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The influence of crude oil on the growth of subantarctic marine bacteria

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Abstract: The short term degradation of 'Arabian light' crude oil was followed under various seasonal conditions in coastal seawater at Iles Kerguelen. Artificial degradation experiments were carried out in 3 m^3 tanks. *In situ* experiments were conducted in free-floating, semi-enclosed chambers permitting direct contact between the crude oil and the marine environment. Daily sampling allowed a regular survey of the bacterial changes of the oil contaminated seawater. All samples were analysed for total bacteria, heterotrophic viable microflora and hydrocarbon utilizing microflora. At the end of experiments, the remaining oil was carefully collected for rough quantitative estimation of hydrocarbon degradation. All the results clearly revealed a significant increase in the three types of bacterial microflora after the addition of crude oil. However, the data suggest that the initial state of the bacterial communities is important. Thus, the seasonal variations in the bacterial responses to hydrocarbon addition can be related to the differences in the natural bacterial populations involved. In all cases the wall effects observed in batch systems were reduced with *in situ* incubations.

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Introduction

Relatively little is known about crude oil degradation processes in cold environments. Several observations have been made in Arctic areas (Horowitz & Atlas 1977, Atlas *et al.* 1978, Sparrow *et al* 1978, Jordan *et al.* 1978, Griffiths *et al.* 1981, Horowitz *et al.* 1983) but few for southern polar areas (Clarke & Law 1981, Platt *et al* 1981). The major contribution of bacteria to hydrocarbon decomposition is unquestionable. Numerous studies deal with the impact of bacteria on petroleum hydrocarbon degradation, but only a few of them deal with the possible regulating effect of hydrocarbons on natural bacteria (McKinley *et al.* 1982). The aim of the present paper is to address this question using subantarctic seawater microbiota.

Materials and Methods

The study was carried out between January 1982 and January 1983 in Morbihan Bay, Iles Kerguelen. Located in the south east of the archipelago, Morbihan Bay (about 30 x 20 km) opens to the Indian Ocean through Royal Pass which is 12 km wide and 40 m deep. The Bay is always free of ice. Batch experiments were carried out on shore near the 'Port aux Français' marine laboratory (Fig. 1, station A). *In situ* incubation systems reduce the confinement effects in batch experiments (Menzel & Case 1977, Tagger *et al* 1983). These studies were conducted in a small sheltered bay (Fig. 1, station B).

Experimental studies

Batch experiments were carried out in 3 m^3 tanks (cylinders of 150 cm diameter and 170 cm depth) exposed to the natural environmental conditions. The tanks were filled with coastal seawater and incubated for eight days at ambient temperature (ranging from 1°C in winter to 8°C in summer). Three tanks were used as replicates and one was used as a control. *In situ* studies were conducted in four free-floating open bottom chambers (rigid cylinders of 60 cm diameter and 90 cm depth) permitting direct contact between the crude oil and the underlying seawater column eight metres deep. Three of the chambers were used as replicates and one was used as a control.

Both sets of experiments were conducted in the same manner but not simultaneously. Initially based on a monthly interval, the timing of the seasonal experiments was greatly influenced by the weather conditions. Experiments started with an addition of a quantity of crude oil (fresh 'Arabian light- oil, provided by ELF-ERAP Society and stocked in sealed containers) corresponding to a thickness of 500 µm (i.e. 500 ml m^{-2}). Daily sampling allowed a regular survey of the bacterial microflora (subsamples taken 2 cm under the surface with a sterile glass pipette). At the end of the

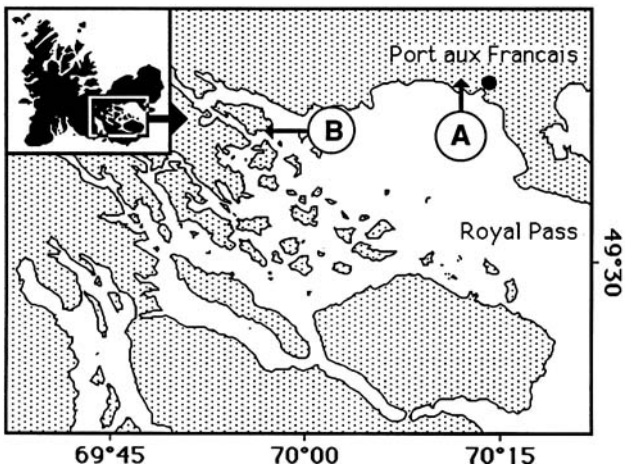

Fig. 1. Map of the Morbihan Bay (Iles Kerguelen) showing the two experimental sites, a: batch systems, b: *in situ*.

experiments the remaining oil was carefully collected and mass determined by gravimetric analysis after dehydration at room temperature.

Determination of bacterial parameters

Total bacteria (A.O.D.C.) were determined by acridine orange direct counts (Hobbie *et al.* 1977). Viable heterotrophic bacteria (V.C.) were counted using the M.P.N. method with 2216 E medium (Oppenheimer & Zobell 1952). After inoculation the six tubes per dilution were incubated at 18°C for 20 days. A majority of the bacteria isolated from subantarctic seawater must be considered psychrotrophic and not truly psychrophilic strains (Delille & Perret 1989). There was no significant difference between Antarctic and subantarctic viable counts obtained after incubation at 4°C and 20°C (Delille *et al.* 1988, Delille & Perret 1989). Thus, the relatively high incubation temperature used in the present study did not bias the data and allowed a substantial reduction in the usual incubation time needed (three months) at 4°C on this medium. Hydrocarbon utilizing bacteria were estimated in the same way using an 'Arabian light' supplemented basal mineral medium without carbon (Mills *et al.* 1978). The precision of the bacteriological counts, based on five replicates during two periods of the year (winter and summer), was 10% for direct counts and 15% for viable counts in logarithmic values (Bouvy 1985, Imbaud 1987). The relative microheterotrophic activity was estimated on duplicates using the single concentration technique (Griffiths *et al.* 1977). Ten ml subsamples of seawater were incubated with 15 µl of either [U-^{14}C] glucose or [U-^{14}C] glutamic acid (1 µCi m^{-1}, specific activity: 250 mCi mM^{-1}). Incubations (2 hours) were carried out in the dark at the *in situ* temperature. The total potential heterotrophic uptake was estimated as the sum of respired and assimilated fractions (Cahet *et al.* 1986). Results were expressed as percentage of initially injected radio-labelled compound (Bouvy 1989).

Results

The time course of changes in bacterial counts during hydrocarbon degradation experiments are shown in Fig. 2 (batch cultures) and Fig. 3 (*in situ* incubation). Addition of crude oil always induced a sharp increase in bacterial numbers. Only very short lag times were observed in the increase in heterotrophic bacterial populations (total and hydrocarbon specialised). Direct count increases appeared later and were often much smaller than viable count increases. Seasonal changes were easily discernible during *in situ* incubations. The largest bacterial response occurred in February, and the smallest responses were observed in July and September. The same seasonal pattern was observed in batch cultures but it was much reduced. The time course of changes in potential microheterotrophic activities are shown in Fig. 4 (batch cultures) and Fig. 5 (*in situ* incubation). These were very similar for oil-contaminated and oil-free seawater. The natural disappearance of hydrocarbons after seven days ranged from 12% in August to 87% in February in batch cultures and from 17% in October to 90% during January in *in situ* experiments.

Discussion

Both sets of experiments allowed evaporation, photo-oxidation and dissolution of the oil. Thus the natural disappearance of petroleum hydrocarbon corresponds to the sum of the physical and chemical weathering and the biodegradation. In spite of consistently low temperatures the disappearance was appreciable. The disappearance (annual mean of 59%) of crude oil from the *in situ* tanks seems greater than the disappearance from batch cultures (annual mean of 26%). Flushing effects may be responsible for the very high value of oil disappearance recorded in some of the *in situ* experiments (25 February–3 March; 24–30 March; 3–9 July). However flushing effects cannot occur in batch experiments, thus the biodegradation effect may contribute significantly to 87% of oil disappearance recorded in the batch experiment conducted in February 1982. Physical and chemical weathering may be more important than the biodegradation effects, particularly for the experiments showing a relatively low percentage of oil disappearance (between 10 and 50%)

Hydrocarbon addition always induces rapid and substantial bacterial increases. This confirms the remarkable activity of southern polar bacterial microflora reported previously (Delille & Cahet 1984. Delille *et al.* 1988). Usually the hydrocarbon addition induced a greater growth of bacteria in the batch rather than in the *in situ* experiments. This difference can probably be attributed to confinement and/or flushing effects. Confinement effects were reduced by *in situ* incubation and

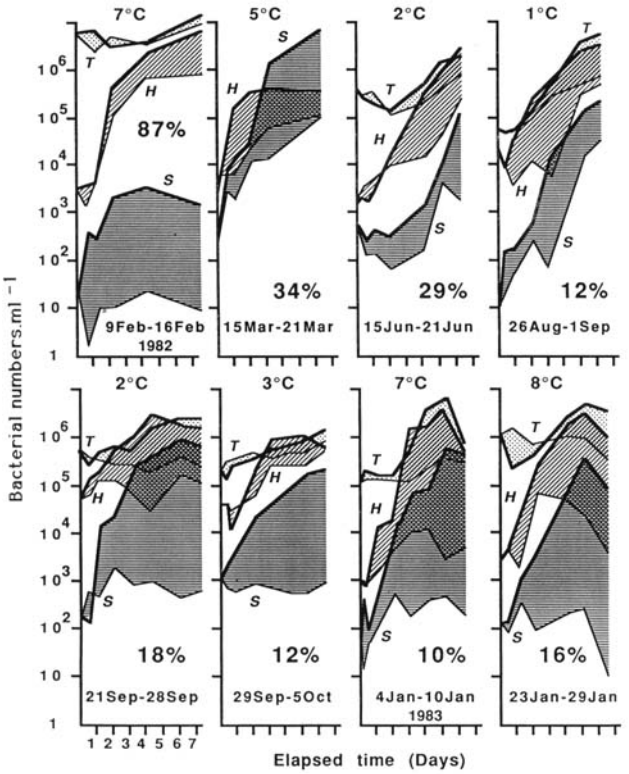

Fig. 2. Time course of changes in bacterial microflora during batch experiments. Thick line – oiled seawater; fine line – control seawater. Shadowed area shows differences between oiled and control seawater. percentage numbers indicate natural disappearance crude oil after 7 days.

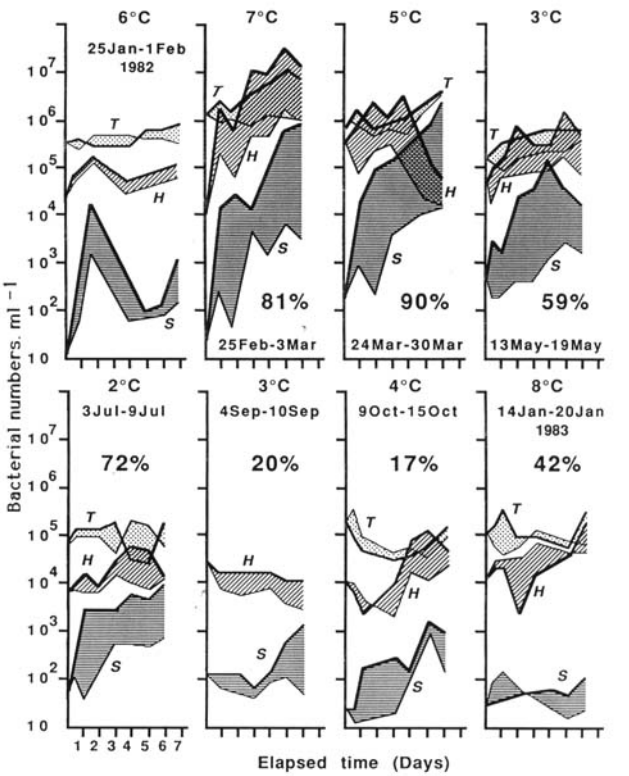

Fig. 3. Bacterial populations during *in situ* experiments (symbols as in Fig.2).

this revealed the relatively low activity of the winter microflora which was not discernible in batch cultures.

Total direct counts reflect the actual bacterial abundance but their variations in time were less pronounced than those of other bacteriological parameters. During growth periods the quantitative difference between direct and viable counts showed a marked decline. Similar findings have been previously reported (Bouvy *et al.* 1986,Delille 1987) and discussed (Delille & Bouvy 1989). Exceptional values of viable counts higher than direct counts were recorded in this study (September and October for batch cultures, March, May and October for *in situ* experiments). These surprising observations may be explained by the presence, in some growth conditions, of extremely small active bacteria. The existence of such bacteria with diameters < 0.2 µm was confirmed by positive results of viable counts conducted after 0.2 µm filtration of the inoculum.

With only a few exceptions (February, March and September for batch cultures, 25 February–3 March for *in situ* experiments) hydrocarbon addition seems to have had no measurable influence on potential microheterotrophic activity. Oil degrading metabolism is not necessarily linked with glucose or glutamate metabolism. Labelled hexadecane and naphthalene were also tried. Unfortunately, field conditions did not allow us to obtain a complete set of data with these compounds.

Seasonal variations of natural marine subantarctic bacterial microflora have been previously observed (Delille 1977. Delille & Cahet 1985, Bouvy & Delille 1988). Results from the winter batch experiments showing sharp bacterial increases seem to indicate, as reported earlier (Delille *et al.* 1988, Delille & Perret 1989), that temperature has only a minor influence on bacterial growth. However temperature could play an important indirect role in crude oil degradation as suggested by the low percentage of natural disappearance reported in winter in spite of sharp increases of bacterial microflora in batch cultures. The rate at which petroleum hydrocarbons are degraded depends largely on how many bacteria are present at the start. In batch culture the largest initial bacterial population (experiment of February 1982 with more than 10^6 cells ml^{-1}) had a degradation percentage more than twice as great as that observed in other batch experiments. Similarly the *in situ* incubation (24 March-30 March) conducted with the more active heterotrophic initial population (activity indicated by the low direct counts/ viable counts ratio) corresponds to the largest degradation

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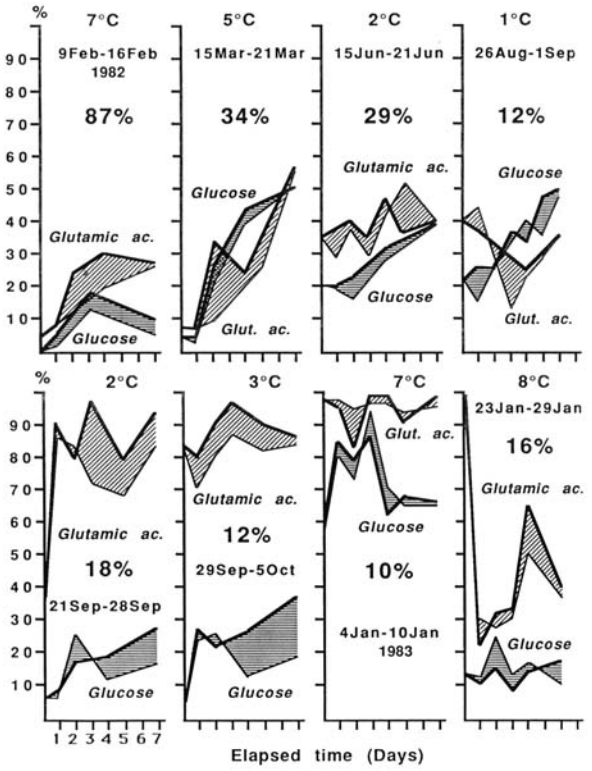

Fig. 4. Changes in potential microheterotrophic activities during batch experiments (symbols as in Fig. 2).

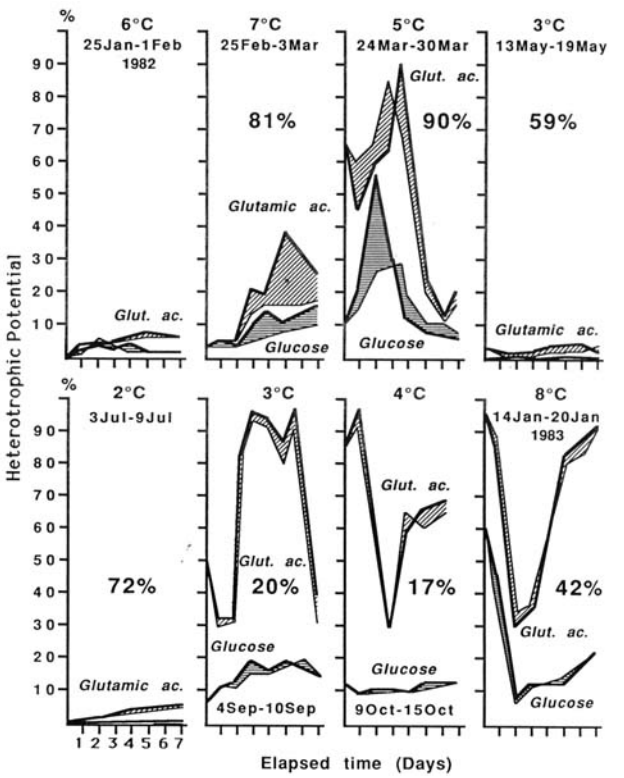

Fig. 5. Changes in potential microheterotrophic activities during *in situ* experiments (symbols as in Fig 2).

percentage recorded *in situ* . The species composition and particularly the initial richness in oleoclastic bacteria may also be important and should be the focus of future investigations.

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