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Effectiveness of bioremediation for oil-polluted Antarctic seawater

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Abstract The effectiveness of a specific fertiliser (INIPOL EAP 22) addition on bioremediation of oil-contaminated Antarctic coastal seawater was determined in the "Terre Adelie" area. Mesocosm studies were conducted to evaluate the effects of "Arabian light" crude oil contamination on coastal bacterioplanktonic communities. After oil addition, regular surveys of the bacterial changes of the oil-contaminated seawater were performed during 5-week periods during the austral summer of 1992/1993 and 1993/1994. All results (total, saprophytic and hydrocarbon-utilising bacterial abundance) clearly revealed a significant response of Antarctic bacterial communities to hydrocarbon contamination. A 1 order of magnitude increase of bacterial microflora occurred in seawater after crude oil contamination. A concomitant enrichment in oil-degrading bacteria was generally observed, from less than 0.001% of the community in uncontaminated samples to up to 50% after 3 weeks of contamination. Addition of fertiliser (INIPOL EAP 22) induced clear enhancement of both saprophytic and hydrocarbon-utilising microflora. Chemical analysis of the residual hydrocarbon fractions confirmed that fertiliser application increased the rate of oil biodegradation.

Introduction

Biodegradation by naturally occurring populations of micro-organisms is a major mechanism for the removal of petroleum from the environment (Atlas et al. 1981; Leahy and Colwell 1990; Bragg et al. 1994). Bioremediation may be defined as a field procedure designed to increase the rate of natural degradative processes (Atlas and Cerniglia 1995; Floodgate 1995). Such treatments have been shown to stimulate the biodegradation of oil on a number of contaminated shorelines (Sveum and Ladousse 1989; Lee and Levy 1991; Bragg et al. 1994). However, little is known about hydrocarbon degradation processes in cold environments. Several observations have been made in Arctic (Horowitz and Atlas 1977; Atlas et al. 1978; Jordan et al. 1978; Sparrow et al. 1978; Griffiths et al. 1981; Horowitz et al. 1983) and Antarctic ice-free seawater (Clarke and Law 1981; Platt et al. 1981; Delille and Vaillant 1990) but relatively few data are available for ice-covered areas (Atlas et al. 1978; Cripps and Priddle 1991; Delille and Siron 1993; Siron et al. 1993, 1995; Delille et al. 1997). Most of these studies concerning ice-covered environments have been conducted in the Northern hemisphere. The shipwrecks of the supply ships *Nella Dan* and *Bahia Paraiso*, which ran aground and subsequently sank near Macquarie Island and the Antarctic Peninsula (Kennicutt et al. 1991; Karl 1992; Smith and Simpson 1995), highlighted the need for research into hydrocarbon contamination of the Southern Ocean ecosystem which has been, until relatively recently, almost uncontaminated by anthropogenic hydrocarbons (Platt and Mackie 1980; Clarke and Law 1981; Reinhardt and VanVleet 1986; Cripps 1990, 1992; Berkman 1992). The objective of this study was to investigate the consequences of crude oil contamination on total and specific bacterial communities in Antarctic seawater and to evaluate the benefit of fertiliser (INIPOL EAP 22) addition for the growth of indigenous microflora. Experimental studies were performed using 220-l tanks since it is very difficult to carry out such an investigation under natural field conditions. Hobbie and Wakeham (1988) have pointed out the usefulness of mesocosms for the study of the degradation of complex substrates, such as crude oil, spilled into the aquatic environment.

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Because numerous factors control enzyme-mediated degradation mechanisms (Lee and Levy 1989; Leahy and Colwell 1990; Atlas and Bartha 1992; Siron et al. 1995), it is still difficult to predict biodegradation rates under various environmental conditions encountered in the natural environment (Mackay and McAuliffe 1988). However, measurements of hydrocarbon-degrading micro-organisms are good indicators of the exposure of natural environments to hydrocarbons (Braddock et al. 1995) and their increase may indicate a stimulation of the degradation potential (Kämpfer et al. 1993).

Materials and methods

This study was conducted each year from January 1991 to December 1995 in the Géologie Archipelago (Adélie Land, 66°40′S; 140°01′E). All the experiments showed the same general trends. For clarity reasons we present only the data collected from March to April 1993 and then from December 1993 to January 1994. Batches of 220 l surface coastal seawater were exposed to natural ambient temperatures. However, to avoid the influence of UV and an increase in temperature, they were sheltered from direct sunlight. The changes in bacterial communities were studied during 40-day periods after contaminant addition. Six experiments were performed: seawater control, fertiliser (INIPOL EAP 22, 0.2 l) control, "Arabian light" crude oil (2 l), "Arabian light" crude oil (2 l) + fertiliser (INIPOL EAP 22, 0.2 l), "Arabian light" crude oil (2 l) + fertiliser (0.1 l) and "Arabian light" crude oil (2 l) + periodic addition of fertiliser (0.1 l). Periodic sampling (triplicates aseptically collected 3 cm under the surface) allowed a regular survey of total, psychrophilic saprophytic and hydrocarbon-degrading bacteria. All bacterial analyses began in the laboratory within 15 min.

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 were counted under oil immersion (× 1000) in a minimum of 10 randomly chosen fields. Cell volumes were estimated using an ocular micrometer.

Viable counts of aerobic saprophytic bacteria were made using the spread plate technique on Marine Agar 2216 (DIFCO). Inoculated plates (six replicates) were incubated for 20 days at 2°C.

Hydrocarbon-degrading bacteria were counted using the most probable number (M.P.N.) method with a basal mineral medium without carbon supplemented with "Arabian Light" crude oil (Mills et al. 1978). Rezasurin was used as a growth indicator. After inoculation (six tubes per dilution), the tubes were incubated at 12°C for 30 days. A large majority of the bacteria isolated from Antarctic seawater must be considered psychrotrophic and not truly psychrophilic strains (Delille and Perret 1989); there was no significant difference between M.P.N. counts obtained after incubation at 4°C and 20°C (Delille et al. 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 M.P.N. need 3 months at 4°C, but such a long period is not always compatible with Antarctic field work).

In order to monitor the fate of the added pollutant, at the end of the experiment the remaining hydrocarbons were carefully collected and stored at −20°C until analysis. After decantation and filtration, they were separated in four different fractions (aliphatics, aromatics, resins and asphaltenes) using a silica gel column. Analysis of aliphatic fractions was performed on a gas chromatograph (Hewlett Packard 5980) fitted with a 50-m CP SIL 5 capillary column and a flame ionization detector. The temperature profile was a start temperature of 50°C, which was kept for 5 min, and then a temperature ramp of 7°C min^{-1} until 320°C was reached. This final temperature was maintained for 15 min. Standard deviations calculated for five sample replicates performed at T60 and T40 ranged between 10 and 12%. Some quantitative ratios of alkanes and aromatic hydrocarbons were calculated from chromatographic data to characterise the weathering processes affecting the added oil. A decrease in the ratios C_{17}/pristane and C_{18}/phytane over time has been reported to reveal the bacterial degradation of oil (Blumer et al. 1973; Atlas et al. 1981).

Results

During the first experiment (March/April 1993), all the batches were rapidly covered by a thick ice layer (more than 30 cm). Seawater samples were taken under this ice cover. In contrast, the batches were always free of ice during the second experiment (December 1993 to January 1994).

The three fertiliser treatments yielded relatively similar results. Only data corresponding to a single 0.2-l addition are shown. Increases of total bacterial abundance occurred in all contaminated batches (Fig. 1). Observed increases were larger in treated than untreated batches. The maximal value of a little less than 10^7 cells ml^{-1} was reached in 1994 in the "crude oil + INIPOL"

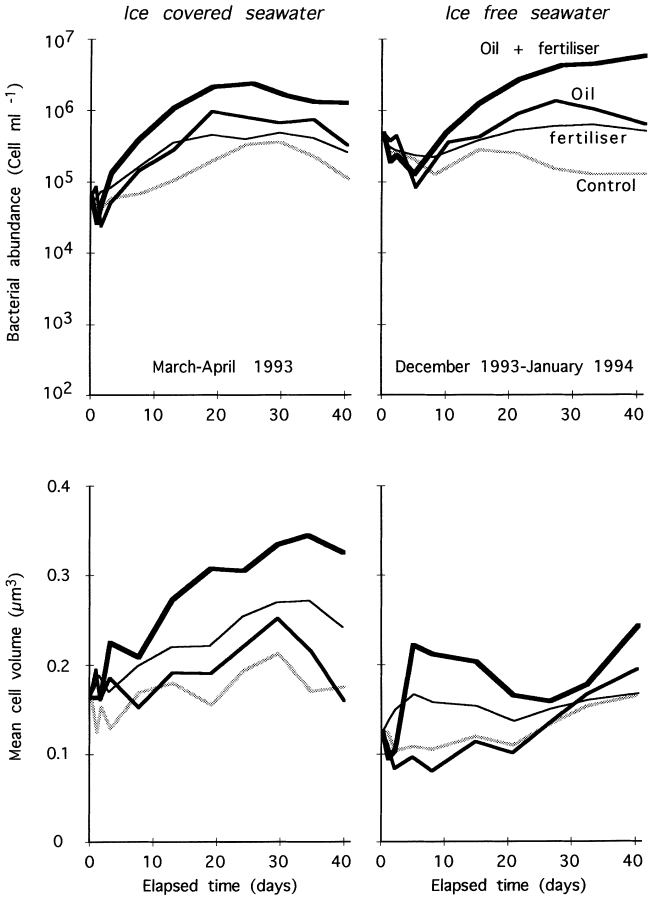

Fig. 1 Changes of total bacterial abundance and mean cell volume in experimental batches

batch. The two experiments yielded relatively similar estimates of total bacterial abundance. In contrast, the large increase of mean cell volume occurring in 1993 in the "crude oil + INIPOL" batch was severely reduced in 1994.

All the results clearly revealed a significant response of Antarctic-specific bacterial communities to hydrocarbon contamination; 3 orders of magnitude increases of saprophytic bacterial abundance occurred after crude oil and fertiliser addition (Fig. 2). The difference between treated and untreated batches can exceed 2 orders of magnitude.

A concomitant enrichment in oil-degrading bacteria was observed. With values generally lower than 10 bacteria ml^{-1}, the hydrocarbon-degrading bacteria (Fig. 3) found in seawater before treatment never represented more than 0.1% of the saprophytic assemblage. After 40 days of contamination this proportion exceeded (in some cases) 95%. The increase of hydrocarbon-degrading bacteria was higher in the second experiment than in the first. In each case, specific bacterial abundances were more than 10 times higher in treated than in untreated batches.

Chemical analyses of residual hydrocarbon fractions show a discernible decrease of the aliphatic fractions in all contaminated batches (Fig. 4). However, this decrease is more pronounced in the 1993 INIPOL-treated batch. Biodegradation rates deduced from the C_{17}/pristane and C_{18}/phytane ratios also show a relatively small but noticeable increase of biodegradation in the INIPOL-treated batches.

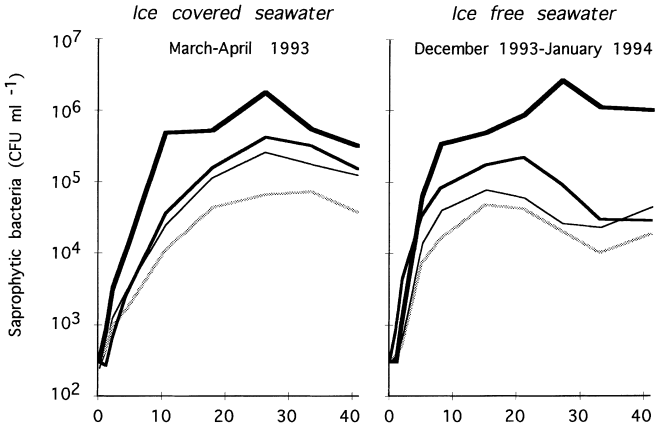

Fig. 2 Changes of saprophytic bacterial abundance in experimental batches

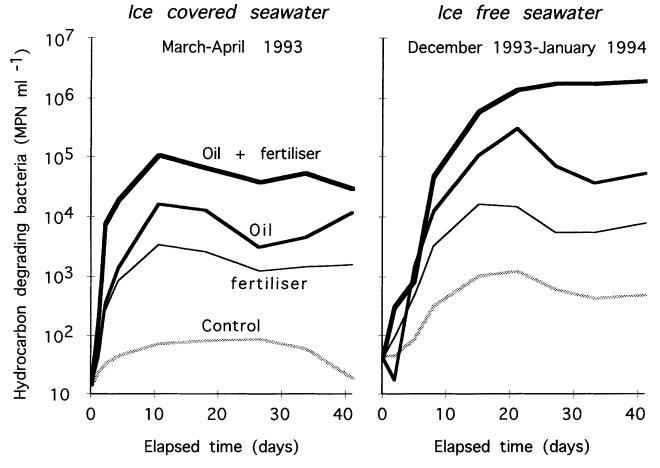

Fig. 3 Changes of hydrocarbon-degrading bacterial abundance in experimental batches

Discussion

Previous work in the Antarctic has shown that sea-ice bacteria actively grow and assimilate dissolved organic substrates (McConville and Wetherbee 1983; Grossi

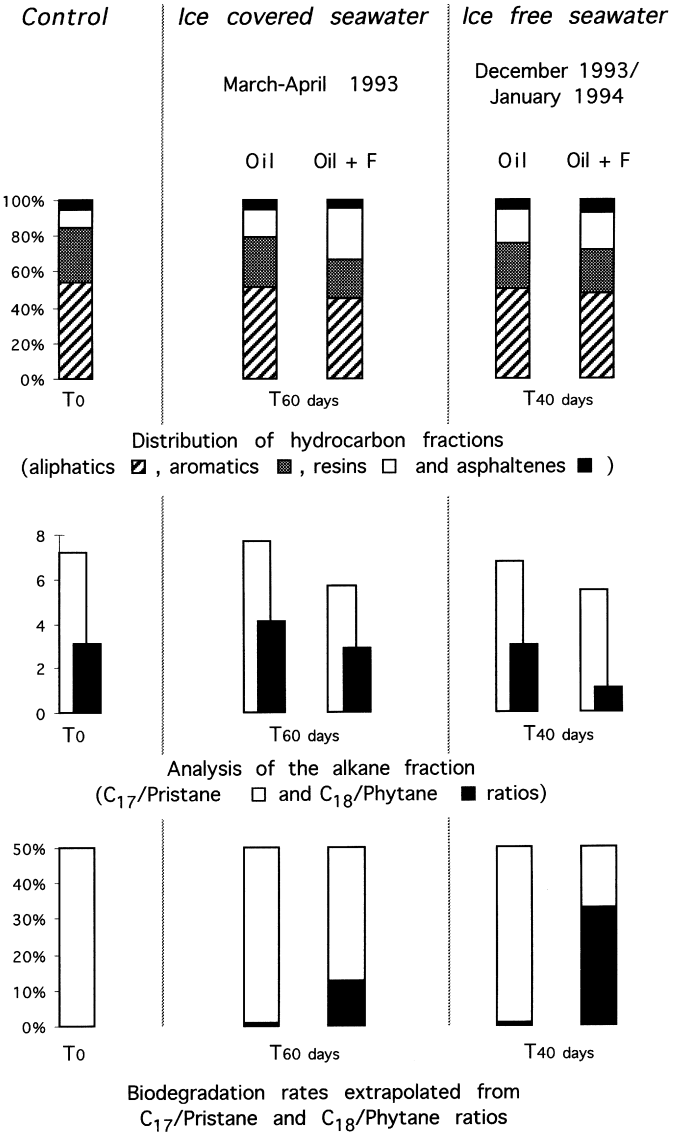

Fig. 4 Changes of biodegradation indicators in experimental batches

et al. 1984; Kottmeier and Sullivan 1987; Kottmeier et al. 1987; Grossmann 1994). The presence of sea ice during the first experiment did not inhibit the development of total and specific bacterial communities in underlying seawater. This is in agreement with previous studies conducted *in situ* at the same location (Delille et al. 1997). The differences observed between cell volume changes occurring during the two experiments could be related to sea ice formation during the first study. Bacteria in sea ice have been reported to be generally larger than those found in seawater (Marra et al. 1982; Delille 1992; Palmisano and Garrison 1993), which may be a reflection of generally higher organic nutrient concentrations in the ice environment (Marra et al. 1982; Sullivan 1985), lower temperature (Wiebe et al. 1992) or lower grazing pressure (Turley et al. 1986; Gonzales et al. 1990; Grossmann and Dieckmann 1994). In any case, addition of fertiliser strongly enhanced this phenomenon.

All results revealed a clear response of Antarctic microbial communities to hydrocarbon contamination. Two orders of magnitude increases of total bacterial abundance were observed after crude oil contamination with added fertiliser.

The biodegradation of hazardous substances is often limited by the antimicrobial action of the pollutants (Heipieper et al. 1992). The potential toxicity of the water-soluble oil fraction to marine bacteria has been demonstrated (Hodson et al. 1977; Griffiths et al. 1981). Such inhibiting effects against bacterial communities seem relatively uncommon. They appear to be very weak and occur only in the early stages of experiments.

It is well established that nutrients are one of the major limiting factors of hydrocarbon biodegradation at sea (Lee and Levy 1989). In this survey the bacterial growth was doubtless improved by the availability of mineral nutrients released by INIPOL EAP 22. As noted by Rivet et al. (1993), some increases in bacterial numbers after INIPOL addition may be attributed to the bacteria growing on the oleic acid contained by the fertiliser. However, stimulating effects detected in control batches after INIPOL addition were always significantly lower than the corresponding increases observed after treatment of contaminants. The considerable enrichment in hydrocarbon-degrading bacteria after a few days of crude oil contamination is a clear indication of possible biodegradation. This is consistent with chemical analysis of residual hydrocarbons, which demonstrated a decrease of the aliphatic fraction. In the studied area temperature has a rather limited influence on bacterial growth (Delille and Perret 1989). Nevertheless, as reported by Siron et al. (1993) under similar conditions of very low temperature, the rates of biodegradation observed during this study were relatively slight. 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). However, biodegradation indicators were weak but perceptible. Long term studies conducted after the *Exxon Valdez* oil spill have shown that pristane and phytane can be biodegraded (Bragg et al. 1994). However, we used only very short investigation times. Furthermore, a potential degradation of these two markers would only increase the estimated biodegradation rates, which correspond to relative but minimum values. The present data demonstrate that bioremediation may be a useful tool in more severe environmental conditions than those of Alaska where its effectiveness has now been clearly established (Bragg et al. 1994; Atlas and Cerniglia 1995).

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