Effects of Biostimulation on Growth of Indigenous Bacteria in Sub-Antarctic Soil Contaminated with Oil Hydrocarbons

F. Coulon[1] and D. Delille[1]

1 *Observatoire océanologique, université Pierre et Marie Curie, UMR-CNRS 7621, 66650 Banyuls-sur-Mer Cedex - France*
e-mail: coulon@obs-banyuls.fr - daniel.delille@wanadoo.fr

Résumé — **Effets de traitements de biostimulation sur la croissance des bactéries indigènes d'un sol subantarctique contaminé par des hydrocarbures** — Une étude en mésocosme, destinée à l'évaluation de l'efficacité de traitements de biostimulation, a été réalisée entre mai et novembre 2001 sur un sol subantarctique prélevé à proximité de la base de Port aux Français (archipel de Kerguelen, 49°21'S, 70°13'E). Deux procédés stimulants potentiels ont été utilisés : élévation de la température et addition d'un agent fertilisant (Inipol EAP 22, *Elf Atochem*). Des bacs contenant 6 l de sol ont été contaminés et incubés à l'obscurité à trois températures différentes (4, 10 et 20° C). Six traitements spécifiques ont été utilisés pour chacune de ces températures : contrôle sans contamination, brut arabe léger "BAL" (100 ml), BAL (100 ml) + Inipol (50 ml), gazole (100 ml), gazole (100 ml) + Inipol (50 ml) et Inipol seul (50 ml). Les mésocosmes ont été échantillonnés régulièrement pendant 7 mois. Les abondances bactériennes totales, hétérotrophes et spécifiques de la dégradation des hydrocarbures ont été évaluées sur chaque échantillon prélevé. Les résultats démontrent une réponse significative du consortium microbien du sol étudié. Les deux types de contamination entraînent une large augmentation du nombre de bactéries hétérotrophes et spécifiques (de 5×10^5 NPP g^{-1} à 10^8 NPP g^{-1} pour les bactéries spécifiques). L'augmentation de la température ne semble pas avoir d'effet marqué sur l'abondance bactérienne totale et hétérotrophe, elle induit néanmoins une légère augmentation de l'abondance des bactéries spécifiques de la dégradation des hydrocarbures (augmentation d'un ordre de grandeur). À l'opposé, l'Inipol ne semble pas avoir d'effet réel sur l'abondance des bactéries spécifiques, mais stimule la croissance des germes hétérotrophes sur les sols contaminés par le gazole.

Abstract — *Effects of Biostimulation on Growth of Indigenous Bacteria in Sub-Antarctic Soil Contamined with Oil Hydrocarbons* — *In order to evaluate the efficiency of biostimulation of soil contaminated with oil hydrocarbons under sub-Antarctic conditions, a mesocosm study was initiated in May 2001 in the Kerguelen Archipelago (49°21'S, 70°13'E). The effects of temperature and fertilizer addition (Inipol EAP-22,* Elf Atochem*) on soil bacterial assemblages contaminated with hydrocarbons were studied in 6-l batches of subantarctic soil incubated in the dark. Six different conditions were used at three temperatures (4, 10 and 20°C): control, fertilizer (50 ml), diesel oil (100 ml), diesel oil (100 ml) + fertilizer (50 ml), "Arabian light" crude oil (100 ml) and crude oil (100 ml) + fertilizer (50 ml). Mesocosms were sampled on a regular basis over a seven-month period. All samples were analyzed for total bacteria, viable heterotrophic assemblages and hydrocarbon-utilising microflora. The results clearly showed a significant response of sub-Antarctic microbial soil communities to hydrocarbon contamination. Large increases in total, heterotrophic and hydrocarbon-utilising bacteria were observed*

(from less than 5×10^5 MPN g-1 to more than 10^8 MPN g^{-1} for hydrocarbon degrading bacteria). Temperature elevation had no significant impact on the total or heterotrophic assemblages but induced a one order of magnitude increase in hydrocarbon-utilising bacteria in contaminated mesocosms. In contrast, fertilizer addition had no clear effect on hydrocarbon-degrading specific bacteria but stimulated heterotrophic growth in diesel oil-contaminated soils.

INTRODUCTION

Petroleum hydrocarbons belong to the most widespread contaminants of water and soil. Antarctica and the sub-Antarctic islands are some of the last remaining pristine zones on Earth, being generally uncontaminated by anthropogenic hydrocarbons (Reinhardt and Van Vleet, 1986). The main human activities undertaken in these regions are scientific research, tourism and fishing (Cripps and Shears, 1997). All these activities require fossil fuels for transport and energy requirements and an increasing use of petroleum hydrocarbons has led to an increase in the probability of major spillages contaminating terrestrial and aquatic environments. Accidental contamination of Antarctic (Bej *et al.*, 2000) and sub-Antarctic (Delille *et al.*, 2001) soil with fuel oil has recently occured. These events highlighted the need for research in hydrocarbon contamination in cold environment.

Hydrocarbon-degrading organisms are widely distributed in nature and have even been found in environments that have not been subjected to high levels of hydrocarbon pollution (Atlas, 1981; Prince, 1993). Biodegradation by naturally occurring populations of microorganisms is a major mechanism for the removal of petroleum from the environment (Atlas *et al.*, 1981 ; Leahy and Colwell, 1990; Bragg *et al.*, 1994). In recent years there has been an increasing interest in developing cost effective *in situ* techniques for restoration of oil-contaminated soils, biostimulation being one of the most extensively studied process (Joergersen *et al.*, 1995; Møller *et al.*, 1996). Biostimulation is a treatment technology for the clean-up of polluted sites that involves the use of indigenous microorganisms to detoxify and degrade environmental contaminants.

The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of cold terrestrial and marine systems, including Arctic soil (Braddock *et al.*, 1997), Alpine soil (Margesin and Schinner, 1997b, 2001a), Antarctic soil (Aislabie *et al.*, 1998; Delille, 2000), sub-Antarctic soil (Delille *et al.*, 2001), sub-Antarctic intertidal sediments (Delille and Delille, 2000, Delille *et al.,* 2002), Arctic seawater (Siron *et al.*, 1993, 1995) and Antarctic seawater and sea-ice (Delille *et al.*, 1998). Temperature plays a significant role in controlling the nature and the extent of microbial hydrocarbon metabolism, which is of special significance for *in situ* bioremediation. Bioavailability and solubility of less soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons, are temperature dependent. A temperature increase decreases viscosity and increases diffusion rates of organic compounds. Therefore, higher reaction rates due to smaller boundary layers are expected at elevated temperatures. The increased volatilization and solubility of some hydrocarbons at elevated temperature affects toxicity and allows biotransformations with high substrate concentrations (Müller *et al.*, 1998; Whyte *et al.*, 1998; Niehaus *et al.*, 1999; Margesin and Schinner, 2001b). This may explain why a delay in the onset of biodegradation can be observed at low temperatures (Atlas, 1981; Leahy and Cowell, 1990; Itävaara *et al.*, 2000). Although microbial activity is generally reduced at low temperatures, many of the components in crude oil can actually be degraded in these conditions (Atlas, 1981; Bossert and Bartha, 1984; Leahy and Colwell, 1990; Margesin and Schinner, 1997a; Whyte *et al.*, 1998; Delille, 2000; Margesin and Schinner, 2001b). Relatively little data are available for Antarctic and sub-Antarctic soils. Indeed, some bioremediation experiments have been conducted on Antarctic soils (Kerry, 1993; Delille, 2000) but, to our knowledge, only bioattenuation observations have been carried out in sub-Antarctic soils (Delille *et al.*, 2001).

The aim of the present mesocosm study is to evaluate and compare the benefits of temperature increase or fertilizer addition (Inipol EAP 22) on total and hydrocarbon-utilising indigenous microbial populations of a sub-Antarctic soil contaminated by crude or diesel oils.

1 MATERIALS AND METHODS

1.1 Soil

The soil was collected from the surface to a depth of about 0.2 m in an approximately 20 m^2 area located near the " Port aux Français " scientific French station in the Kerguelen Archipelago (49°21'S, 70°13'E). This area has no known history of hydrocarbon contamination. A water content of 40% (w/w) was determined from measurement of the weight loss after heat treatment (24 h at 60°C). The soil pH was 6.4 (determined after mixing 1 part of soil with 2.5 parts of sterile water). The organic matter content was 340 g/kg (dry weight). Sieve analysis gave nearly 2.3% fine ground (< 40-63 μm), 22.6% foam ground (63-250 μm), 36% medium ground (250-800 μm) and 22.3% coarse ground material (800-2000 μm), as well as 16.8% gravel and plant residues (2000-3150 μm). After removal of plant residues and soil aeration, 5 kg of soil was placed in each mesocosm.

1.2 Incubation Experiment

Mesocosm experiments were conducted in containers of dimensions $27 \times 24 \times 13$ cm. The artificially contaminated soil was prepared by direct application of hydrocarbons (diesel or crude oil). For each temperature of incubation (4, 10 and 20°C), six conditions were used: control, Inipol EAP 22 (50 ml), diesel oil (100 ml), diesel oil (100 ml) + Inipol EAP 22 (50 ml), Arabian light crude oil, "BAL", (100 ml) and BAL (100 ml) + Inipol EAP 22 (50 ml). The mesocosms were incubated in the dark under aerobic conditions and homogenised twice a month. The changes in bacterial communities were studied during a 6-month period after contaminant addition. Sampling dates were 1, 3, 6 and 9 weeks and 3, 5 and 6 months. Triplicate samples aseptically collected in the surface layer of the soil (from surface to 2 cm under the surface) allowed a regular survey of total, saprophytic and hydrocarbon-degrading bacteria.

1.3 Bacteriological Counts

Total bacteria were determined by acridine orange direct count (AODC) enumerations. After coloration, total bacteria were filtered on black nucleopore filters (0.2 µm). The filters were analysed 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 were counted under oil immersion ($\times$ 1000) in a minimum of 10 randomly chosen fields.

Viable counts of aerobic saprophytic bacteria were made using the spread plate technique on Nutrient Agar 2216 (Oppenheimer and Zobell, 1952, using distilled water in place of seawater). Inoculated plates (6 replicates) were incubated for 10 days at 15°C. Saprophyte counts are representative of a small group of active bacteria that react immediately to changes in nutrient supply; thus, they are useful bacterial indicators (Delille and Bouvy, 1989; Reinheimer *et al.*, 1989).

Hydrocarbon-degrading bacteria were counted using the most probable number (MPN) method, using a basal mineral medium without carbon and supplemented with *Arabian light* crude oil (Mills *et al.*, 1978). Rezasurin (1 mg·l⁻¹) was used as a growth indicator. After inoculation (3 tubes per dilution) the tubes were incubated at 15°C for 30 days. The standard deviation calculated from 3 replicates was found $\leq 20\%$ for both CFU (Colony-Forming Unit) and MPN estimations.

2 RESULTS

There were only slight changes in total microbial abundance after contamination. The total number of microorganisms was roughly constant throughout all the mesocosm experiments *(Fig. 1, 2 and 3),* with values higher than 10^8 cells ml⁻¹ in each case.

Before contamination, the initial concentration of saprophytic bacteria was 3.2×10^6 CFU ml⁻¹. This number remained relatively constant in most of the uncontaminated mesocosms *(Fig 1)*. The only significant exception is a one order of magnitude increase observed in the Inipol amended mesocosm incubated at 20°C. Positive responses of saprophytic bacterial assemblages to crude and diesel oil contamination were observed during the course of the experiment *(Fig. 2* and *3)*. An increase greater than one order of magnitude in heterotrophic bacterial abundance could occur after contamination. The largest increases were observed in diesel oil + Inipol contaminated samples *(Fig. 2)*.

Prior to contamination, the number of hydrocarbon-utilising microorganisms was estimated at 2.7×10^5 MPN ml⁻¹. Even in the absence of diesel or crude oil contaminants, Inipol amendment induced an increase in numbers of these micro-organisms *(Fig. 1)*. A significant increase in hydrocarbon-utilising bacteria was observed within the first month of oil addition, with no apparent lag phase except for 3 batches incubated at 10°C (diesel, BAL and BAL + Inipol). At the end of the experiment, the hydrocarbon-utilising microbial numbers in diesel oil-contaminated soil were two orders of magnitude higher than those in the pristine soil (4.6×10^7 *versus* 1.4×10^5 MPN g⁻¹) while, in the crude oil contaminated soil, they were three orders of magnitude higher (2.8×10^8 *versus* 2.1×10^5 MPN g⁻¹). However, bacterial growth seemed to become limited at the end of the mesocosm experiment since maximum values were often observed after 100 days of contamination.

Temperature elevation had a slight and irregular impact on saprophytic assemblage. It had a positive effect in the Inipol amended mesocosm, a negative effect in the crude oil contaminated mesocosms and no visible influence in the other mesocosms *(Fig. 4)*. In contrast, temperature increases induced a one order of magnitude increase in hydrocarbon-degrading microbial numbers in diesel and crude oil contaminated mesocosms *(Fig. 5)*. The optimum temperature for hydrocarbon-utilising microorganisms was between 4 and 10°C in the diesel oil-contaminated mesocosm and between 10 and 20°C in the crude oil contaminated mesocosm.

Except for the "diesel mesocosm" incubated at 4°C, fertilizer addition (Inipol EAP 22) had no clear effect on hydrocarbon-utilising bacteria in the contaminated micro-cosms *(Fig. 5)*. Nevertheless, Inipol seemed to slightly stimulate heterotrophic growth in diesel oil-contaminated and uncontaminated soils *(Fig. 4)*.

DISCUSSION

The present observations demonstrate the clear stimulating effect of oil addition on indigenous bacteria in sub-Antarctic soil. A two and three-orders of magnitude increase in
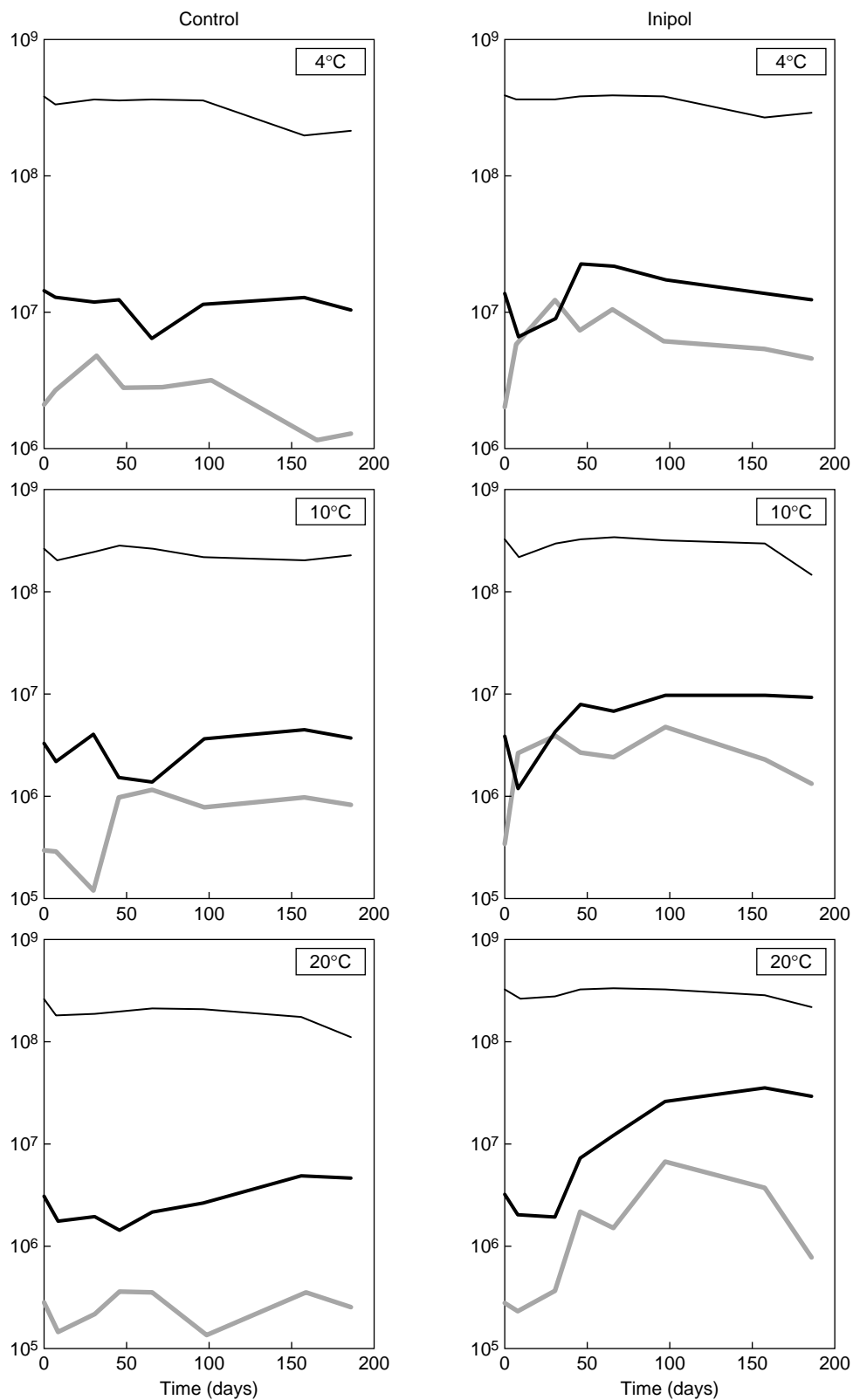
Figure 1

Changes of microflora composition during incubation of the uncontaminated mesocosms (thin line: total cells in cell ml^{-1}, bold line: viable cells in CFU ml^{-1}, gray line: hydrocarbon-degrading cells in MPN ml^{-1}).
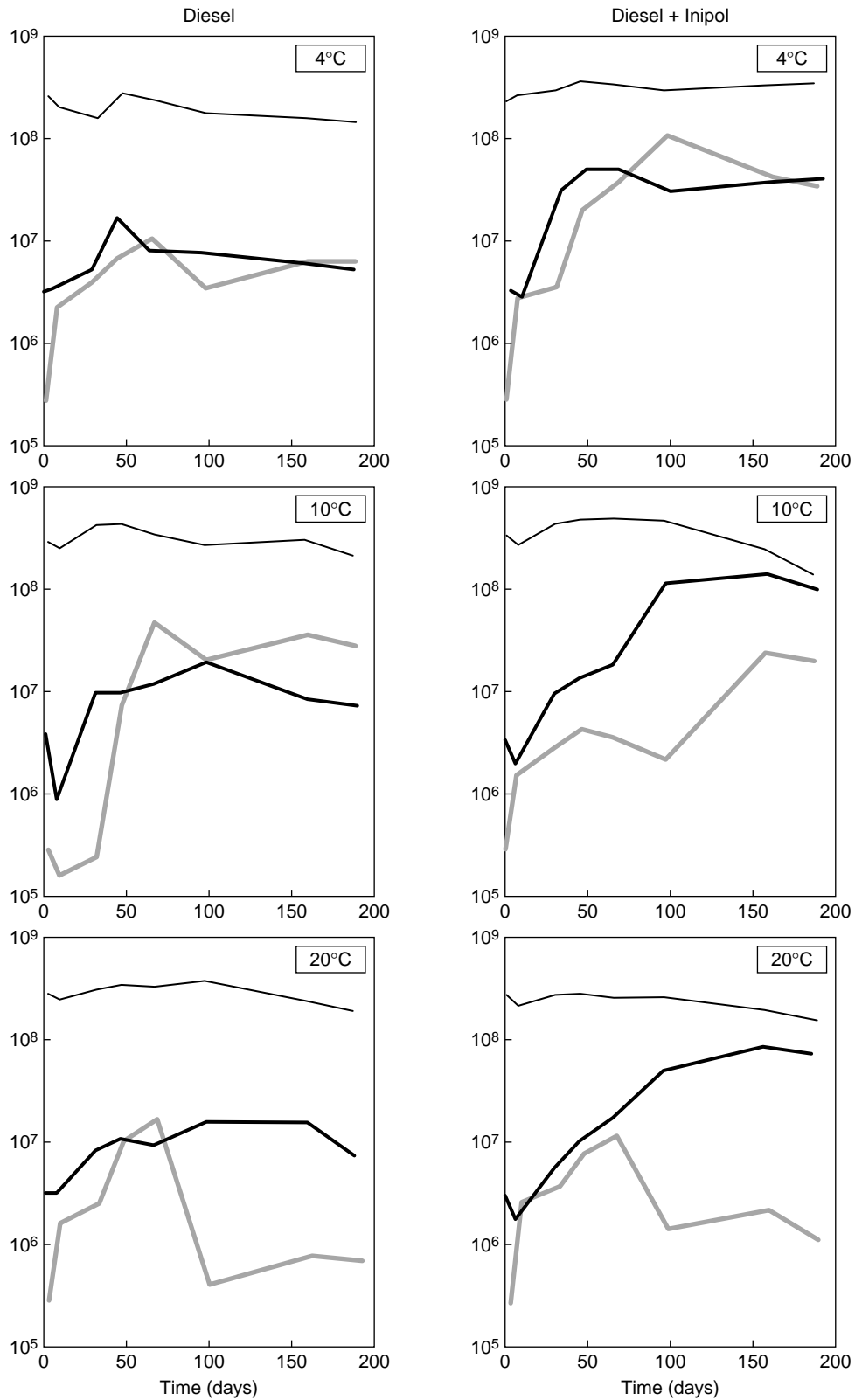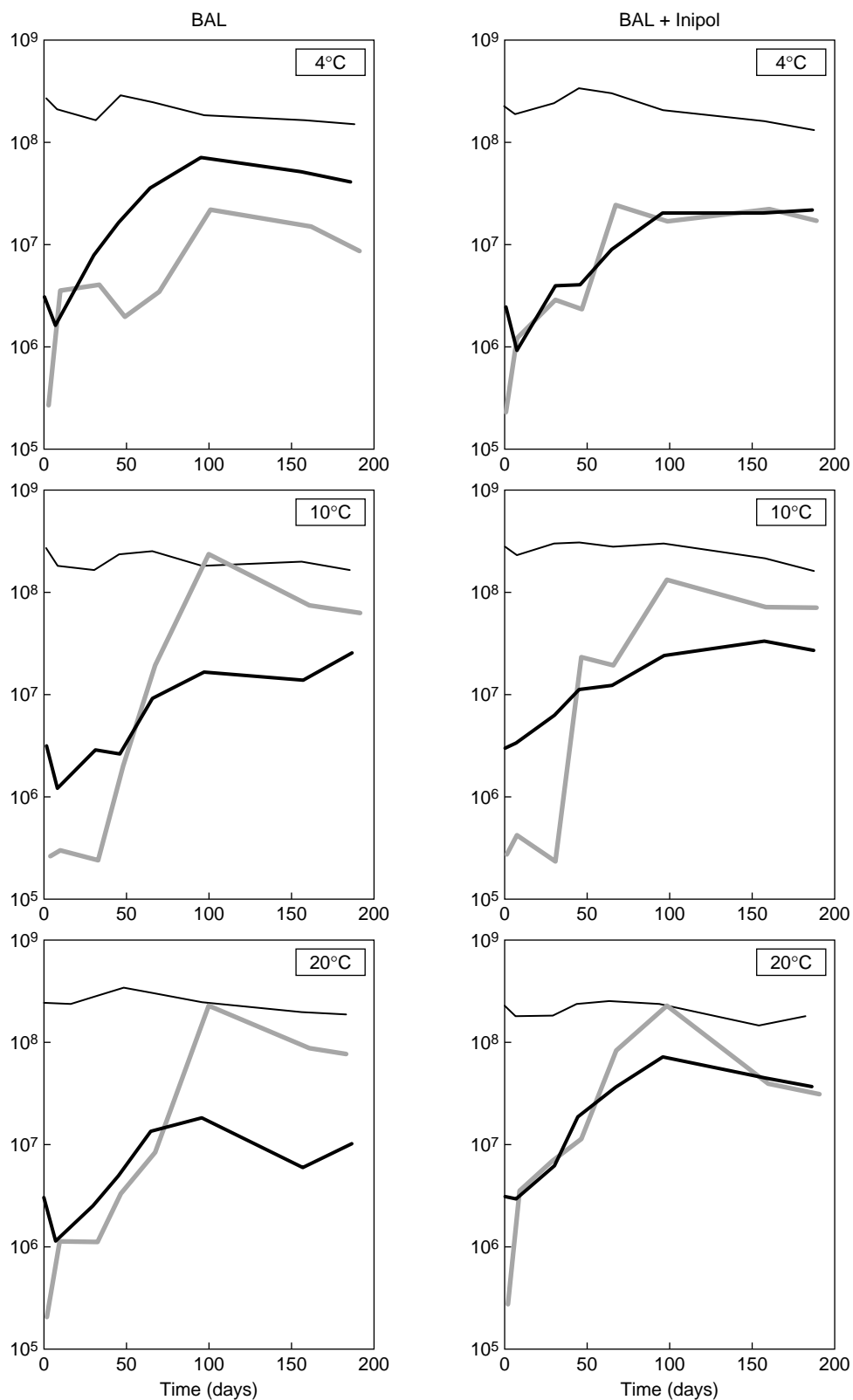
Figure 2

Changes of microflora composition during incubation of the diesel oil-contaminated mesocosms (thin line: total cells in cell mL^{-1}; bold line: viable cells in CFU ml^{-1}; gray line: hydrocarbon-degrading cells in MPN ml^{-1}).

Figure 3

Changes of microflora composition during incubation of the crude oil contaminated mesocosms (thin line: total cells in cell ml^{-1}; bold line: viable cells in CFU ml^{-1}; gray line: hydrocarbon-degrading cells in MPN ml^{-1}).
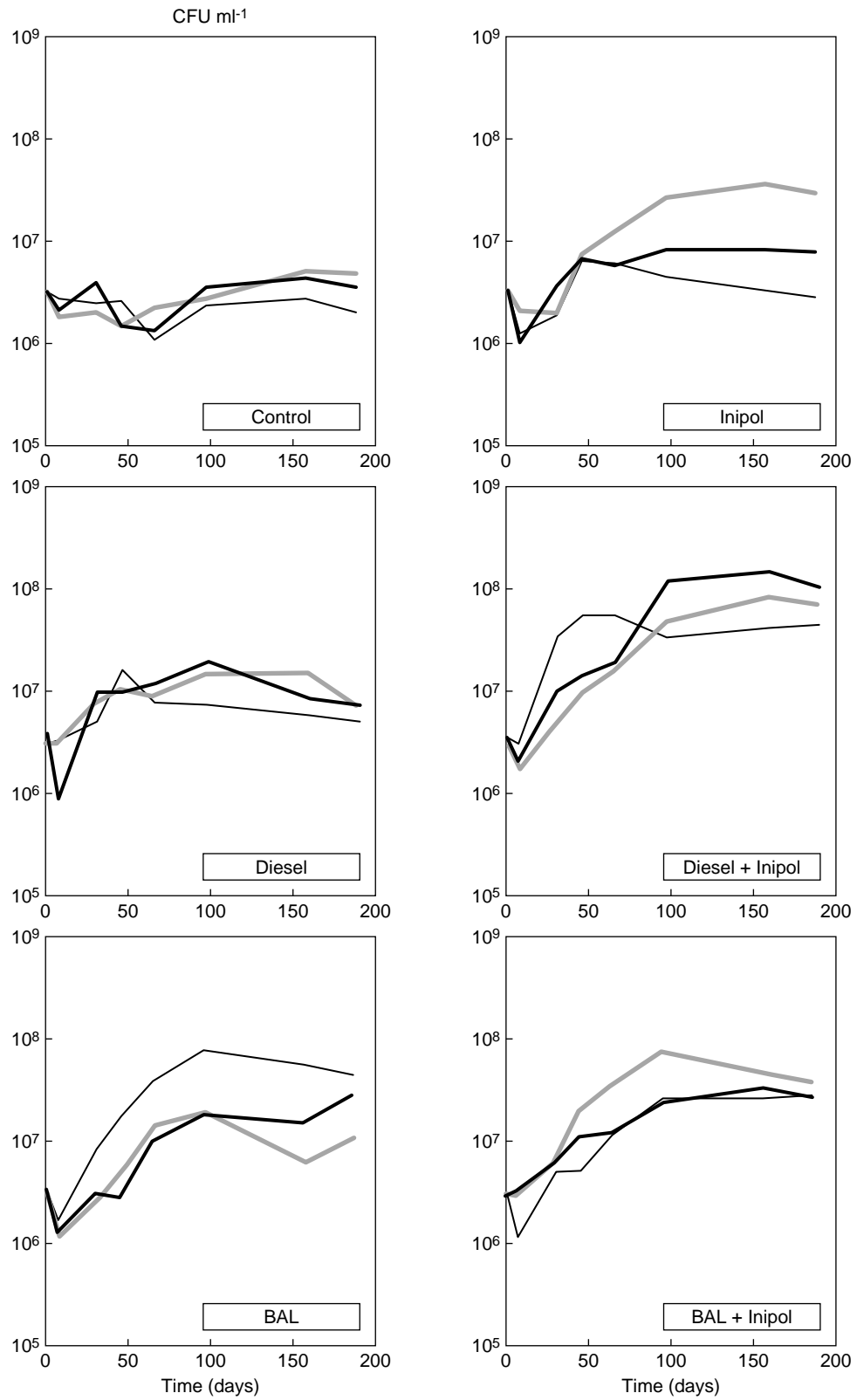
CFU ml⁻¹

Figure 4

Temperature effect on the abundance of saprophytic microorganisms (thin line: 4°C; bold line: 10°C; gray line: 20°C).
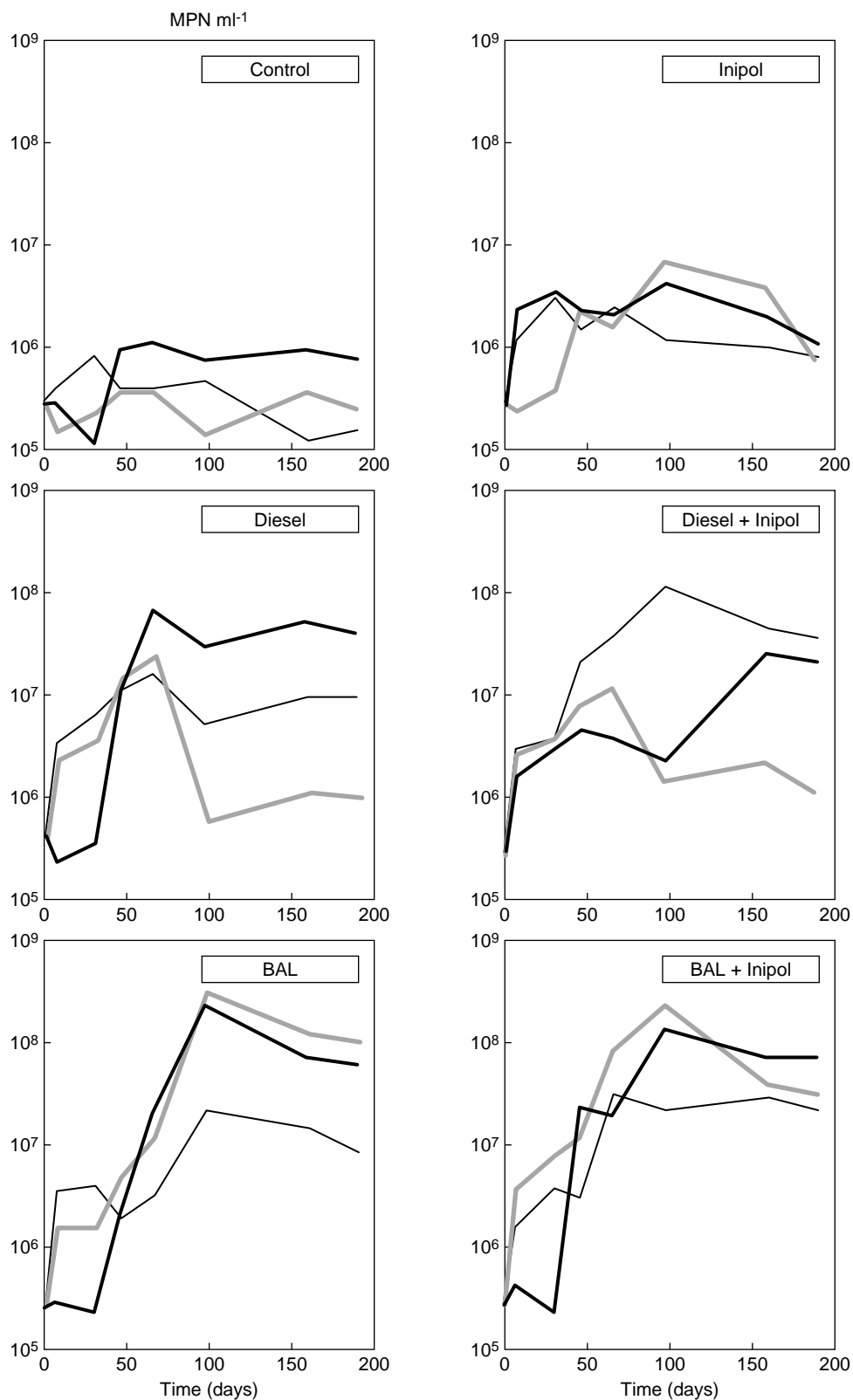
MPN ml^{-1}

Figure 5

Temperature effect on the abundance of hydrocarbon-degrading microorganisms (thin line: 4°C; bold line: 10°C; gray line: 20°C).

bacterial abundance occurred after diesel and crude oil addition, respectively. The hydrocarbon-degrading microbial numbers were significantly higher in contaminated than reference mesocosms, indicating a fast acclimatization of the resident microbial communities. These observations are in good agreement with previous results obtained from *in situ* studies in sub-Antarctic soils (Crozet Island) (Delille *et al.*, 2001).

Microbial metabolism is usually considered as a direct function of the temperature of the environment (Bossert and Bartha, 1984; Leahy and Colwell, 1990). However, some previous studies have shown that temperature has only a minor influence on bacterial growth in south polar areas (Dellile and Perret, 1989; Delille and Vaillant, 1990). Results of the present mesocosm experiments indicate that a temperature increase can stimulate the microbial development in sub-Antarctic soils. However, one exception can be noted (diesel oil + Inipol mesocosm) where the optimum temperature was below 20°C. It is reasonable to assume that the limiting factor was the temperature characteristics of the enzymes that carry out the initial oxidative steps (Floodgate, 1995). In the present study, similar bacterial numbers were observed at three different temperatures. Such observations reflect the adaptation of the indigenous soil microorganisms to the low temperatures that prevail in the sub-Antarctic environment. These temperatures are significantly below the optimum for mesophilic microorganisms. Cold adapted biodegraders could be of particular importance for bioremediation treatments of environments where low temperatures prevail.

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, stimulating effects detected in a control batch after Inipol addition was always significantly lower than the corresponding increases observed after treatment with contaminants.

Adding surfactants to soil contaminated with hydrophobic contaminants may increase the bioavailability of these materials to hydrocarbon-degrading microorganisms. This approach to biostimulation has been widely recognized, and many investigators have evaluated the effects of adding surfactants along with inorganic nutrients to soils (Aronstein *et al.*, 1991; Abecassis and Bartha, 1993; Rousse *et al.*, 1994). Inipol EAP 22 is a commercially available example of a product based on this approach. Although Inipol EAP 22 has been advertised primarily for marine oil spills, Abecasis and Bartha (1993) described successful application of this material in soil contaminated with diesel fuel. However, this study suggests that Inipol EAP 22 addition had no clear effect on indigenous bacteria in sub-Antarctic soil contaminated with oil hydrocarbons. It is well established that nutrients are one of the major factors limiting hydrocarbon biodegradation in soil and sea (Lee et Levy,

1989; Walker *et al.*, 1997; Santas *et al.*, 1999; Head and Swannell, 1999; Mohn and Stewart, 2000). Several studies have reported favourable effects of fertilizers on oil biodegradation at low temperatures in Arctic (Braddock *et al.*, 1997; Piotrowski and Aaserude, 1992; Whyte *et al.*, 1999; Mohn and Stewart, 2000), Alpine (Margesin and Schinner, 1997b; Margesin and Schinner, 1999) and Antarctic soils (Kerry, 1993; Wardell, 1995; Aislabie *et al.*, 1998; Delille 2000). Nevertheless, in the present study, it is not clear if fertilization has a beneficial effect. A possible reason for the inability of Inipol EAP 22 to enhance hydrocarbon-specific microbial growth is that nitrogen and phosphorus are not the major limiting factors in the experimental soil used.

A general decrease in microorganism populations was observed at the end of the experiments (generally after 3 months of contamination). As reported by Long *et al.* (1995), petroleum contaminants could exert toxic effects on the active microbial assemblages. Furthermore, during the initial steps of the biodegradation process, an important amount of contaminant is incompletely oxidized to CO_2 and H_2O, thus several oxidation products could accumulate in the soil during the course of the experiment and eventually lead to inhibition of microbial growth. It has been shown that, in the intertidal zone of the same sub-Antarctic area of the present study, residual toxicity of contaminated sediments did not enhance the oil degradation process (Delille *et al.* 2002). It appears that the less toxic material was degraded first and a large part of the toxic material remained in place for a much longer period. The rate of oil degradation was improved by bioremediation treatment, but residues with high toxicity were unchanged and biotreatment did not induce a complete disappearance of toxicants. A similar phenomenon could explain the observed final decline of the microbial assemblage during the present mesocosm study.

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