Microbial Ecology, Vol. 56, August 2008, pp243-252

Comparative mesocosm study of biostimulation efficiency in two different oil-amended sub-Antarctic soils

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Abstract

Biological treatment has become increasingly popular as a remediation method for soils and groundwater contaminated with petroleum hydrocarbon, chlorinated solvents, and pesticides. Bioremediation has been considered for application in cold regions such as Arctic and sub-Arctic climates and Antarctica. Studies to date suggest that indigenous microbes suitable for bioremediation exist in soils in these regions. This paper reports on two case studies at the sub-Antarctic Kerguelen Island, in which indigenous bacteria were found that were capable of mineralizing petroleum hydrocarbons in soil contaminated with crude oil and diesel fuel. All results demonstrate a serious influence of the soil properties on the biostimulation efficiency. Both temperature elevation and fertilizer addition have a more significant impact on the microbial assemblages in the mineral soil than in the organic one. Analysis of the hydrocarbons remaining at the end of the experiments confirmed the bacterial observations. Optimum temperature seems to be around 10°C in organic soil while it was higher in mineral soil. The benefit of adding nutrient was much stronger in mineral than in the organic soil. Overall, this study suggests on the basis of microbiological and physicochemical parameters, that biostimulation treatments were driven by soil properties and that ex-situ bioremediation for treatment of cold contaminated soils will allow greater control over soil temperature, a limiting factor in cold climates.

Key words: sub-Antarctic, soil, biostimulation, crude oil, diesel fuel, oil-degrading bacteria

Introduction

The increasing use of petroleum hydrocarbons in high latitude regions led to a growing probability of major spillages threatening terrestrial and aquatic environments. A number of studies have focused on chronic hydrocarbon contamination near the Antarctic and sub-Antarctic research stations, revealing the presence and persistence of these human-derived contaminants (Cripps and Shears, 1997; Snape et al., 2001; Delille and Pelletier, 2002; Aislabie et al., 2004; Rayner et al., 2007). Of all the different types of contamination reported up to now in Antarctica, petroleum has been identified as the most significant problem to be addressed (Snape et al., 2001). The need for research into hydrocarbon degradation process at low temperatures is therefore without question in order to preserve the vulnerable and extreme Antarctic environments. Many physical, chemical and biological technologies have been developed to remove hydrocarbon pollutants from soils and restore the environmental integrity. However, these techniques are often unfeasible in remote areas where accessibility of heavy equipment and materials is limited or prohibited by the high sensitivity of threatened environments. Bioremediation, including biostimulation and bioaugmentation, has proven to be an effective method for cleaning up residual oil in a variety of environments (Van Hamme et al., 2003) and has been proposed as the only viable management option that can be implemented on a large scale in Antarctic environments (Snape et al., 2001). In many instances, however, the rate of petroleum biodegradation in cold environments is severely limited by temperature fluctuation and the available concentrations of fixed forms of nitrogen and phosphate (Walworth and Reynolds 1995; Coulon et al., 2004; Delille et al., 2007). Low temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism (Gerdes et al., 2005, Nedwell, 1999) and directly affects both the rate of biodegradation and the physicochemical behaviour of oil hydrocarbons, such as viscosity, diffusion and volatilization (Enell *et al.*, 2005; Maliszewska-Kordybach, 2005). Additionally,

nutrient supplementation (so-called biostimulation) of polluted sites has been shown to be an effective means of stimulating hydrocarbon biodegradation activities of indigenous microbial populations and thereby of reducing the ecological impact of oil pollution (Delille et al., 2004; Aislabie et al, 2006). However, monitoring studies conducted in oil-contaminated polar soils and experimental research during the last decades have documented the complexity of cold ecology and the parameters that affect the severity of impacts to these systems (see for review Aislabie et al., 2006). This knowledge complicates decisions regarding cleanup in cold environments, because parameters such as substrate type, oil type, season of impact and climate may all affect the eventual recovery of oil-contaminated polar soils. Limiting factors need to be overcome if microbial breakdown of contaminants is to be used effectively (Alexander 1999, Margesin et al., 2007). However, the conditions when cleanup is desirable in a polar soil are not clearly delineated yet, and several important questions remain unresolved. Among them two of the more decisive are: which methods should be employed and at what point intervention is no longer useful? The aim of the present mesocosm study is to evaluate and compare the benefits of temperature increase and slow release fertilizer addition on biodegradation of diesel fuel and crude oil in two different sub-Antarctic soils.

Materials and methods

Field site, sampling and mesocosm set-up

Two soils were collected from areas with no past exposure to hydrocarbon contamination. Both areas were located near the scientific research station “Port aux Français” in Kerguelen Archipelago (49°21’S, 70°13’E). Soil samples were collected from the surface to a depth of about 0.2 m in approximately 20 m^2 areas. The first selected soil was an organic soil supporting an abundant vegetal cover (*Acaena magellanica*), while the second one was a mineral soil, completely dry and desert. The general physical, chemical and biological

properties of the two sub-Antarctic soils have been previously described (Coulon et al., 2004). Mesocosm experiments were conducted in polyethylene containers of dimensions 27 x 24 x 13 cm. Plant residues have been removed for the organic soil before use. Soils have been aerated and homogenized before placing 5 kg ($^{w}/_{w}$) of soil in each mesocosm. The artificially contaminated soils were prepared by direct application of either 100 ml Arabian light crude oil or diesel fuel (initial contaminant concentration = 30 mg g^{-1} soil dry mass). The composition of Arabian light crude oil and diesel fuel was 52 and 71% of saturated linear and cyclic alkanes, 45 and 28% of aromatics (2 to 5 rings PAH) and 3 and 1% of polar compounds, respectively. The slow release fertilizer used was Inipol EAP 22® (CECA S.A., France, C: N: P = 62:7.4:0.7), which is a stable microemulsion consisting of a urea core (the nitrogen source) surrounded by oleic acid carrier, lauryl-phosphate (surfactant and the source of phosphorus), and butoxyethanol (as viscosity reducer). The initial concentration of the nutrients obtained were 1.2 mg N and 0.1 mg P g^{-1} soil dry mass. For each temperature of incubation (4°C, 10°C and 20°C), six conditions were used: control, Inipol (50 ml), crude oil (100 ml), crude oil (100 ml) + Inipol (50 ml), diesel (100 ml), and diesel (100 ml) + Inipol (50 ml). The mesocosms were incubated in the dark under aerobic conditions during 2 months period. They were homogenized twice a month. Samples for chemistry analysis were collected in the surface layer of the soil and were stored at – 20°C until analysis.

Bacteriological counts

The changes in bacterial community abundance, comprising total, heterotrophic and hydrocarbon-degrading microorganisms were studied during a 42 days period after contaminant addition. Sampling dates were 7, 15, 30, and 42 days. Triplicate samples were aseptically collected in the surface layer of the soil (from surface to 2 cm under the surface). Total bacteria were determined by acridine orange direct count (AODC) on black nuclepore

filters (0.2 µm) using an Olympus BHA epifluorescence microscope according to the method of Hobbie *et al.* (1977). A minimum of 500 fluorescing cells with a clear outline and definite cell shape cells were counted under oil immersion (x 1000) in a minimum of 10 randomly chosen fields. The standard deviation calculated from 3 replicates was found ≤ 15 %. Heterotrophic microorganisms in each soil sample was made using the spread plate technique on Nutrient Agar 2216 (Oppenheimer and ZoBell 1982), using distilled water in place of seawater. Inoculated plates (six replicates) were incubated for 10 days at 15°C. Hydrocarbon-degrading microorganisms were determined by the most probable number (MPN) method using tubes containing 9 ml of a basal mineral medium (NH_4Cl: 2.0 g L^{-1}, KH_2PO_4 : 0.89 g L^{-1}, NA_2HPO_4: 1.25 g l^{-1}, $FeCl_3$: 0.6 mg l^{-1}), supplemented with 0.2 ml of Arabian light crude oil and 1 mg l^{-1} resazurin indicator (Mills et al., 1978). After inoculation (3 tubes per dilution), the tubes were incubated at 12°C for 30 days. The standard deviation calculated from 3 replicates was found ≤ 20 % for both CFU and MPN estimations.

Hydrocarbon extraction and analysis

Before extraction, soil samples were freeze-dried and homogenized by screening through a 1-mm sieve. Extraction procedure and gas chromatography-mass spectrometry (GCMS) analysis setting have been previously described (Coulon and Delille, 2006). Each dry soil sample was extracted with a mix hexane:dichloromethane (1:1) overnight and the solution was evaporated under a mild stream of nitrogen on an ice bath to minimize losses of light alkanes and PAH. Very light hydrocarbons such as benzene, pentane and hexane could be partly lost during freeze drying. These light hydrocarbons are not accounted in total alkanes (calculated from C10 to C36 linear alkanes and including iso-alkanes) and total aromatics (from naphthalene to 4-ring aromatics including alkyl-substituted compounds). Analytes of Arabian light crude oil were normalized to the conservative biomarker 17α(H), 21β(H) C30-

hopane naturally present in crude oil (Butler *et al.*, 1991) while analytes of diesel fuel were normalized to chrysene, considered to be one of aromatics most resistant to biodegradation (Bossert and Bartha, 1986). Hopane was absent from diesel fuel samples. For quality control, a 1.0 ng μl^{-1} diesel standard solution (ASTM C_{12}-C_{60} quantitative, Supelco) and a 1.0 ng μl^{-1} PAH Mix Standard solution (Supelco) were analyzed every 20 samples. The variation of the reproducibility of the extraction and quantification protocol was determined by successive extractions and injections of 8 replicates of the same field sample and estimated to be ± 10%.

Statistical analysis

Measures analysis of variance (ANOVA) in Statview F-5.0 PPC (SAS Institute Inc.) was used to analyze the response variables (CFU, MPN, fertilizer and oil analytes) in the two soils separately. When the ANOVA indicated significant differences ($p < 0.05$), univariate ANOVAs were run on data at each time point. Where significant differences were indicated at a specific time point ($p < 0.05$), protected least significant difference (LSD) mean separations were used to assess treatment differences

Results

There were only slight changes in total microbial abundance after contamination (data not shown). With values comprised between 10^8 and 10^9 cells g^{-1}, the total number of micro-organisms was roughly constant throughout all the mesocosm experiments.

The basal level of heterotrophic microbes in the mesocosms, prior to oil addition, was estimated to be 3.2 x 10^6 CFU g^{-1} in organic soil and 2.6 10^5 CFU g^{-1} in mineral soil. These number remained relatively constant in most of the uncontaminated mesocosms. Following diesel fuel (Figure 1) or crude oil (Figure 2) addition, the number of heterotrophic microbes did not change significantly in both soils ($P = 0.659$ in organic soil and $P = 0.317$ in mineral

soil). Inipol amendment did not induce significant enhancement of the heterotrophic assemblage in organic soil. In contrast significant increases of the number of saprophytic bacteria occurred in all Inipol amended mesocosms of mineral soil ($P < 0.001$). Enhancements reach three orders of magnitude and seems relatively independent from temperature.

Before contamination, initial level of oil-degrading microbes estimated by the most probable number (MPN) procedure was 2.7×10^5 MPN g^{-1} in organic soil and 3.0×10^5 MPN g^{-1} in mineral soil (Figures 3 and 4). After crude oil amendment, the number of oil-degrading microbes increased by one order of magnitude in both kind of soils ($P < 0.082$ in organic soil and $P < 0.056$ in mineral soil) over a period of 7 days. In contrast, these numbers did not increase after diesel fuel amendment, except in organic mesocosms incubated at 4 and 20˚C. According to the type of oil used, the comparison of the oil-degrading microbe's number for the same soil did not revealed significant difference between diesel fuel and crude oil ($P > 0.05$). The efficiency of the biostimulation treatments was much higher in the mineral soil (more than three orders of magnitude) than in the organic one (less than two orders of magnitude) for both kinds of oil contaminants. At the end of the experiment, the numbers of oil-degrading microbes in diesel amended mineral soil microcosms can be four orders of magnitude higher than those determined in corresponding pristine soil whilst this difference is less than two orders of magnitude in organic soil. Inipol amendment alone induced also a significant increase in numbers of oil-degrading microbes in all mesocosms. This increase was significantly greater in mineral soil mesocosms ($P < 0.001$) than in organic ones ($P = 0.056$). Temperature elevation had a slight but positive effect on biostimulation of oil degrading microbes. With the exeption of diesel contaminated organic soil, the maximum numbers were always observed in mesocosms incubated at 20˚C.

Overall, the total extractable petroleum hydrocarbons content (TPH) of both oil contaminants have reduced by more than 70% and 80% in organic soil mesocosms and more than 76% and

96% in mineral soil mesocosms incubated at 4 and 20˚C, respectively. Detailed changes of oil hydrocarbon fraction of both crude oil and diesel fuel are shown in Table 1. After fertiliser application, the differences in extent of degradation were most pronounced for both aliphatic ($P < 0.001$) and aromatic ($P = 0.098$) fractions in mineral and organic soil mesocosms (Table 1; Fig 5). However, the benefit of adding nutrient was higher in mineral than in organic soil. The mass fraction of aromatics relative to aliphatic hydrocarbons has increased of more than 20% in crude oil amended mesocosms and more than 24% in diesel fuel amended ones, regardless the soil type. These mass balance shifts were even higher in fertilised mineral soil mesocosms: more than 35% in crude oil amended mesocosms ($P < 0.001$) and more than 42% in diesel fuel amended ones ($P < 0.05$). The benefit of increasing temperature is particularly obvious in diesel contaminated mineral soil mesocosms. In contrast, the best results of oil hydrocarbons degradation were observed in organic soil mesocosms incubated at 10°C.

Discussion

The present observations demonstrate the clear stimulating effect of oil addition on indigenous bacteria in sub-Antarctic soil. Several orders of magnitude increase in bacterial abundance occurred after both diesel and crude oil addition. These observations are in good agreement with previous results obtained from *in situ* studies in sub-Antarctic soils (Crozet Island) (Coulon & Delille 2006, Delille et *al.*, 2001, 2004). Biostimulation induced a clear increase of the number of hydrocarbon-degrading microbes. As noted by Rivet et *al.* (1993), some increases in bacterial numbers after Inipol EAP 22 addition may be attributed to the bacteria growing on the oleic acid in the fertilizer. However the concomitant reduction of the residence time of the contaminants demonstrates the efficiency of the biostimulation. Despite that the same general pattern was observed in the two soils, the results indicate a serious influence of the soil properties on the observed biodegradation efficiencies.

The intensity of hydrocarbon biodegradation in soil is influenced by a number of site-specific factors (e.g. low temperature, low nutrient availability, low oxygen levels, soil structure, etc…). Among them, it is well established that nutrients are one of the major factors limiting hydrocarbon metabolization in soils (Mohn and Stewart, 2000). Inputs of large quantities of carbon sources (i.e., hydrocarbon contamination) tend to result in rapid depletion of the available pools of major inorganic nutrients, such as nitrogen and phosphorus. Several studies have reported favourable effects of fertilizers on oil biodegradation at low temperatures in Arctic (Braddock et *al.*, 1997, Whyte et *al.*, 1999; Mohn and Stewart, 2000), alpine (Margesin and Schinner 1997a,b; Margesin and Schinner 1999, Margesin et al. 2007) and Antarctic soils (Kerry 1993; Wardell 1995; Aislabie et *al.*, 1998; Delille 2000, Powell et al., 2006).

A possible reason for the inability of Inipol EAP 22® to greatly enhance hydrocarbon-degrading microbes growth in organic soil is that nitrogen and phosphorus are not the major limiting factors in this soil. Initial nitrogen concentrations naturally present were probably high enough to sustain rapid intrinsic rates of biodegradation without addition of fertiliser. In addition, particle size distribution, elemental analysis and water content of both sub-Antarctic soils were strongly different from each other. Thus, differences in the observed degradation rates in both studied soils may be associated with the availability of the contaminants. Furthermore, long-term in situ experiments demonstrated that the wet organic soil seems to be more efficient to retain some toxic compounds than the mineral one (Delille et al. 2007). This difference could have a direct influence on the mineralisation rate of hydrocarbons.

Microbial metabolism is usually considered as a direct function of the temperature of the environment (Leahy and Colwell 1990). Results of the present mesocosm experiments indicate that a temperature increase can stimulate the microbial hydrocarbon degradation in sub-Antarctic soils. However, the influence of temperature can differ greatly from a soil to another. We have no clear explanation of the difference of temperature sensitivity observed

between the two soils used in the present experiment. However, these differences exist and cannot be forgotten.

In conclusion, the results obtained under the same experimental design differ greatly from one soil to another, demonstrating a serious influence of the soil properties on the biostimulation efficiency. While spill management requires the development of a quick action in response planning, the finding of this study reinforces the need to evaluate firstly the factors that control both the microbial activity and the degradation of organic compounds at a specific site. Whilst acknowledging the fundamental limitations of ex-situ bioremediation approach in cold environments, this study demonstrated that ex-situ bioremediation is likely to be the strategy of choice for remediation of hydrocarbon-contaminated sub-Antarctic soils.

Acknowledgements

This research was supported by the French Polar Institute (IPEV) and the European Project FACEiT (Fast Advanced Cellular and Ecosystems Information Technologies).

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Legends

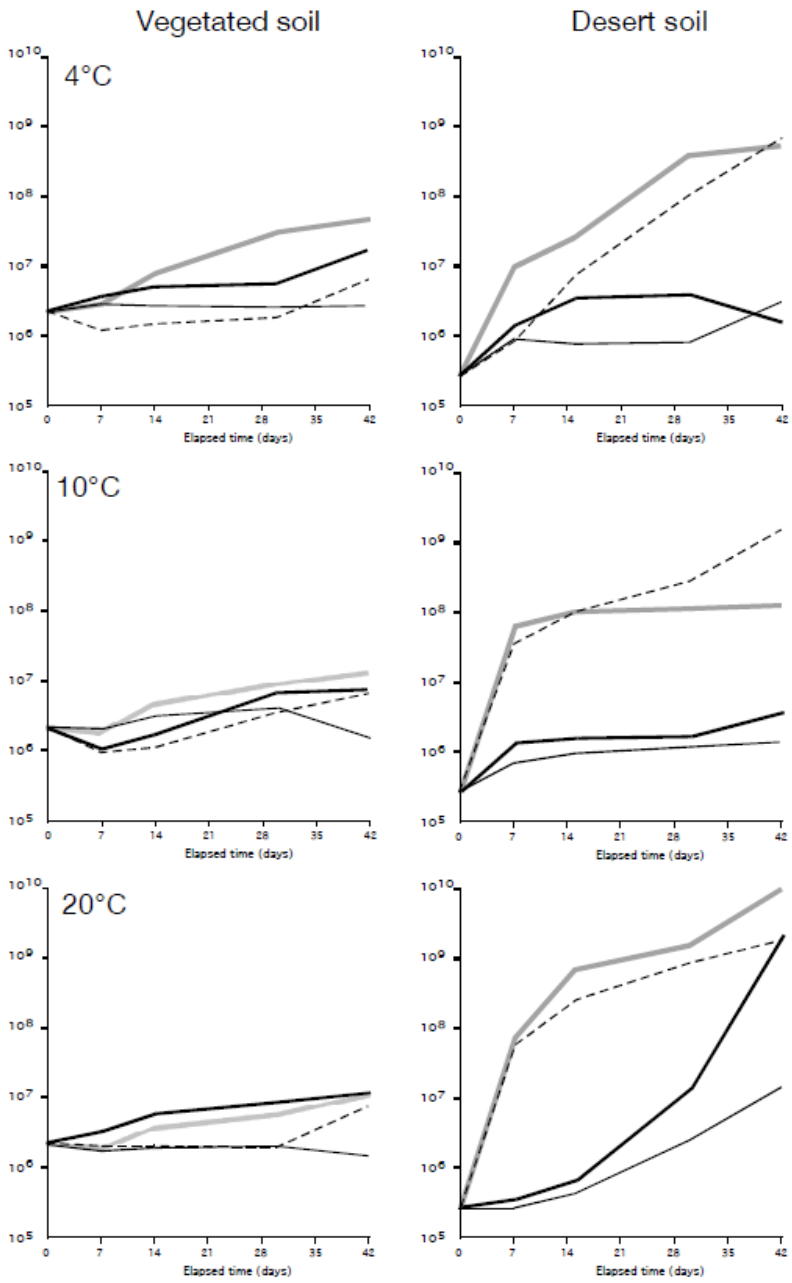
Figure 1: Changes of heterotrophic bacterial abundance during incubation of the diesel amended mesocosms (thin line: control, doted line: control + fertiliser, bold line: diesel, gray line: diesel + fertiliser).

Figure 2: Changes of heterotrophic bacterial abundance during incubation of the crude oil contaminated mesocosms (thin line: control, doted line: control + fertiliser, bold line: crude oil, gray line: crude oil + fertiliser).

Figure 3: Changes of hydrocarbon-degrading bacterial abundance during incubation of the diesel contaminated mesocosms (thin line: control, doted line: control + fertiliser, bold line: diesel, gray line: diesel + fertiliser).

Figure 4: Changes of hydrocarbon-degrading bacterial abundance during incubation of the crude oil contaminated mesocosms (thin line: control, doted line: control + fertiliser, bold line: crude oil, gray line: crude oil + fertiliser).

Figure 5: Changes in oil hydrocarbon fractions concentration (as % of initial values) over 180 days. Each percentage represents the mean of hydrocarbons fractions from duplicate samples and bars indicate standard deviation.



1

2 Figure 1

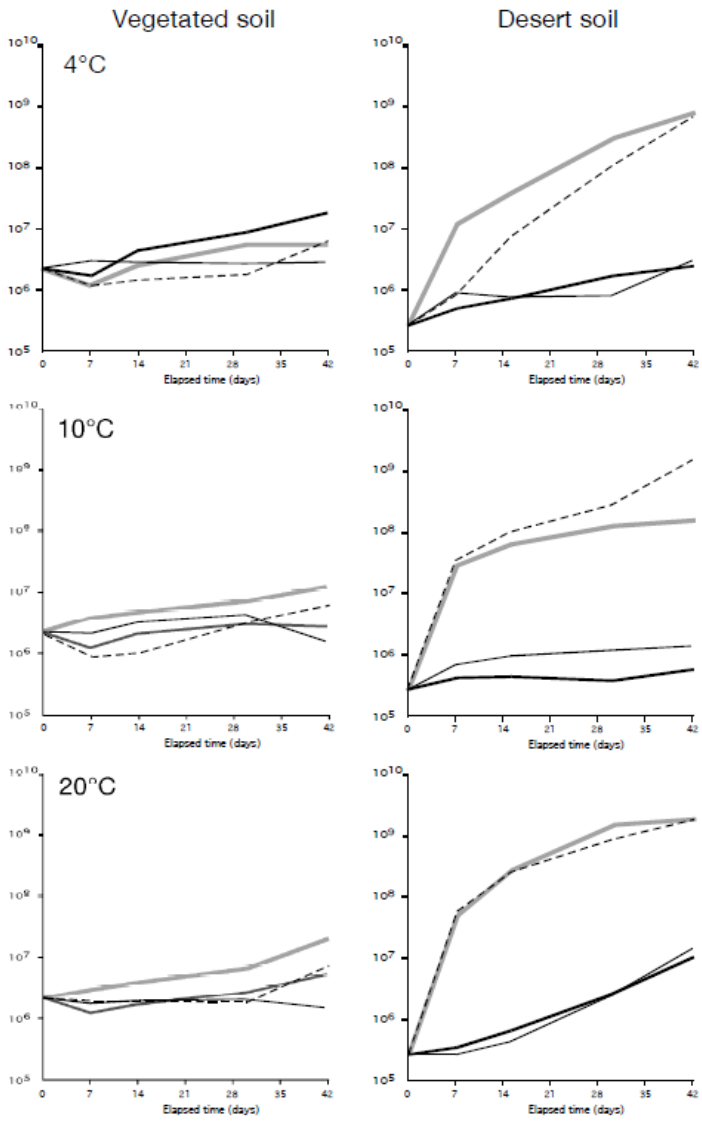
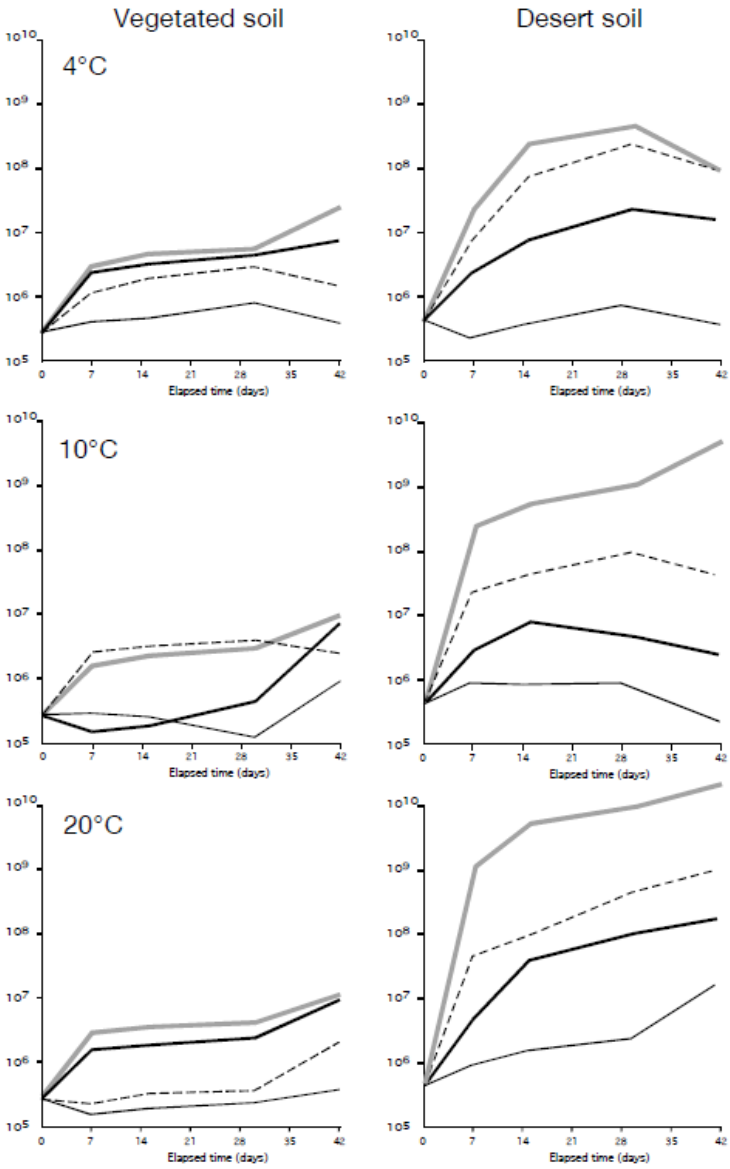


Figure 2



1

2 Figure 3

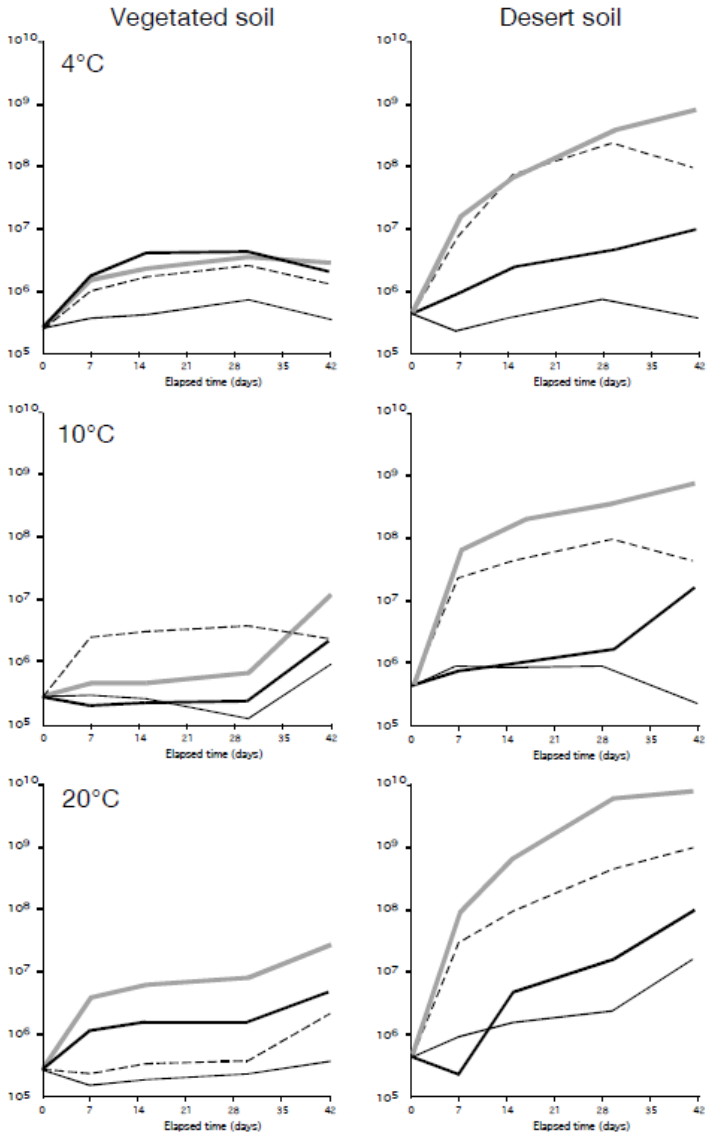
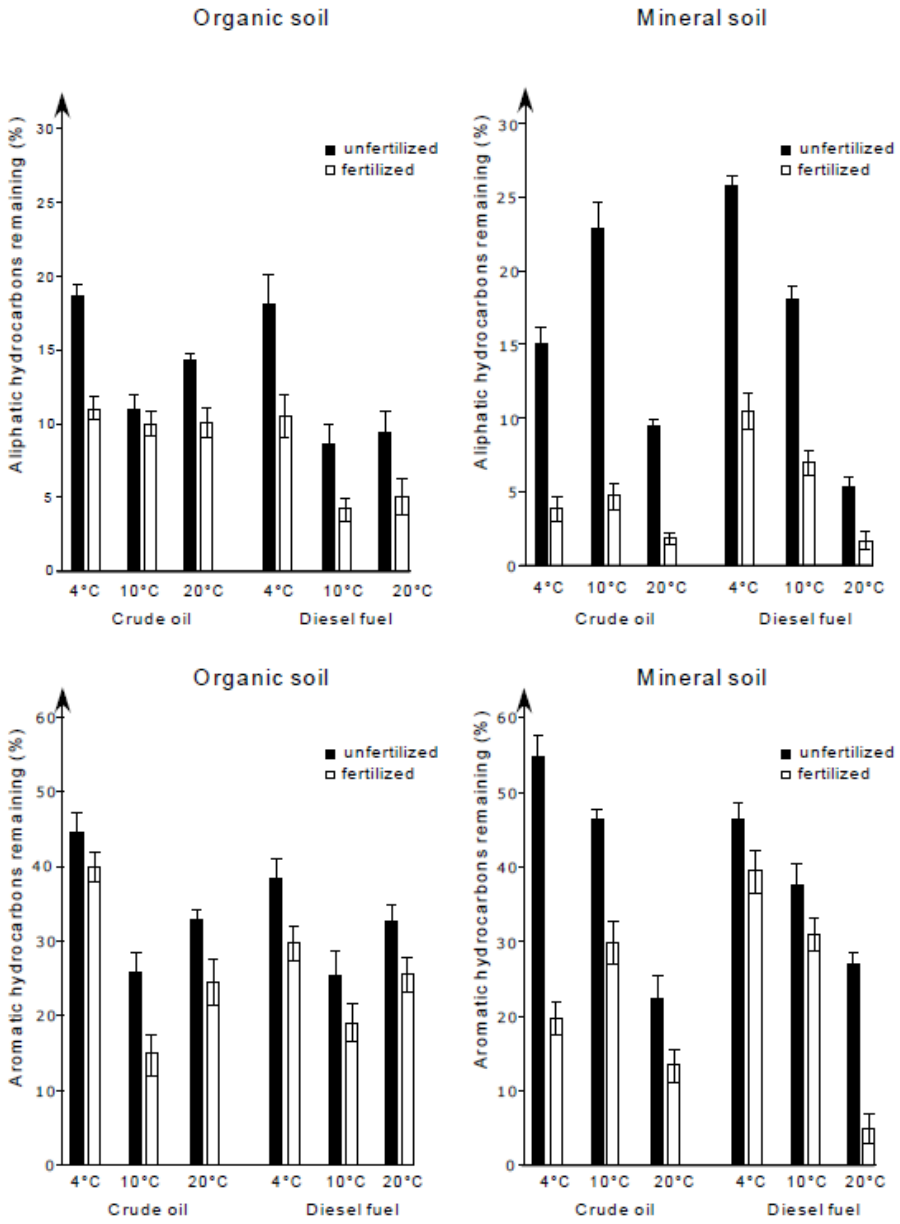


Figure 4


Organic soil
Mineral soil
Aliphatic hydrocarbons remaining (%)
Aromatic hydrocarbons remaining (%)
unfertilized
fertilized
4°C
10°C
20°C
Crude oil
Diesel fuel

1

2 Figure 5

Table 1: Changes in oil hydrocarbon fractions concentration (as % degradation of initial value) over 180 days. Each percentage represents the mean of hydrocarbons fractions from duplicate samples

Oil type/ treatment	Hydrocarbon fraction	Organic soil			Mineral soil		
		4°C	10°C	20°C	4°C	10°C	20°C
Crude oil	***Aliphatic***						
	EC >10 - 12	65	82	73	82	75	91
	EC > 12 -16	59	79	75	73	70	84
	EC > 16 -35	45	56	53	67	61	71
	Aromatic						
	EC >10 - 12	63	65	77	76	79	87
	EC > 12 -16	55	69	62	54	70	89
	EC > 16 -21	23	39	42	38	46	51
Crude oil + inipol	***Aliphatic***						
	EC >10 - 12	81	84	89	96	96	> 99
	EC > 12 -16	76	79	85	90	95	98
	EC > 16 -35	67	53	53	86	90	95
	Aromatic						
	EC >10 - 12	73	85	77	80	87	89
	EC > 12 -16	55	69	62	70	74	79
	EC > 16 -21	39	42	49	65	69	72
Diesel	***Aliphatic***						
	EC >10 - 12	82	94	98	79	90	91
	EC > 12 -16	69	77	89	75	89	84
	EC > 16 -35	45	51	53	58	66	71
	Aromatic						
	EC >10 - 12	67	74	67	73	81	88
	EC > 12 -16	58	61	59	65	69	77
	EC > 16 -21	39	45	42	43	56	63
Diesel + Inipol	***Aliphatic***						
	EC >10 - 12	85	98	> 99	94	> 99	> 99
	EC > 12 -16	78	86	89	88	97	98
	EC > 16 -35	56	71	69	67	71	82
	Aromatic						
	EC >10 - 12	75	82	76	84	89	94
	EC > 12 -16	63	71	62	68	71	85
	EC > 16 -21	51	65	59	49	58	71

Comparative mesocosm study of biostimulation efficiency in two different oil-amended sub-antarctic soils

Delille, Daniel

2008-08

Daniel Delille and Frédéric Coulon, Comparative mesocosm study of biostimulation efficiency in two different oil-amended sub-antarctic soils, Microbial Ecology, Vol. 56, August 2008, pp243-252
http://hdl.handle.net/1826/3023