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Bioremediation of a petroleum hydrocarbon-contaminated Antarctic soil: Optimization of a biostimulation strategy using response-surface methodology (RSM)

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ABSTRACT

Bioremediation is a biotechnological approach to clean up contaminated soils. Among bioremediation strategies, biostimulation is a simple method which involves the modification of the soil physicochemical conditions in order to enhance the biological degradation of contaminants. One of the most common ways to do this is by the addition of macronutrients, mainly Nitrogen (N) and Phosphorus (P). Optimization of the amounts of N and P for a soil biostimulation strategy represents a key step prior to its application to a full-scale process. In this work, the response-surface methodology (RSM) was applied to optimize a biostimulation process for a hydrocarbon-contaminated Antarctic soil, considering a Carbon:Nitrogen:Phosporus (C:N:P) ratio of 100:10:1 as a reference. A faced-centered central composite design was used to determine the levels of the variables that lead to the optimum response values. Flasks containing contaminated soil and receiving different N and P amounts were incubated at 15 °C for 80 days. Biological activity and hydrocarbon concentration were evaluated. Results predicted that for the soil used in this experiment, the addition of 0.183 g N/kg and 0.0179 g P/kg leads to the highest hydrocarbon removal efficiency. The resulting C:N:P ratio (100:17.6:1.73) was different from that taken as reference (100:10:1), highlighting the usefulness of such an optimization. The hydrocarbon concentration decreased from 1042 (±73) mg kg^{-1} to 470 (±37) mg kg^{-1} in the most efficient combination tested.

1. Introduction

Soil contamination caused by fuel spills occurs worldwide. In Antarctica, this problem affects both frozen and thawed soils near the stations, mainly in the areas adjacent to fuel storage tanks (Aislabie et al., 2004; Schafer et al., 2009). Considering that *ex-situ* remediation of these soils is a cost-prohibitive and environmentally harmful procedure, alternative strategies must be developed. Bioremediation is a biotechnological approach which allows the recovery of a contaminated soil, causing a lesser environmental impact than non-biological remediation methods (Lehr, 2004). It is a simple, low-cost and environmentally friendly methodology (Bhatnagar and Kumari, 2013; Subhash et al., 2013). Among bioremediation strategies, bioaugmentation (Mrozik and Piotrowska-Seget, 2010), biostimulation (Tyagi et al., 2011) and phytoremediation (Zhou et al., 2011) have been used to clean up contaminated soils. The success of bioremediation strategies depends on several environmental variables such as O_2 availability or the concentrations of nutrients which are required by the fraction of inhabiting microbial community adapted to metabolize the contaminants. Providing these key factors to the biodegradation system is a relatively simple and cheap procedure (Bento et al., 2005). However, the variables need to be adequately optimized, because the addition of either an excess or a low amount of these nutrients could lead to an inhibition of biological activity, resulting in removal efficiencies far away from the optimum (Liu et al., 2011).

A Carbon:Nitrogen:Phosphorus (C:N:P) ratio of 100:10:1 has been frequently reported as a reference level for biostimulation approaches (Cheng and Mulla, 1999; Dibble and Bartha, 1979). Though, this ratio should be considered only as guide value. The optimum nutrient concentration should be tested whenever a bioremediation process is considered, as universally applicable solutions are not valid for biological systems as complex as soils (Ruberto et al., 2013). For instance, Liu et al. (2011) reported high hydrocarbon removal efficiencies using C:N:P ratios which were quite different from those considered as reference. For these reasons, optimization of this strategy is a key operation prior to the full-scale field application.

The response-surface methodology (RSM) is a combination of mathematical and statistical tools used to predict the optimum values

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for a multivariable system in order to obtain the maximum response. The method requires a limited number of experiments, turning it into a simple and feasible optimization procedure. It is widely used for the optimization of industrial and productive biological processes (Chauhan et al., 2013; Wang et al., 2014; Wu and Ahn, 2014) and in biological sciences to study the effect and interaction between factors on a certain response, such as the interaction between N and P on plant growth (Kuehl, 2001). The application of this statistical method provides a tool to examine the response of a variable at different factor levels. It is also a useful tool for the optimization of bioremediation processes for sediments (Mohajeri et al, 2011) and soils (Gomez and Sartaj, 2014).

The effectiveness of bioremediation strategies has been reported for soils in cold regions (Aislabie et al, 2006; Sanscartier et al, 2009). In previous studies, we have shown the effectiveness of bioremediation techniques to reduce hydrocarbon contamination in Antarctic soils (Mac Cormack and Fraile, 1997; Ruberto et al., 2003, 2006, 2009; Vázquez et al., 2009). However, as the coastal Antarctic soils of Potter Peninsula contain low levels of nutrients and organic matter, addition of N and P is an obligate step for the bioremediation of these soils. In this work, the levels of N and P required for a biostimulation treatment of the petroleum hydrocarbon-contaminated soils of Carlini Station (25 de Mayo Island, South Shetlands, Antarctica) were optimized using RSM. These optimized levels and the corresponding C:N:P ratio would allow the development of a pilot-scale field assay for the bioremediation of the diesel fuel-contaminated soil of Carlini Station.

2. Materials and methods

2.1. Soil analysis and characterization

The soil used for the RSM was obtained during the 2011–2012 Argentine Antarctic Expedition, from the area surrounding the diesel fuel storage tanks at Carlini Station, 25 de Mayo Island, South Shetlands, Antarctica. Contaminated soil was taken from the surface layer (up to 20 cm depth) and sieved (10 mm mesh) to remove stones, concrete, large paint residues and any other rough material that may interfere with analytical determinations, and then stored at −20 °C. 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 Kurt, 1945) and total Kjeldhal nitrogen. Water content was determined gravimetrically by drying samples at 105 °C for 24 h. For pH measurements, 10 mL of sterile saline solution (NaCl 8.9 g L^{-1}) was added to 1 g of soil and vortexed for 1 min. pH of the resulting suspension was measured using a Docu pH+ meter probe (Sartorius).

2.2. Microcosm design

The sieved soil was dispensed into cylindrical glass flasks (65 mm diameter, 80 mm height), each containing 150 g of soil. Different N and P concentrations were applied and codified according to Table 1. Nutrients were added to each system following a faced-centered central composite design distribution and considering N and P concentrations leading to a C:N:P of 100:10:1 as central points. A solution containing 286 g L^{-1} ammonium nitrate (NH_4NO_3, Alpha SA, analytical grade) was used as the N source, whereas a 580 g L^{-1} sodium dihydrogen phosphate ($NaH_2PO_4.12H_2O$) solution (Anedra SA, analytical grade) was used as the P source. Flasks were kept at 15 °C in a culture chamber (New Brunswick Scientific Co., Inc.) during the experimental period (80 days). The content of each flask was aseptically mixed three times a week to ensure a homogeneous distribution of nutrients and contaminants as well as appropriate aeration of the soil. Moisture was monitored with a data logger (Decagon Devices Inc.) and controlled around 15% by water addition when necessary (Table 1).

Table 1
Faced-center central composite design (CCD). Actual and coded values for each experimental run.

Run	Independent variables			
	g N added/kg		g P added/kg	
	Coded value	Actual value	Coded value	Actual value
1	1	0.2	−1	0
2	0	0.1	0	0.01
3	0	0.1	0	0.01
4	1	0.2	1	0.02
5	−1	0	1	0.02
6	0	0.1	−1	0
7	0	0.1	0	0.01
8	1	0.2	0	0.01
9	−1	0	−1	0
10	0	0.1	0	0.01
11	0	0.1	0	0.01
12	0	0.1	0	0.01
13	−1	0	0	0.01
14	0	0.1	1	0.02

2.3. Biological activity

Spilled petroleum hydrocarbons in soils represent a direct resource for hydrocarbon degrading bacteria (HDB). However, they could be also an indirect resource for other bacteria which are not able to degrade hydrocarbons but can take hydrocarbon metabolites or other molecules coming from HDB. Such activity favors petroleum hydrocarbon removal, pulling stoichiometric balances towards degradation. The determination of microbial activity and its comparison among treatments is a useful tool to follow the biological process going on in the contaminated soil. In this way, the fluorescein diacetate (FDA) method (Adam and Duncan, 2001) and R2A agar counts are adequate methods to estimate biological activity in soils undergoing bioremediation treatments. Heterotrophic counts could be considered as indicators of both soil health and availability of organic nutrients (Pepper and Gerba, 2015) and are used to monitor bioremediation processes (Cai et al., 2010; Zhang et al., 2010).

To determine biological activity more accurately, both methods were applied: FDA hydrolysis and bacterial counts.

Total microbial activity was followed by the FDA method (Adam and Duncan, 2001). Briefly, 2 g of soil was 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 FDA. After 30 min, 15 mL of the reaction terminator (Chloroform:Methanol 2:1) was added. Solutions were centrifuged for 3 min at 479 g, and the absorbance of the aqueous phase was measured spectrophotometrically (490 nm). The number of culturable heterotrophic bacteria was determined by plating serial dilutions of the samples on a half-strength R2A agar (Oxoid), to provide an oligotrophic medium, 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. The mix was shaken for 15 min in a vortex to allow an efficient detachment of microbial cells from soil particles. The R2A halved medium contained (in g L^{-1}): 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; and Agar, 15.0.

2.4. Hydrocarbon extraction and quantitation

For hydrocarbon quantification, soil samples (5 g) were extracted with 40 mL of hexane:acetone solution (1:1) in 40 mL Teflon tubes (Nalgene). A spoon tip of sodium sulfate was added to the organic phase to remove any remaining water. After that, 1 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, 7656 *g*) to avoid the presence of soil particles in the supernatant. 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. Data were collected using the PC-Chrome software (Buenos Aires University, 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). Hydrocarbon removal (HR) was calculated using the following equation:

$$HR(\%) = 100 \times \frac{\text{initial hydrocarbon-finalhydrocarbon concentration}}{\text{initialhydrocarbon concentration}}.$$

2.5. Statistical analysis

The experiment was performed following a three-factor face-centered central composite design considering two independent variables (N concentration and P concentration). The design diagram is shown in Fig. 1. Hydrocarbon removal efficiency (percentage) was analyzed as response. Each independent variable was studied at three levels (+1, 0 and −1). Four central points (0, 0) were included to analyze model robustness, resulting in 12 experiment runs. The levels were chosen around a C:N:P ratio of 100:10:1, which is considered as a reference value for the growth of most microorganisms (Cheng and Mulla, 1999) and for biomass synthesis from hydrocarbons (Dibble and Bartha, 1979; Ron and Rosenberg, 2010).

To predict the optimal condition and the existence of interactions, experimental data were fitted to a second-order polynomial regression model (Eq. 1), containing two linear, one interaction and two quadratic terms (Mason, 2003):

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} {X_i}^2 + \beta_{jj} {X_j}^2 \quad (1)$$

where β_0 is the value of the fixed response at the center point of the design, β_i and β_j are linear coefficients, β_{ii} and β_{jj} are quadratic coefficients, and β_{ij} is the interaction effect coefficient. X_i and X_j represent the independent variables (N and P amounts added to the soil expressed as g/kg respectively), X_iX_j represent the effect of the interaction between the independent variables and X_i^2 and X_j^2 are the quadratic effects of each independent variable. The significance of each coefficient in the equation was determined by F-test and P-values.

For statistical calculation, independent variables were coded as described in Eq. (2):

$$X_i = (X_i - X_o)/\delta X_i \quad (2)$$

where X_i is the experimental value of the variable, X_o is the mid-point of X_i, δX_i is the step change in X_i, and X_i is the coded value for X_i; $i = -1, 0, +1$.

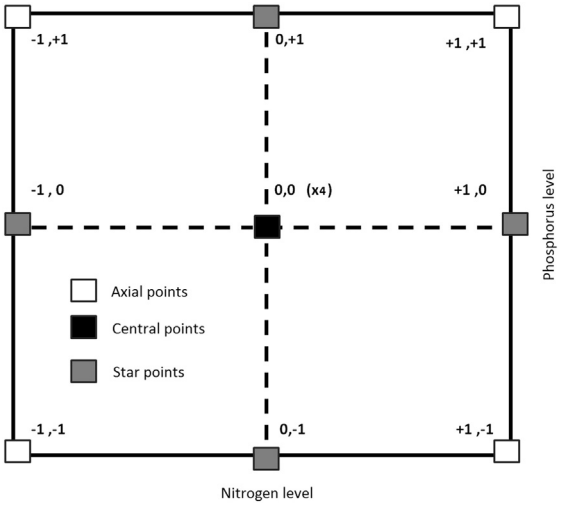

Fig. 1. Faced-centered central composite design. The plot shows the central points (0,0) and the distribution of the axial and star points.

3. Results

3.1. Soil analysis

The soil had a sandy texture, containing 1.8% clay, 3.8% silt, and 94.4% sand. Organic carbon, total Kjeldhal nitrogen and extractable phosphorous levels were 10.21 g kg^{-1}, 0.32 g kg^{-1} and 5.0 mg kg^{-1}respectively. The pH was 6.8 and the water content 10%. Quantitation of hydrocarbons showed that this soil contained a total petroleum hydrocarbon concentration of 1042 ± 73 ppm.

3.2. Statistical analysis

F-test indicated that in this experimental design only X_1, X_2 and X_1^2 factors were statistically significant at the 95% confidence level. The effects (factors and interactions) with P-values higher than 0.05 were considered as insignificant. All terms, regardless of their significance, are included in the following Eq. (3):

$$Y = 48.57 + 9.03X_1 + 4.54X_2 + 1.99X_1X_2 - 6.38{X_1}^2 - 3.92{X_2}^2 \quad (3)$$

where X_1 and X_2 are the N and P amounts added to the soil (expressed as g/kg) respectively. The model F value of 15.24 implies that the model is significant and that there is only 0.23% of chance that this model F-value occurs due to noise ($p < 0.0023$). The determination coefficient ($R = 0.9270$) suggests that more than 92.70% of the variance is attributable to the variables and indicates a high significance of the model. Consequently, only 7.30% of the total variance cannot be explained by this model.

The lack-of-fit test gives information about how well the model fits the response of our system. The F-statistic, which is calculated as the ratio between the lack-of-fit mean square error (MSE_{LOF}) and the pure mean square error (MSE_P) values, tests the hypothesis that the model is correctly specified. A large F-ratio indicates that the variation given by center replicates is too large and that the model is not fitting the response. In our case, a value of 0.72 ($p < 0.05$) implies that the lack-of-fit is not significant relative to pure error, therefore indicating that the model fits the regression. All the statistical data obtained are shown in Table 2.

Table 2
ANOVA for hydrocarbon removal.

Source	Sum of squares	df	Mean square	F value	Pvalue Prob > F	
Model	845.42	5	169.08	15.24	0.0023	Significant
A-A	487.80	1	487.80	43.96	0.0006	
B-B	124.21	1	124.21	11.19	0.0155	
AB	16.00	1	16.00	1.44	0.2751	
A^2	108.80	1	108.80	9.80	0.0203	
B^2	40.30	1	40.30	3.63	0.1053	
Residual	66.58	6	11.10			
Lack of fit	27.98	3	9.33	0.72	0.6012	Not significant
Pure error	38.61	3	12.87			
PRESS	254.24	1				
Correlation total	912.00	1				

Both the adjusted coefficient and the predicted coefficient were assessed to evaluate the quality of fit of the polynomial model for hydrocarbon removal (Montgomery, 2008). The predicted R-squared value of 0.7212 is in agreement with the adjusted R-squared value of 0.8661. Adequate Precision measures the signal-to-noise ratio, being a ratio greater than 4 desirable. The ratio of 11.519 obtained indicates an adequate signal.

The coefficient of variation (CV) is a measure of the residual variation of the data relative to the size of the mean, and therefore, a measure of the reproducibility of the model. It is divided by the dependent mean and expressed as a percentage. A model is generally considered as reasonably reproducible if its CV is lower than 10%. The low CV value obtained (CV = 7.67%) indicates a high precision and reliability of our experiments.

3.3. Hydrocarbon concentration

Hydrocarbon concentration (Fig. 2) decreased from an initial value of 1042 ± 73 mg kg^{-1} to a final value of 470 ± 37 mg kg^{-1} in the most efficient treatment (54.91% removal), and to a final value of 753 ± 37 mg kg^{-1} in the less efficient one (27.75%). It should be noticed that some abiotic factors contributing to hydrocarbon elimination (such as rain, wind and lixiviation) were absent or not significant during this assay, because it was performed in closed flasks incubated in a temperature-controlled chamber. Hydrocarbon losses due to evaporation during the experiment were assessed as i-C_{14} removal showed to be 33% (±6.4). Isoprenoid hydrocarbons are thought to be less sensitive to biodegradation than n-alkanes (Pond et al, 2002). For this reason it is possible to assess evaporation using them as markers. Nonetheless, some extent of isoprenoids biodegradation could not be neglected, especially in soils inhabited by members of the Pseudomonadaceae family (Cantwell et al, 1978), commonly predominant in Antarctic soils. In any case, the system in which no nutrients were added (−1,−1) presented only 27.75% removal. Assuming the most unfavorable possibility, this removal may be entirely caused by evaporation, with no biological degradation present.

The GC-FID analysis of the soil before any treatment showed a pattern in which the C11–C14 range of aliphatic hydrocarbons prevailed in the sample. There was also a non-resolved mixture. At the end of the experiment, systems where hydrocarbon removal was maximum presented chromatographic patterns very similar to the initial ones, but with a much lower area under the peaks, suggesting that all the hydrocarbons existing in that range were metabolized to a similar extent.

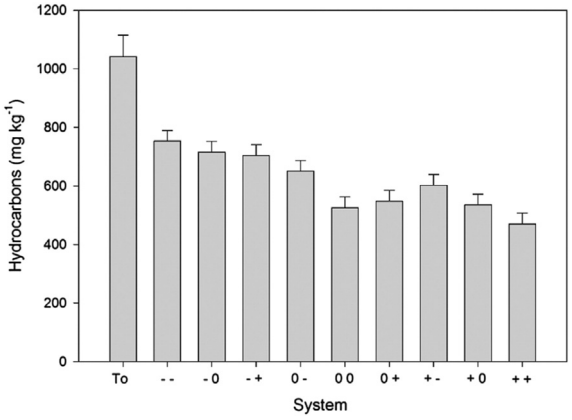

Fig. 2. Comparison of the hydrocarbon concentration (mg kg^{-1}) for each system at the beginning of the experiment and after the treatment. Error bars indicate standard deviation.

3.4. Biological activity

Biological activity showed significantly higher values in the systems in which N was added (0 and +1) than in the systems without addition of N (−1). Phosphorus (P) on the other hand, seemed to have no significant effect on biological activities. Fig. 4 shows that, in all the N-fertilized systems, the amount of fluorescein released was approximately four times higher than in the systems where no N was added (over 2 μg versus 0.5 μg/gram of dry soil). On the other hand, the systems where no N was added (−1) exhibited lower enzymatic activity, proving that this nutrient is essential for biological activity and suggesting that the soil used in this experiment did not provide enough nutrient to promote an optimum catabolic rate. This low biological activity was accompanied by low hydrocarbon removal efficiency in the systems.

Bacterial counts also showed higher values in the systems where N was added (independently of the level of this nutrient) (data not shown). These results are in agreement with those obtained by the FDA methodology. The positive effect of N addition on total and hydrocarbon degrading bacteria was clearer during the first 30 days of the experiment, in which counts increased only in those experimental units where this compound was added. The effect of P addition showed no significant effect on bacterial counts when not combined with N.

3.5. Response surface method

The face-centered central composite design showed an adequate relation with the predicted values, suggesting that hydrocarbon removal for these soils would be maximum when X1 = 0.83 and X2 = 0.79. The resulting response-surface and the predicted versus actual plots are shown in Fig. 5. Therefore, biostimulation consisting in the addition of 0.183 g N/kg soil and 0.0179 g P/kg soil would be required in this soil to reach maximum removal efficiency. These values suggest an optimum C:N:P ratio of 100:17.6:1.73, which significantly differs from those considered as reference in most biostimulation processes. According to these results, one ton of this Antarctic soil would require the addition of 523 g NH_4NO_3 and 80 g NaH_2PO_4 to undergo an optimized bioremediation procedure.

4. Discussion

The experimental design applied in this work showed to be suitable to assess the optimum N and P levels for a biostimulation strategy of contaminated soil at Carlini station, Antarctica. It was possible to define an N and P combination able to maximize biological hydrocarbon removal using only 12 experimental units. The experiment provided a statistical model which predicts a maximum removal of 54.12% (for a soil containing 1042 ± 73 mg kg^{-1} of hydrocarbons) when 0.183 g N/kg soil and 0.0179 g P/kg soil are added. It is important to consider that without the implementation of the statistical approach provided by RSM, the optimum N and P levels obtained would have been quite different, being the values corresponding to the +1;+1 experimental run (0.2 g N/kg soil and 0.02 g P/kg soil), different from those obtained with this mathematical model. From a cost-effectiveness perspective, this would have implied the use of approximately 20% more nutrients for the biostimulation process, making the process implementation more expensive and less efficient, and the possibility of other undesired biological processes due to this nutrient excess. As previously reported, RSM is useful to optimize bioremediation processes (Lim et al., 2013; Singh et al., 2013), because it allows achieving a higher removal efficiency and the consumption of lower nutrient levels.

Although the RSM results in removal efficiencies lower than those obtained in some field assays, the predicted value is in accordance with the absence of abiotic hydrocarbon elimination processes. Abiotic elimination is responsible for the removal of a significant fraction of hydrocarbons when the soil is exposed to environmental conditions. This is also true for Antarctica, where wind and snow are important factors

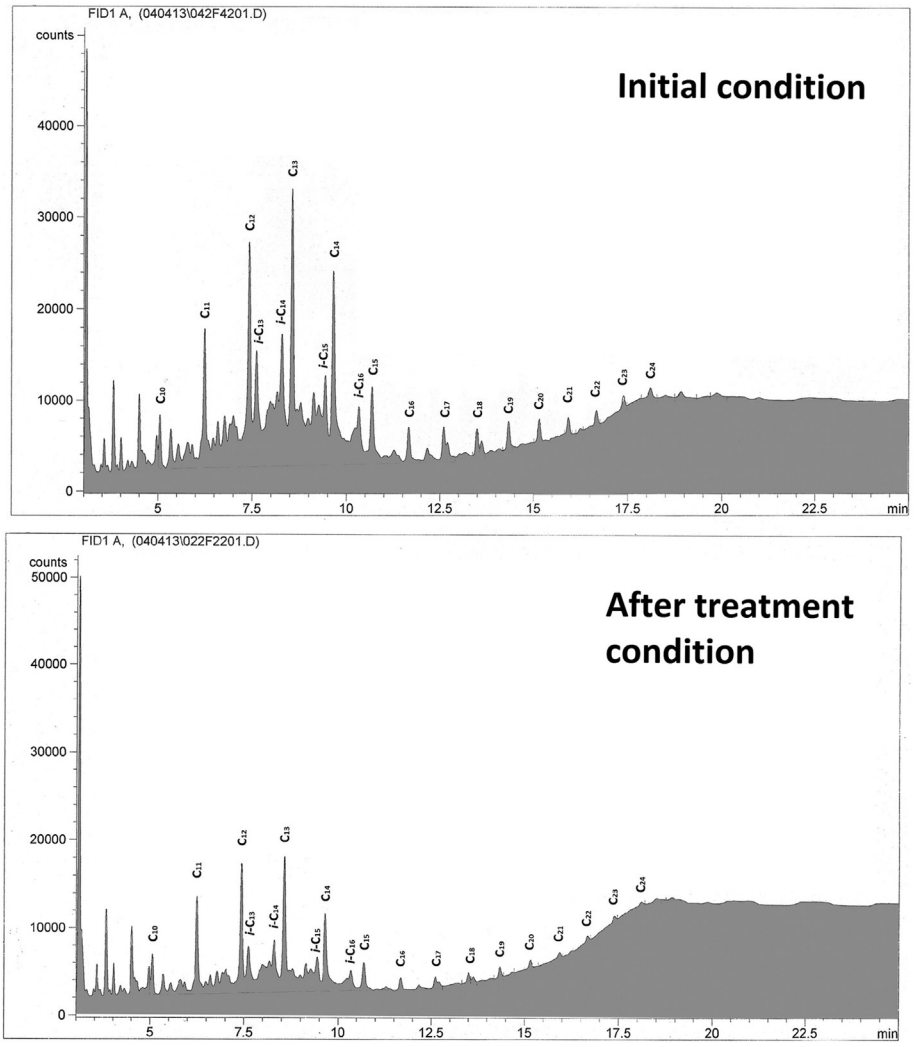

Fig. 3. GC-FID chromatograms for initial condition and after treatment condition in system +1 + 1.

to consider. In this case, as experimental units were screw-capped flasks isothermally incubated in a chamber, the systems were not exposed to wind, rain, snow or lixiviation. However, abiotic loss caused by evaporation is always considerable. During this experiment, hydrocarbon loss due to evaporation was around 30%. This value was similar to those obtained for the system without nutrient addition where biological activity remained almost unchanged during the experimental period. Previous experimentation reported by our group showed that 60% of hydrocarbon removal was attributable to biological activity (total removal − abiotic removal) during a 45-day field assay in Antarctica, applying a biostimulation strategy to a soil containing an initial hydrocarbon concentration of almost 12,000 mg kg^{-1} (Ruberto et al., 2009). The results obtained during the RSM experiment are in accordance with these levels of biological removal. As stated by Adam and Duncan (2001), soil properties are fundamental to obtain suitable results, as soil variability may cause lack of robustness. Our experience with Antarctic soils showed that the use of this technique to monitor biological activity through bioremediation processes is proper to obtain remarkably reproducible results, which also proved to be in accordance with those obtained by bacterial counts. The FDA methodology evidenced a high correlation between hydrocarbon removal efficiency and fluorescein released levels, establishing that the decrease in hydrocarbon concentration could be attributable to the biological activity present in the soil.

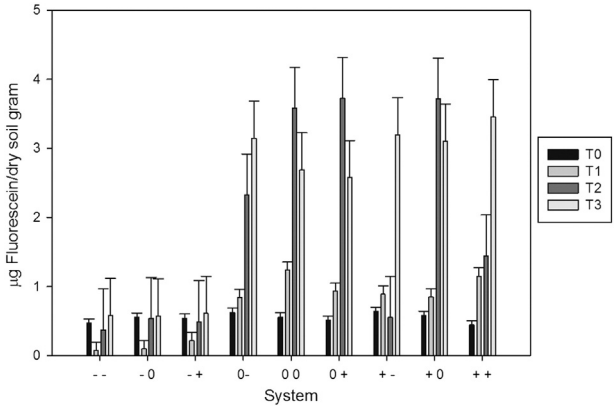

Fig. 4. Biological activity in the systems studied expressed as fluorescein released at different sampling times.

The optimized C:N:P ratio obtained as a consequence of the combination of these values (hydrocarbon, N and P concentration) resulted in 100:17.6:1.73. This C:N:P ratio differs from that referred as optimum for microbial growth (Cheng and Mulla, 1999) and hydrocarbon biodegradation (Dibble and Bartha, 1979; Ron and Rosenberg, 2010). In a previous work, Ferguson et al (2003) reported the evaluation of the addition of different amounts of N sources for a biostimulation strategy of an Antarctic petroleum hydrocarbon contaminated soil at Old Casey Station. In that case, the removal of almost 9000 mg kg^{-1} from an Antarctic soil was obtained using a C:N:P ratio of 100:1.4:0.09. In

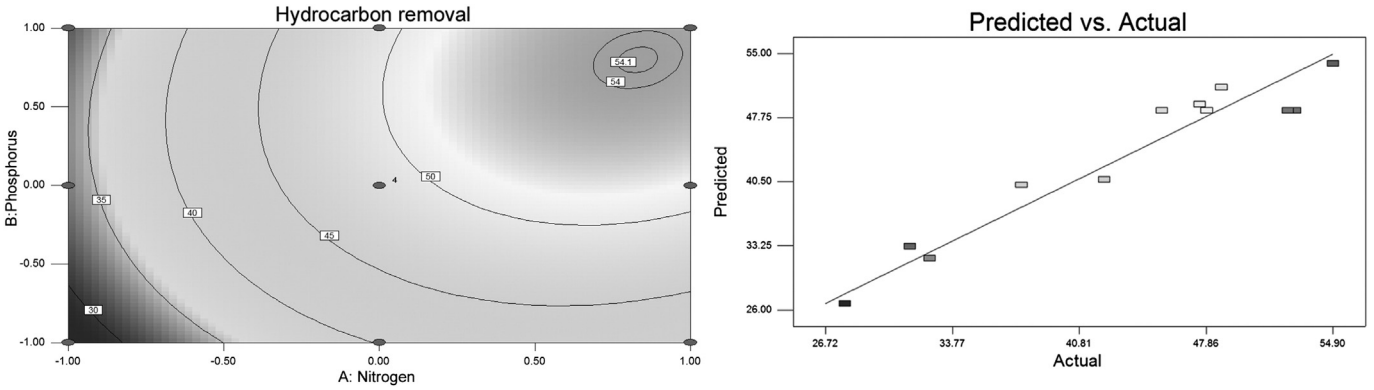

Fig. 5. Surface response graph, showing the maximum response value (A). Predicted vs actual values plot (B).

other report, Liu et al. (2011), working with a petroleum hydrocarbon-contaminated soil (14,000 mg kg^{-1}), reported 69.8% of hydrocarbon removal after 35 days of biostimulation with inorganic nutrients (NH_4NO_3 and K_2HPO_4) at a C:N:P ratio of 100:11:3.7. Only 46.7% of removal was achieved for the same soil when the C:N:P ratio was 100:27:6.5. These results clearly exemplify how important the optimization of nutrient level is, and how different the optimum combination could result in comparison with the reference value of 100:10:1. It has also been reported that higher nutrient amounts can cause biological inhibition or a reduction in soil water potential (Aislabie et al, 2006), achieving lower removal levels when excess N and P are added to the soil. Walworth et al. (2007) also reported an inhibition of biological hydrocarbon removal for a sub-Antarctic soil attributable to an excess of N and/or P. Chong et al (2009) studying the bacterial diversity in different soils from the surroundings of Casey Station (Antarctica), reported a trend to high soil bacterial diversity with elevated N and water levels. All these results reinforced the role of N as a vital nutrient for bacteria in Antarctic soils.

The differences between this optimized value and those obtained by other authors under different conditions could be caused by several factors. Basal N and P levels in the soil under treatment seem to be the most obvious one. However, such a situation would result in lower amounts of nutrient addition than a soil without basal N and P. The necessity of adding higher levels of N and P to the soil could be related to physical phenomena affecting bioavailability. Huygens et al. (2008) demonstrated that a volcanic rainforest soil presented a minimal loss of bioavailable N and attributed this capacity to a synergy among abiotic processes, soil microbes and plant roots, leading to stable N sinks. Antarctic soils in general are far away from this situation, because they usually lack plants and significant levels of organic matter. Microbial populations are also smaller than those present in other soils. All these factors could result in a matrix with a low ability to retain the bioavailable form of nutrients required by degrading microorganisms. If N and P are less available for microbial biomass, it could be necessary to add higher amounts of these nutrients than the levels only based on stoichiometric calculations, resulting in C:N:P ratios higher than 100:10:1.

Another point to analyze is the effectiveness of adding nutrients to Antarctic soil, in order to emulate or replicate the RSM behavior in a field assay. Even though the identification of suitable nutrients concentrations could contribute to improve a biostimulation strategy, it would be worthless if the nutrients are finally washed out from soil under treatment. Soil from Carlini's station surrounding area presents a low water holding capacity and for this reasons a high draining nature. This feature, as was previously reported by Ferguson et al (2003) for Old Casey Station soils, could result in a rapid wash-out of the added nutrients due to rain or snow melting. The use of slow release nutrient sources or the designs of biopiles contained in geomembranes could be alternatives to deal with this kind of challenge.

When a chamber and a field assay are compared, it is important to consider another question: what is being favored when a complex matrix containing a complex community is incubated isothermally? It is possible (and expected) for a chronically contaminated soil exposed to natural weather conditions to be inhabited by different microorganisms able to take advantage of different environmental situations. If we focus on temperature, it would be possible to define some thermal niches as stated by Magnuson et al. (1979) for fishes and by Bronikowski et al. (2001) for enteric pathogen bacteria. For a naturally exposed Antarctic soil, the oscillation in daily temperature offers microorganisms with different optimal growth temperatures and potentially different and/or complementary catabolic capabilities the opportunity to be metabolically fully active. Isothermal incubation will allow the optimal growth of only a limited group of microorganisms. What could result from these two situations is difficult to assess. The incubation at 15 °C could allow the development of some microorganisms at a faster rate than the Antarctic natural daily temperature range, resulting in a higher removal rate. In this sense, Franzmann (1996) pointed out that many of Antarctic species present an optimal growth temperature of 23 °C. Whyte et al (1999) hypothesize the idea that 23 °C is the optimal temperature for cold-regions bioremediation because it represents a trade-off between significant hydrocarbon bioavailability and low indigenous microflora inhibition. However, Ferguson et al. (2003) demonstrated that microbial flora from Old Casey Station (Antarctica) is able to mineralize more ^{14}C-octadecane at 42 °C than at other lower temperatures. Nonetheless, crucial catabolic activities could be inhibited or limited at this temperatures, and consequently removal potential underestimated. In any case, all these considerations were made for isothermal incubations. In order to reveal the effect of irregular cycles of temperature on the final efficiency of bioremediation field assays, further experimentation simulating natural daily temperature changes would be necessary to be carried out.

It is interesting to highlight that the GC profile of the hydrocarbon fraction remaining in the system showing the highest removal showed a pattern similar to that of the soil before the treatment, although the area below the peaks was significantly lower in the first case (Fig. 3). According to this, all the fractions present in the contaminated soil were degraded at similar rates despite their chemical structure. However, this recalcitrant fraction evidences the presence of some limitation in the degradation activity, due to hydrocarbon availability, concentration, experimental time or inadequate microbial catabolic activity. To improve the degradation of this remaining fraction, a sequential bioremediation process with an initial stage of biostimulation to remove the bulk of the contaminant, followed by a specific bioaugmentation strategy focused on the removal of the remaining hydrocarbons, could be useful.

5. Conclusion

In our opinion, the present work provides a useful tool to infer the optimum amounts of N and P required by a hydrocarbon-contaminated Antarctic soil for biostimulation by means of a simple but powerful experimental approach. Further research will be necessary to find out the range of hydrocarbon concentrations in which the prediction adequately adjusts to the experimental results.

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