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Effect of nutrient enrichments on the bacterial assemblage of Antarctic soils contaminated by diesel or crude oil

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ABSTRACT. There is an urgent need to develop new technologies to address the problem of soil remediation in high-latitude regions. A field study was initiated in January 1997 in two contaminated soils in Terre Adélie (Antarctica) with the objective of determining the long-term effectiveness of two bioremediation agents on total and hydrocarbon-degrading microbial assemblages under severe Antarctic conditions. This study was conducted in two steps, from January to July 1997 and from February to November 1999 in the Géologie Archipelago (Terre Adélie, 66°40′S, 140°01′E). Changes in bacterial communities were monitored *in situ* after crude oil or diesel addition in a series of 600 cm^2 soil sectors (20×30 cm). Four contaminated sectors were used for each experiment: diesel oil (10 ml), diesel oil (10 ml) + fertilizer (1 ml), Arabian light crude oil (10 ml), and crude oil (10 ml) + fertilizer (1 ml). Two different bioremediation agents were used: a slow release fertilizer Inipol EAP-22 (Elf Atochem) in 1997 and a fish compost in 1999. Plots were sampled on a regular basis during a three-year period. All samples were analysed for total, saprophytic psychrophilic, and hydrocarbon-utilising bacteria. A one order of magnitude increase of saprophytic and hydrocarbon-utilising micro-organisms occurred during the first month of the experiment in most of the contaminated enclosures, but no clear differences appeared between fertilized and unfertilized plots. Diesel-oil contamination induced a significant increase of all bacterial parameters in all contaminated soils. Crude-oil contamination had no clear effects on microbial assemblages. It was clear that the microbial response could be rapid and efficient in spite of the severe weather conditions. However, microbial growth was not clearly improved in the presence of bioremediation agents.

Contents

Introduction

As a result of twentieth-century industrialization, many harmful substances have been discharged into terrestrial and aquatic environments. The most widely distributed environmental pollution can be attributed to the spill of crude oil and various oil residues (Margesin 2000). With increasing public attention regarding the preservation of the environment, the development of oil clean-up technologies has gained considerable interest. In recent years, a number of cost-effective *in situ* techniques for remediation of oil-contaminated soil have been proposed (Joergensen and others 1995; Møller and others 1996; Eweis and others 1998). Although physical methods of oil removal may cause more damage to soil than spilled oil itself, biological methods, such as bioremediation, may be more effective in removing oil without undue damage to the environment. Bacterial degradation, by weathering processes, is the ultimate fate of oil spills in nature (Atlas 1981; Leahy and Colwell 1990; Madsen 1991; Michaelsen and others 1992; Bragg and others 1994). Specific micro-organisms generally degrade a limited number of crude-oil components (Floodgate 1984), but the activity of mixed microbial populations often results in a higher level of biodegradation (Komukai-Nakamura and others 1996). Thus, microbial clean-up can be advantageous, when compared to other remediation techniques (Blackburn and others 1993; Venosa and others 1996; Salanitro and others 1997).

Human activities in high-latitude regions rely heavily on fossil fuels for transportation and generation of heat and electricity. Until recently, the Antarctic ecosystem was almost uncontaminated by anthropogenic hydrocarbons (Platt and Mackie 1980; Clarke and Law 1981; Reinhardt and VanVleet 1986; Cripps 1990, 1992; Berkman 1992). However, the accidental contamination of soils with fuel oil has occurred frequently in recent

years, and little is known about hydrocarbon-degradation processes in cold environments. A number of studies have been made in the Arctic (Atlas and others 1978; Sparrow and others 1978; Griffiths and others 1981; Horowitz and others 1983; Braddock and McCarthy 1996), Antarctic ice-free sea waters (Clarke and Law 1981; Platt and others 1981; Delille and Vaillant 1990), and ice-covered marine areas (Atlas and others 1978; Cavanagh and others 1998; Cripps and Priddle 1991; Delille and Siron 1993; Siron and others 1993), but relatively few data are available for Antarctic soils (Kerry 1990, 1993; Tumeo and Wolk 1994; MacCormack and Fraile 1997; Aislabie and others 1998, 2001; Bej and others 2000). There is a need to understand the fate and behaviour of hydrocarbon contaminants in these extreme systems. A previous study conducted with ornithogenic soils of Dumont d'Urville station (Terre Adélie, Antarctica) demonstrated the presence of indigenous hydrocarbon-utilising micro-organisms in these Antarctic soils. Despite their strong reactivity to contamination by diesel and crude oil, their biodegradation rates were very low, even after one year of contamination (Delille 2000).

In the present work, the objective was to evaluate the benefit of adding fertilizers (Inipol EAP 22 and fish compost) to oil-contaminated soils on the total and specific bacterial communities. Previous information on oil-decontamination processes in cold terrestrial ecosystems has been relatively limited and mainly related to Arctic (Sparrow and Sparrow 1988; Braddock and others 1997; Whyte and others 1999; Mohn and others 2001) and alpine soils (Margesin 2000; Margesin and Schinner 1997, 2001).

Materials and methods

This study was conducted in two steps: from January to July 1997 (soils 1 and 2) and from February to November 1999 (uniquely soil 2) in the Géologie Archipelago (Terre Adélie, Antarctica: 66°40′S, 140°01′E). Both soil stations (Fig. 1) were located on Petrel Island in the vicinity of the French permanent Antarctic base, Dumont d'Urville. The mean monthly air temperature in the study area rises above freezing point only during December and January and reaches −16°C in July–August. Thus, the resulting annual hydrologic cycle can be described in terms of four periods: winter, snowmelt, summer, and freeze-up. Both soils were submitted to penguins guano during the summer season. However, bird influence was strongly reduced in soil 1 when compared to soil 2. Despite the bird guano, the total natural nitrogen concentrations determined by CHN analysis were less than 1 g kg^{-1} (dry weight) in both soils.

The changes in bacterial communities were studied *in situ* after the addition of crude or diesel oil to the surface of 600 cm^2 soil sectors (20 × 30 cm). Two different bioremediation agents were added: a slow-release fertilizer, Inipol EAP-22 (Elf Atochem), in 1997, and a fish compost in 1999. Four contaminated sectors were used for each experiment: diesel oil (10 ml); diesel oil (10 ml) + fertilizer (1 ml Inipol EAP 22 or 1 g of fish compost); Arabian light crude oil, known as BAL (10 ml); and BAL (10 ml) + fertilizer (1 ml Inipol EAP 22 or 1 g of fish compost). Weekly sampling allowed a regular survey of total, saprophytic psychrophilic, and hydrocarbon-utilising bacteria. Samples were collected with a sterile spatula on the surface of the frozen soils after removal of the snow cover. All bacterial analyses began in the laboratory within 15 min. Colony-forming units (CFU), spread plating, and most probable number (MPN) inoculation were achieved in a few hours and acridine orange direct-count (AODC) enumerations in a few days.

Total bacteria were determined by AODC on black nuclepore filters (0.2 μm) using an Olympus BHA epifluorescence microscope according to the method of Hobbie and others (1977). A minimum of 500 fluorescing cells with a clear outline and definite cell-shape cells were counted under oil immersion (×1000) in a minimum of 10 randomly chosen fields.

Viable counts of aerobic heterotrophic micro-organisms were made using the spread plate technique on Nutrient Agar 2216 (Oppenheimer and ZoBell 1952, using distilled water in place of sea water). Inoculated plates (six replicates) were incubated for 20 days at 2°C.

Hydrocarbon-utilising bacteria were counted by the MPN method, using a basal mineral medium without carbon, supplemented with Arabian light crude oil (Mills and others 1978). Rezasurin was used as a growth indicator. After inoculation (three tubes per dilution) the tubes were incubated at 12°C for 30 days. A large majority of the bacteria isolated from Antarctic sea water must be considered psychrotrophic and not truly psychrophilic strains (Delille and Perret 1989). There was no significant difference between MPN counts obtained after incubation at 4°C and 20°C (Delille and others 1988; Delille and Perret 1989). Thus, the relatively high incubation temperature used in the present study had no significant effect on the data and allowed a substantial reduction of the incubation time (incubation of MPN needs three months at 4°C; such a long time is not compatible with all Antarctic fieldwork).

Results

A small initial increase of total bacterial abundance occurred in all contaminated soils (from 4.8 10^8 to 2.1 10^9 cells ml^{-1}). However, these stimulating effects were generally less than one order of magnitude and they were always limited in time (data not shown).

During the 1997 experiment in soil 1 (Fig. 2), diesel-oil contamination induced a one order of magnitude increase of both saprophytic and hydrocarbon-utilising microbial assemblages. In contrast, crude-oil contamination had no effect on saprophytic micro-organisms and only a late (after three months) stimulating effect on specific micro-organisms. Inipol fertilization had no significant effect on saprophytic microbial abundance, but induced an increase of the hydrocarbon-utilising microbial abundance for both kinds of contamination.

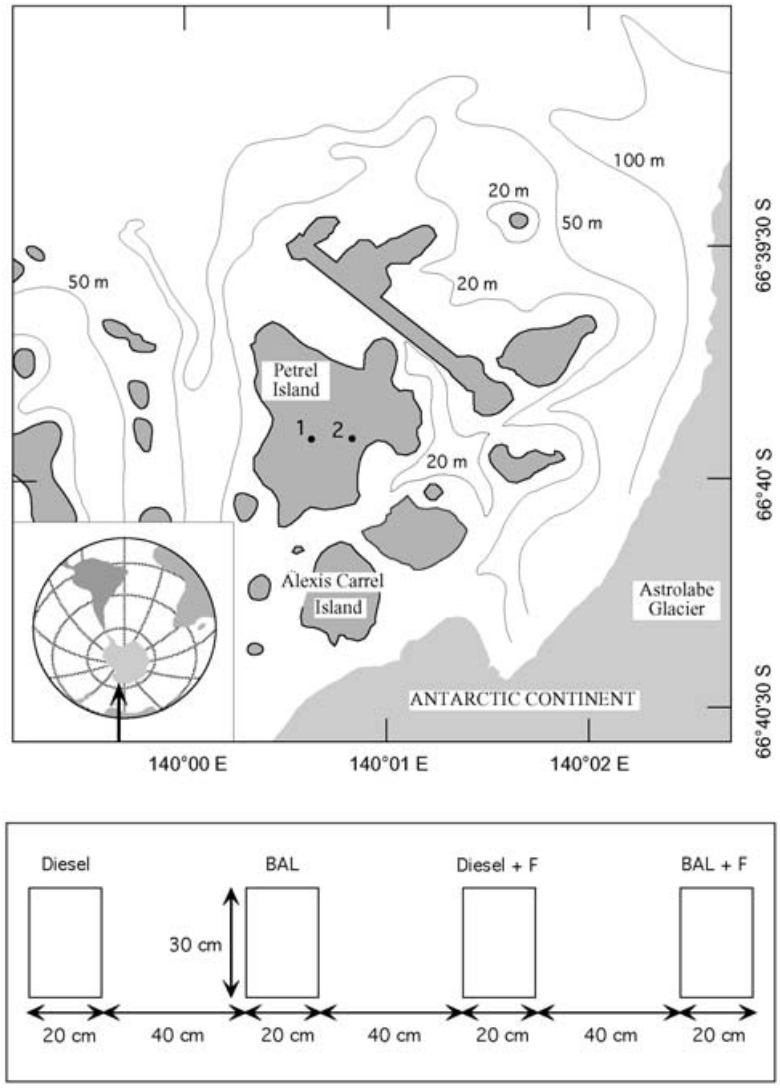

Fig. 1. Map of Petrel Island (Terre Adélie, Antarctica), showing the location of the contaminated soils. The bottom part shows the relative position of the four sampling sectors used during each contamination experiment. BAL = Arabian light crude oil; F = fertilizer.

Observations made during the same period in soil 2 are shown in Figure 3. As seen for soil 1, diesel-oil contamination induced a one order of magnitude increase of hydrocarbon-utilising microbial assemblages, but crude-oil contamination had no clear effect on both saprophytic and hydrocarbon-utilising microbial assemblages. There was relatively little difference between fertilized and unfertilized sectors. The only noticeable increase of specific microbial abundance occurred in February–March after diesel-oil contamination.

During the 1999 experiment, fish compost was used in place of the Inipol fertilizer. The survey was conducted in duplicate in soil 2. A first experiment was conducted in sectors previously used in 1997 (soil 2a); a parallel experiment was conducted at a short distance (4 m, soil 2b). The only noticeable physical difference between the two sites was the presence during sunny days of summer of a small stream coming from the penguin rookeries and running closer to soil 2b than soil 2a. Regarding this stream, soil 2b is located slightly upstream of soil 2a.

It is interesting to note that the global differences between the two sites could be more important than differences observed between the different sectors of each site (Figs 4, 5). Contamination by diesel and crude oil had a strong stimulating effect on both saprophytic and hydrocarbon-utilising microbial assemblages in soil 2b. These positive effects were not so clear in soil 2a, which had been previously submitted to hydrocarbon contamination. Addition of fish compost often gave a toxic effect on microbial abundance, particularly on hydrocarbon-utilising bacterial assemblages of soil 2b. The only noticeable stimulating effect of the fish compost was recorded for hydrocarbon-utilising bacterial assemblages of soil 2a (from March to April in diesel-contaminated sectors and from August

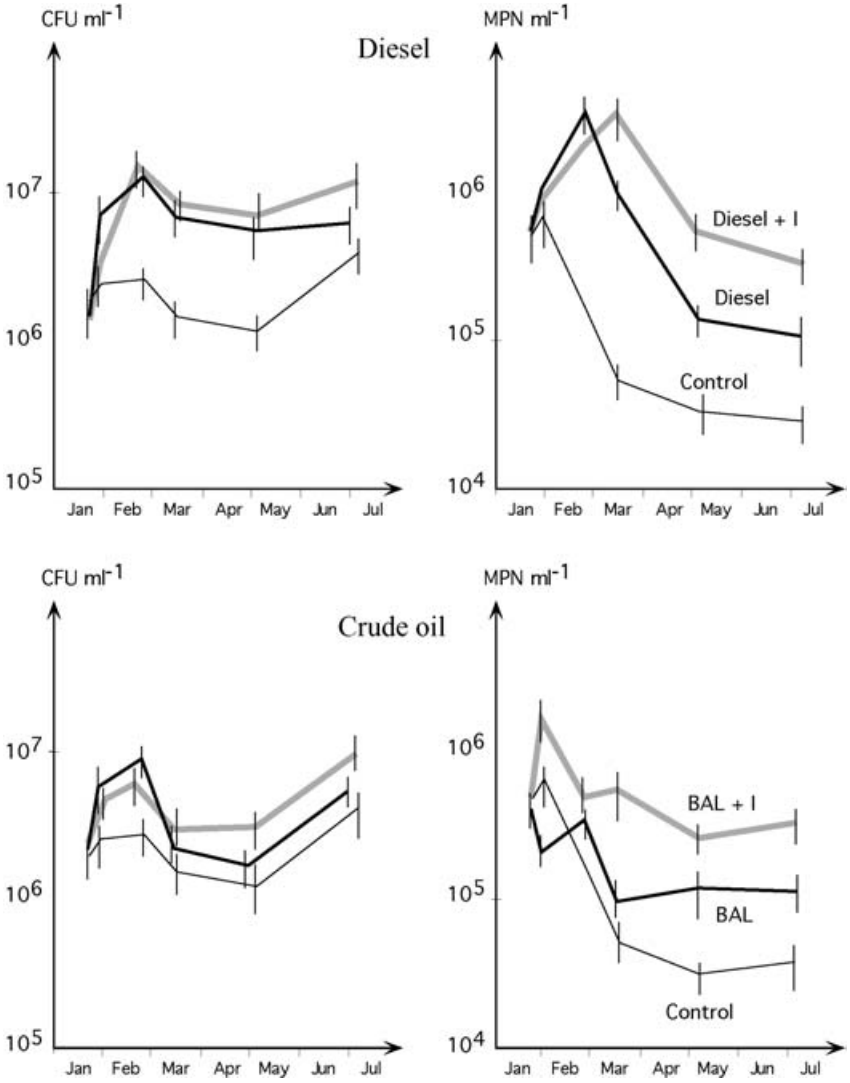

Fig. 2. Changes of saprophytic (left) and hydrocarbon-utilising (right) microbial abundance in soil 1 during the 1997 contamination experiments. Thin line = control; thick line = contaminated soil; gray line = fertilized contaminated soil.

to November in sectors contaminated by crude oil).

Discussion

Total direct counts reflect the actual bacterial abundance, but their variations in time are less pronounced than the changes observed with other bacteriological parameters. During growth periods, the quantitative difference between direct and viable counts showed a marked decline. Similar findings have been previously reported and discussed (Delille and Bouvy 1989). In the present study, direct-count estimates were not sensitive enough to give valuable information concerning the differences between the various treatments. Except in special circumstances where one may be looking for specific microbes, the knowledge gained by viable counts is minimal for biomass estimations. However, saprophyte counts are representative of a small group of active micro-organisms that react immediately to changes in nutrient supply; thus, they are a useful microbial indicator (Delille and Bouvy 1989; Rheinheimer and others 1989).

In uncontaminated soils, saprophytic bacterial counts agree well with those reported by Aislabie and others (1998, 2001). In contrast, the corresponding MPN values of hydrocarbon-utilising micro-organisms were higher than those reported by the same authors for pristine Antarctic soils. The hydrocarbon-utilising bacterial abundance observed in uncontaminated soils of Dumont d'Urville station seems to correspond more to slightly contaminated soils than to true pristine soils. In contrast with the works of Aislabie and others, both soils studied in the present investigation are of ornithogenic nature (Delille 1987). The enrichment by organic and inorganic nutrients may explain the observed differences, but previous accidental oil contamination cannot be excluded after 40 years of human activities

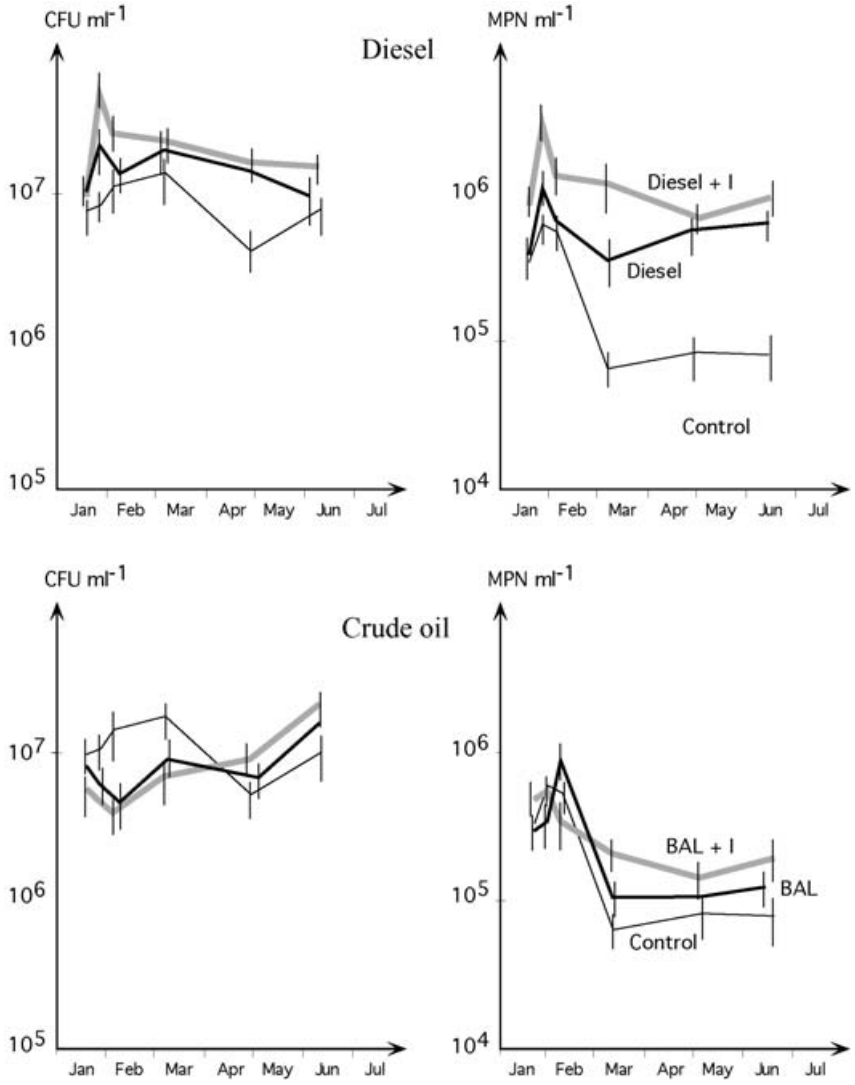

Fig. 3. Changes of saprophytic (left) and hydrocarbon-utilising (right) microbial abundance in soil 2 during the 1997 contamination experiments. Thin line = control; thick line= contaminated soil; gray line = fertilized contaminated soil.

on Petrel Island. In pristine soils, hydrocarbon-utilising micro-organisms typically represent less than 2% of the total number of heterotrophic bacteria. These results are similar to those of Atlas (1981) and Wright and others (1997), who reported that hydrocarbon-utilising micro-organisms comprised 1–10% of the total number of heterotrophic bacteria. After an oil contamination, concentration of hydrocarbon-utilising bacteria can reach values $>10^6$ MPN ml^{-1}, corresponding to those observed in highly contaminated soils by Aislabie and others (1998, 2001). The present data set provides further evidence of the presence of indigenous hydrocarbon-utilising bacteria in Antarctic soils and their potential for oil bioremediation.

Both diesel and crude oil contain thousands of aliphatic and aromatic hydrocarbons, and related heterocyclic compounds. Many of these chemicals can alter the community structure through selection of pollutant degraders or through acute toxicity to micro-organisms. On the other hand, readily biodegradable hydrocarbons can increase population densities by providing carbon and energy for growth to micro-organisms otherwise in oligotrophic environments. The rate at which carbon can be utilised is limited by the physical characteristics of the environment, the lability of the carbon sources, and the availability of other nutrients (Piehler and Paerl 1996). Inipol EAP 22 is a microemulsion consisting of a urea core surrounded by oleic acid, lauryl-phosphate, and butoxyethanol. Its utilisation has enhanced hydrocarbon degradation in numerous Antarctic environments: sea water (Delille and others 1998a, 1998b), sea ice (Delille and others 1997), intertidal sediments (Delille and others, in press), and salt marches (Wright and others 1997). Fish compost has also been used with success in a sub-Antarctic oiled beach (Delille and others 2002). In the present study, in contrast with the observations of Kerry (1993) in Vestfold Hills, fertilization did not

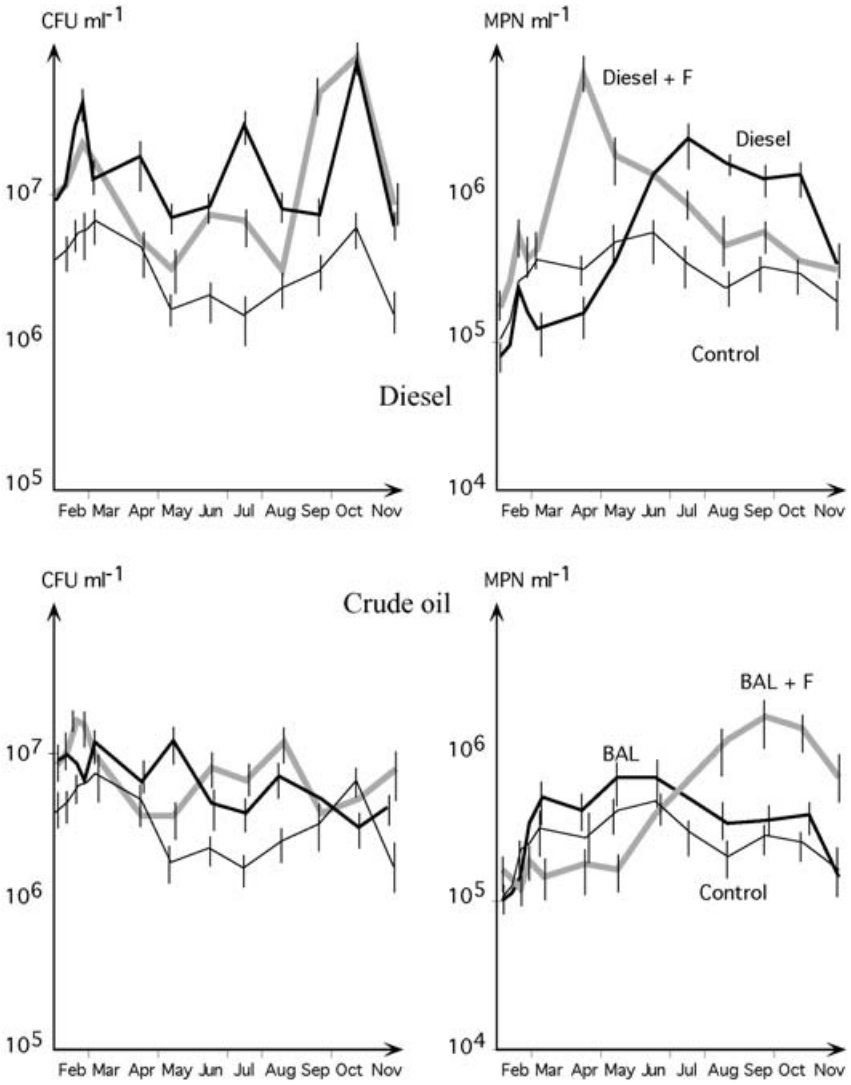

Fig. 4. Changes of saprophytic (left) and hydrocarbon-utilising (right) microbial abundance in soil 2a during the 1999 contamination experiments. Thin line = control; thick line = contaminated soil; gray line = fertilized contaminated soil.

clearly enhance the hydrocarbon-degrading microbial abundance.

Active bioremediation by addition of exogenous nutrient might not be appropriate in cases where background nutrient levels are already sufficiently high to support high intrinsic rates of hydrocarbon biodegradation (Venosa and others 1996). The authors first suspected that the mineralization of penguin detritus supplied microorganisms with enough nitrogen for the degradation of applied hydrocarbons. However this hypothesis is not supported by the CHN analysis of the studied soils (total natural nitrogen concentrations <1 g kg^{-1}).

In periglacial soils, it had been reported that excessive levels of nitrogen could inhibit biodegradation by decreasing soil-water potentials (Walworth 2002). However, in the present experiments, the fertilizer contribution to soil osmotic potential, expressed as nitrogen levels in soil solution, was close to 100 mg N kg H_2O^{-1}. This value is more than 10-fold lower than the maximum nitrogen dose (2500 mg N kg H_2O^{-1}) that can be added without causing any adverse impact (Walworth 2002).

An alternative possible explanation for the lack of stimulation of the bacterial assemblage is the presence of an additional limiting factor. Microbial metabolism increases as temperature increases (Leahy and Colwell 1990), usually doubling for each 10°C increase in temperature from 10 to 40°C (Bossert and Bartha 1984). However, the metabolisms of psychrophilic and psychrotrophic bacteria are adapted to work and function at low temperature (Gounot 1991; Margesin and Schinner 1997; Feller and others 1996; Whyte and others 1996). ZoBell (1973) reported mineral-oil degradation at temperatures below 0°C in a marine environment. In the area presently studied, temperatures close to 0°C have been shown to allow oil biodegradation in

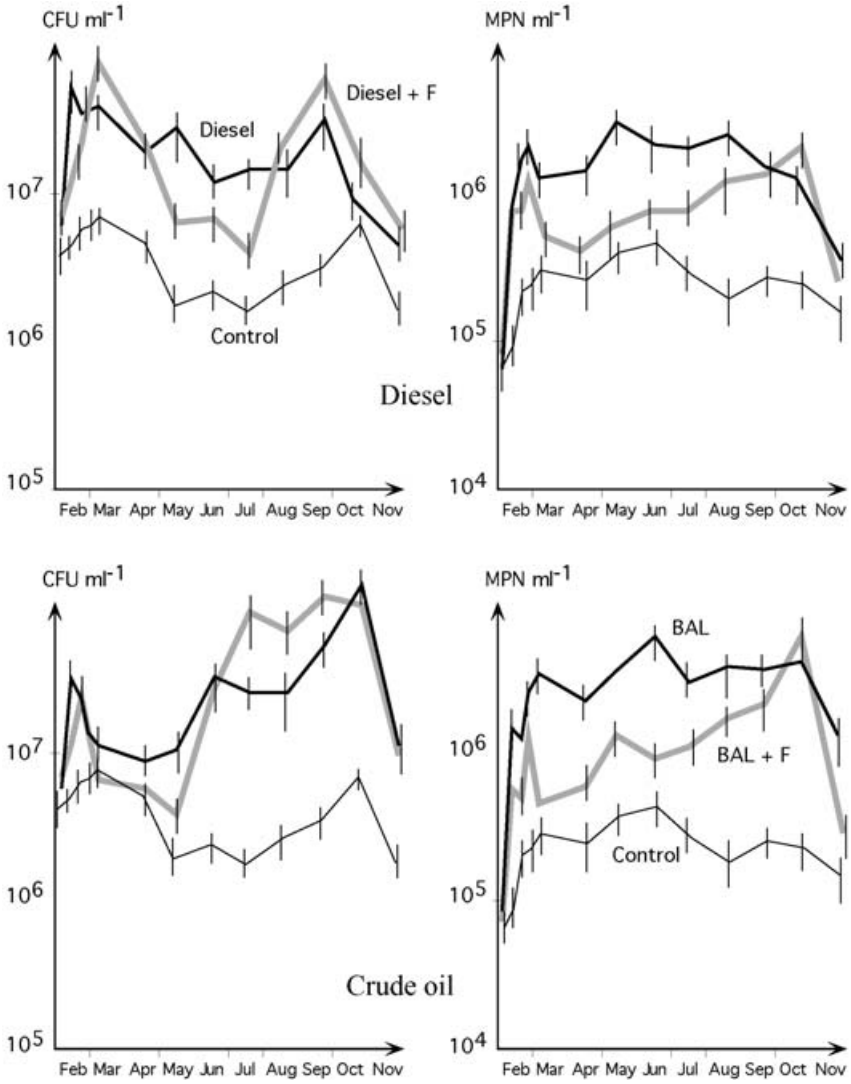

Fig. 5. Changes of saprophytic (left) and hydrocarbon-utilising (right) microbial abundance in soil 2b during the 1999 contamination experiments. Thin line = control; thick line = contaminated soil; gray line = fertilized contaminated soil.

sea water (Delille and others 1998a, 1998b) and sea ice (Delille and others 1997). In coastal Antarctica, a typical summer surface soil temperature is 12°C (Wilson and others 1997; Bej and others 2000). Antarctic soils are thermally unstable, experiencing large temperature fluctuations with temperatures dropping well below 0°C at night and reaching over 20°C during a sunny afternoon (Balks and others 1995; Tibbles and Harris 1996; Harris and Tibbles 1997). Thus, these soils suffer frequent summer freeze–thaw cycles. Antarctic maritime soils are also reported to experience other extreme environmental stresses, principally desiccation and freezing (Wynn-Williams 1990; Davey and Clarke 1991). All these extreme fluctuations may seriously affect the overall bacterial activity. Bacteria must acclimate continuously and be able to rapidly switch activity on and off. Relative temperature stability (as in sea ice or seawater) is more favourable to bacterial growth than the high but fluctuating temperatures experienced in Antarctic soils during summer.

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