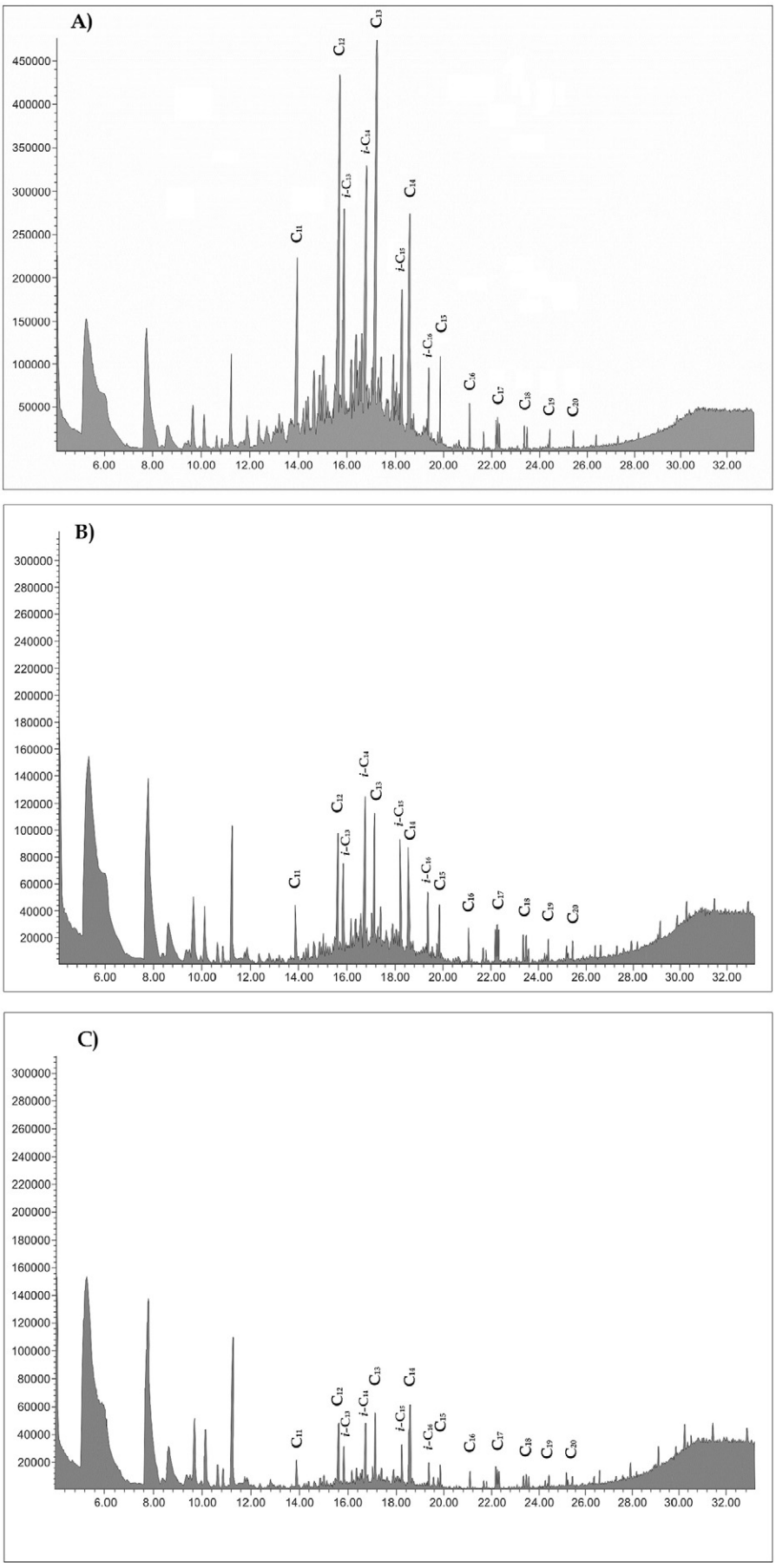

Fig. 7. GC-FID chromatograms of soil hydrocarbons at initial time (A), and after treatment for control (CC) system (B) and biostimulated (BS) system (C).

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Table 1
Isoprenoid hydrocarbons removal per system.

Isoprenoid hydrocarbon	CC percentage removal	BS 304 percentage removal
iC_{14}	43	81
iC_{15}	31	77
iC_{16}	18	71

that most of the removal (69.8%), occurred during the first 35 days, with a resulting elimination rate of 273.5 mg kg^{-1} per day. They suggest the existence of a first removal stage followed by a plateau, during which degradation seems to be limited. A similar pattern was observed during our field experiment at Carlini station. Hydrocarbon removal efficiency plot clearly shows different slopes: one for the BS system from day 0 to day 20 (65.1 mg kg^{-1} day^{-1}, 2.99% day^{-1}) other for CC at the same period (38.2 mg kg^{-1} day^{-1}, 1.75% day^{-1}); and a last one, similar for both systems, from day 20 to day 40 (17.4 mg kg^{-1} $day^{-1}$and 15.7 mg kg^{-1} day^{-1}, 0.80 and 0.73% day^{-1} respectively). The removal rate, expressed as absolute values (mg kg^{-1}), showed by BS system during the first twenty days was significantly lower than those reported by Liu et al. (2011), but was comparable when it was expressed as removal percentages (2.99% versus 1.95% day^{-1}). This information is particularly useful when considering biostimulation strategies in Antarctica, as most of these processes are limited to summer months and its success may depend on the rate of degradation of the contaminant.

The experimental design used in this study (geomembrane-covered biopiles) generated different effects. On the one hand, it provided protection against snow, rain and wind. On the other hand, as was mentioned previously, black membrane coverage showed a temperature buffering effect with some warming outcome, similar to that previously reported by Delille et al. (2004, 2008). In our assay, the temperature buffering effect seemed to be more evident during the night, when the biopile received no solar radiation, and differences up to 6 °C were recorded when soil temperature in and outside the membrane was compared. In contrast, the sun-lighted hours proved to have a higher warming effect on the uncovered soil in comparison with the soil in the biopile, in which the temperature elevation was slighter, showing narrower thermal amplitude inside them. This temperature buffering effect caused by the geomembrane protection could be providing a gentler environment for the growth of soil microbiota. From the point of view of optimal temperature for growth, this mild thermal change may be favoring development of psychrophilic bacteria, avoiding temperature peaks and minimums that could cause growth inhibition or even detention of a fraction of the microbial population and, therefore, be affecting the bioremediation efficiency. However, it could also be true that community members with higher optimal temperatures are not favored by the buffering effect. Also, as it was mentioned before, the fact that this process was not carried out under isothermal conditions, may be favoring the development of microbial species with different optimal growth temperatures; in contrast to the selective pressure being imposed on the community when it is incubated at a single temperature. These observations raise the question about what is convenient to select in order to favor biodegradation: temperature buffering or temperature peaks? The thermal pattern obtained in this field assay, caused merely by environmental conditions -but attenuated by the biopile conformation and the presence of the geomembrane- could be favoring the development of a more diverse microbial flora, with a higher catabolic capacity, able to enhance hydrocarbon removal efficiency in comparison to an isothermal process, as described by Chang et al. (2011). It is important to highlight that Delille et al. (2008) reported a slight effect of temperature (4, 10 and 20 °C) on hydrocarbon biodegradation in sub-Antarctic seawater. These results did not agree with the assumption of the dependence of biological rates with temperature increases. Another way of analysis could be those related to Q_{10} coefficient as reported by Mikan et al. (2002) for Arctic tundra or Bagi et al. (2013) for marine environments. Q_{10} represents the ratio between the reaction rates at temperatures differing in 10 °C. In accordance to Brakstadt et al. (2009), a Q_{10} ranging between 2 and 3 could be considered as a reference value or rule of thumb for calculation of biodegradation rates at two different temperatures. For polar regions in particular, this reference value is considered closer to 3, but no generalizations should be made when studying polar soils. For example, Chang et al. (2011) calculated a Q_{10} of 2.2 for a sub-arctic soil, but also reported that an exponential microbial activity blast occurred at temperatures over 4.7 °C. From this perspective, a temperature rise of only 1.5 °C (provided by the geomembrane envelopment) could be determining a significant increase on hydrocarbon degradation rate, especially considering that almost 70% of the registered temperatures inside the biopiles were higher than 4.7 °C, versus only 49% for uncovered soil. Further research upon microbial community shifts and the influence of thermal protection proportioned by the geomembrane on the overall efficiency of this process may help to confirm or discard these hypotheses.

Soil conductivity showed a regular pattern, presenting peaks every two days in the biostimulated system which correlated with soil mixing. This observation suggests that soil mixture is relevant for solubilization and, consequently, availability of nutrients for the microbial flora. Aeration is the main reason for mixing in most large-scale bioremediation processes. Mechanical aeration using pipes and tubes could be considered as an alternative for mixing. However, this approach only considers availability of oxygen, but not other substrates, such us N and P. Changes in conductivity evidenced during this experiment suggest that soil mixing produces additional effects beyond aeration. Hydrocarbon contaminated soil is a highly complex matrix, composed of several phases (including soil particles, microorganisms, air, water, insoluble liquids and solid hydrocarbons), and containing contaminants in different physical states (soluble, insoluble, gaseous, adsorbed to soil particles or to microorganisms). This feature may cause undesired unpredictability and lack of robustness on bioremediation schemes, leading to the results of an experiment being unrelated to those of replicates or scaled-up processes. For this reason, mixing and homogenization of soil (when possible) is a crucial step in this kind of experimental trial. The high removal efficiency obtained using our previously optimized C:N:P ratio suggests that predictability during scale-up is possible when using adequately homogenized contaminated soil.

5. Conclusions

Hydrocarbon removal efficiency obtained through this pilot scale field assay (over 75% within 40 days) confirms the advantage of optimizing the biostimulation conditions by RSM methodology, as a previous step to the field assays. The results obtained in this work suggest that the use of on-site geomembrane covered biopiles combined with an optimized biostimulation strategy represents a low-cost, simple and efficient approach for bioremediation of diesel fuel contaminated soils during summer months at Antarctic Stations and other extremely cold regions.

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