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# Field observations on the variability of crude oil impact on indigenous hydrocarbon-degrading bacteria from sub-Antarctic intertidal sediments

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**Abstract**

Oil pollution of the oceans has been a problem ever since man began to use fossil fuels. Biodegradation by naturally occurring populations of micro-organisms is a major mechanism for the removal of petroleum from the environment. To examine the effects of crude oil pollution on intertidal bacteria, we repeated the same contamination experiments on nine different sub-Antarctic intertidal beaches using specifically built enclosures (PVC pipe, 15 cm in inner diameter and 30 cm in height). Despite the pristine environmental conditions, significant numbers of indigenous hydrocarbon-degrading bacteria were observed in all the studied beaches. Introduction of oil into these previously oil-free environments resulted in several orders of magnitude of increase in hydrocarbon-degrading micro-organisms within a few days in some of the studied sites but has no obvious effects on two others. The physical environment of the bacterial assemblage seems to play a major role in the biodegradation capacities. After 3 months of contamination, both remaining oil concentrations and biodegradation indexes differ strongly between the different stations. Thus, chemical and biological parameters reveal a strong heterogeneity of biodegradation capacities between the different sites. 

*Keywords:* Antarctica; Oil contamination; Hydrocarbonoclastic bacteria; Biodegradation; Intertidal zone

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## 1. Introduction

Antarctica is one of the largest and most pristine wilderness areas left on Earth. The main human activities undertaken in the region are scientific research, tourism and fishing (Cripps & Shears, 1997). All of these activities require fossil fuel for transport and energy requirements. Such increasing use of petroleum hydrocarbons has led to an increase in the probability of major spillages contaminating terrestrial and aquatic environments. Oil pollution of oceans and coastal environments has been a problem ever since man began to use fossil fuels. As pointed out by Minas and Gunkel (1995), since hydrocarbons are natural products, it is not surprising to find organisms that are able to degrade these energy-rich substrates. While it is recognized that micro-organisms play a critical role in the breakdown of hydrocarbons, the impact of petroleum pollutants on the metabolism and abundance of natural microbial communities is poorly understood (Bartha & Atlas, 1987). Petroleum hydrocarbons, for example, have been shown to enhance (Bunch, 1987), reduce (Griffiths, Caldwell, Broich & Morita, 1981) or have no effect (Bauer & Capone, 1985; Carman, Means & Pomarico, 1996; Wyndham, 1985) on total abundance of sedimentary bacteria. Bacterial communities vary considerably in their metabolic response to hydrocarbons (Alexander & Schwarz, 1980). Therefore, measurements of microbial populations are an important component of contaminated site assessment studies (Braddock, Lindstrom & Brown, 1995).

Increased attention to pollution control has led to a greater demand for remediation technologies. Bioremediation may be defined as a field procedure designed to increase the rate of natural cleaning processes (Atlas & Cerniglia, 1995; Delille, Bassères & Dessommes, 1998; Floodgate, 1995). Most of the strategies proposed generally try to modify the factors known to limit biodegradation rates. Inoculation with hydrocarbon-degrading micro-organisms (Liu & Suflita, 1993) and/or addition of nutrients are the most studied. Nutrient addition has been shown to stimulate the biodegradation of oil on a number of contaminated shorelines (Bragg, Prince, Harner & Atlas, 1994; Delille, Bassères & Dessommes, 1997; Lee & Levy, 1991; Pritchard & Costa, 1991; Rosenburg, Legmann, Kushmaro, Taube, Adler & Ron, 1992; Sveum & Ladousse, 1989). However, the major problem in the analysis of the results of such treatments is the complexity of the physical and biological processes involved in oil biodegradation. Unknown and uncontrollable variables (unidirectional along-shore currents, spatially distinct underground flows, prevailing winds, etc.) may impart bias in experimental design (Venosa et al., 1996). This report is part of a study in which mesocosm experiments were performed to examine the effects of petroleum contaminants on the benthic food web of a sub-Antarctic intertidal sediment. Its main aim was to consider the spatial variability of the physical and bacteriological responses of intertidal beaches to crude oil contamination. Future papers will examine the impact of crude and diesel oil contamination and bioremediation treatments on bacterial production, microalgal abundance and meiofaunal community structure.

## 2. Materials and methods

### 2.1. Study sites

Short-term experiments were conducted on nine different beaches located in Kerguelen Archipelago (Fig. 1). All these beaches were located in relatively sheltered areas; general information concerning these beaches is given in Table 1 and Fig. 2. Except for the station 1, located in the vicinity of the permanent base of Port-aux-Français, all study sites are located in pristine regions with very low boat traffic and no commercial activity. These combined factors lead to a high probability that the sediments have never experienced chronic exposure to either refined or crude hydrocarbons.

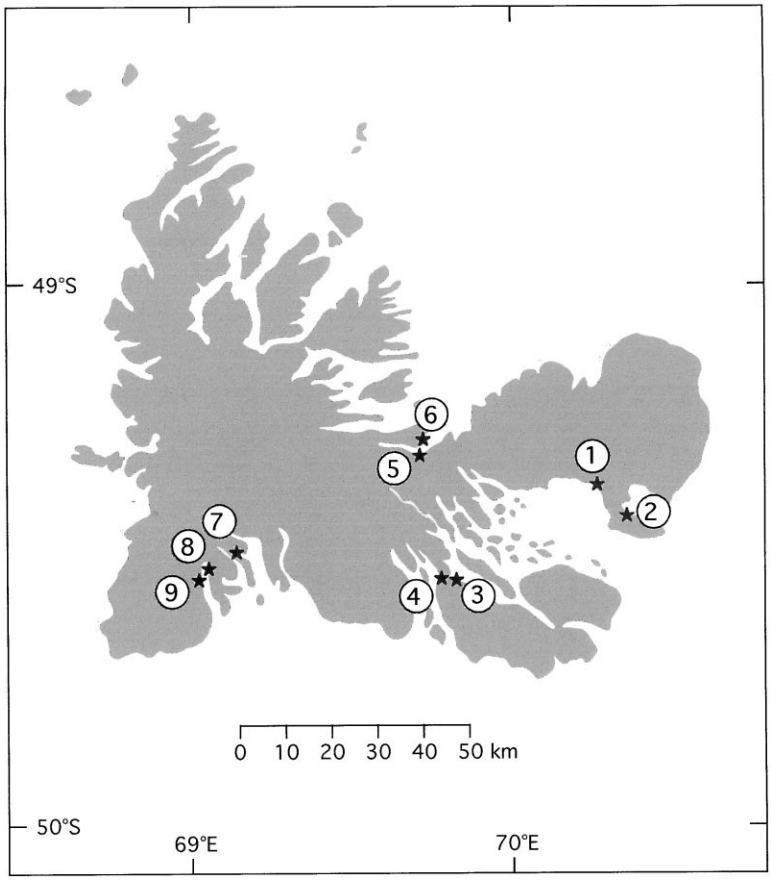


Fig. 1. Map of the Kerguelen Archipelago showing the location of the nine studied sites. 1, Aurore Australe bay; 2, Norwegian bay; 3, Jeanne d'Arc harbour; 4, Halage cove; 5, Sandy cove; 6, Couvreux harbour; 7, Table bay; 8, Mouche bay; 9, Cottage cove.

Table 1
General description of the nine studied beaches

| Site No. | Location | General exposure[a] | Wave exposure | Median grain size (μm) |
|---|---|---|---|---|
| 1 | Aurore Australe bay | West | Very high | 118 |
| 2 | Norwegian bay | Northeast | Low | 172 |
| 3 | Jeanne d'Arc harbour | East | Low | 132 |
| 4 | Halage cove | East | Very low | 240 |
| 5 | Sandy cove | East | High | 413 |
| 6 | Couvreux harbor | North | Very low | 535 |
| 7 | Table bay | South | High | 247 |
| 8 | Mouche bay | South | Low | 504 |
| 9 | Cottage cove | Southeast | High | 411 |

[a] Strong western winds are dominant in the studied area.

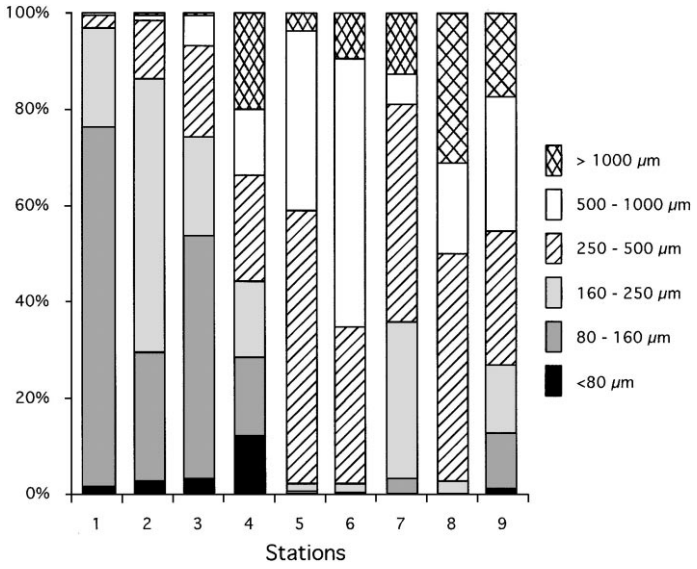


Fig. 2. Relative proportion of substratum components at the nine studied beaches.

## 2.2. Experimental design

To examine the effects of crude oil pollution on intertidal bacteriological assemblages we repeated the same experiments in the nine different sites in specifically built enclosures. The enclosures were made of PVC pipe, 15 cm in inner diameter and 30 cm in height. Approximately 20 cm of each enclosure was buried into the sediment. After addition of the contaminant, which was simply poured in from above, the unburied part of the enclosure was covered with a 200-μm plankton net. We set up two experimental treatments on each beach: (1) a short-term experiment

(5 days) with an Arabian light crude oil addition (100 ml); and (2) a long-term experiment similar to the former one but with a duration of 90 days. We used the sediments outside the enclosures as natural controls. We used three replicates of treatment and natural control at each site.

### 2.3. Bacteriological counts

Total bacteria were determined by acridine orange direct counts (AODC) on black Nuclepore filters (0.2 μm) using an Olympus BHA epifluorescence microscope according to the method of Hobbie, Daley and Jasper (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. Biovolumes were estimated using an ocular micrometer.

The number of viable psychrotrophic aerobic saprophytic micro-organisms in each sediment sample was estimated using the most probable number (MPN) technique. Saprophytic bacteria were defined as those microbes capable of growth on Marine Broth 2216 (DIFCO). After inoculation (three tubes per dilution) the tubes were incubated at 12°C for 30 days. A large majority of the bacterial strains isolated from Antarctic seawater must be considered psychrotrophic and not truly psychrophilic strains (Delille & Perret, 1989), and there was no significant difference between MPN counts obtained after incubation at 4 and 20°C (Delille, Bouvy & Cahet, 1988; Delille & 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 3 months at 4°C; such a long time is usually not compatible with Antarctic field work).

Hydrocarbon-degrading bacteria were counted using the MPN method using a basal mineral medium without carbon supplemented with Arabian Light crude oil (Mills, Breuil & Colwell, 1978). Rezasurin was used as a growth indicator. After inoculation (three tubes per dilution) the tubes were incubated at 12°C for 30 days.

The standard deviation calculated from five replicates was found to be $\leqslant 20\%$ for both MPN estimations.

### 2.4. Hydrocarbon analysis

Sediment samples were analysed for remaining petroleum hydrocarbons. Dry sediments (10 g) were extracted for 4 h with 50 ml of hexane–acetone mixture (1:1). Extracts were fractionated on deactivated silica gel (60–120 mesh). All aliphatic and aromatic fractions were analysed by gas chromatography using a 30 m×0.25 mm i.d. fused silica column (DB5) and a flame ionisation detector. Total oil present in sediments was obtained by the summation of all aliphatic hydrocarbons from C14 to C36 *n*-alkanes and all polycyclic aromatic hydrodrocarbons (PAHs). Recovery for this method was 86% for C14–C36 and 80% for PAHs, respectively (Siron, Pelletier, Delille & Roy, 1993). Gravimetric determinations of concentrations of total remaining oil are reported on sediment dry weight basis.

## 3. Results

Total bacterial biomass did not change significantly during the course of all experiments. The only noticeable observation was a slight increase of mean cell volumes after contamination in sites 5 and 6 (data not shown).

Before contamination the number of saprophytic bacteria was between $5.0\times10^6$ and $2.0\times10^7$ MPN $ml^{-1}$. Stations 5, 6 and 9 had the higher initial concentrations ($>10^7$ MPN $ml^{-1}$). Oil addition induced no clear changes in saprophytic bacterial counts (Fig. 3). After 3 months of contamination saprophytic bacterial numbers were of the same order of magnitude as the initial numbers. The only exception was station 6 where a sharp decrease was observed at the end of the experiment. Analysis of variance (ANOVA) failed to establish any significant relationship between saprophytic bacterial numbers and contamination times ($p>0.5$).

Before contamination the number of hydrocarbon-degrading bacteria was between $2.0\times10^4$ and $5.0\times10^5$ MPN $ml^{-1}$. Stations 1, 2 and 4 had the higher initial concentrations ($>10^5$ MPN $ml^{-1}$). In contrast with total and saprophytic bacterial counts, oil contamination induced obvious increases of numbers of hydrocarbon-degrading bacteria during the initial 30 days of experiments. These increases were much more marked at sites 5 and 6. At these stations, differences between days 0 and 30 reached three orders of magnitude (Fig. 4). The minimum increase (80%) was observed at station 4. With the exception of station 8, a general decrease of hydrocarbon-degrading bacterial numbers occurred at the end of all experiments. ANOVA found a significant relationship between hydrocarbon-degrading bacterial numbers and contamination times for three stations (station 8, $p<0.001$; station 6, $p<0.01$; station 5, $p<0.02$) and no significant relationship for the six other stations ($p>0.4$). Before contamination, hydrocarbon-degrading micro-organisms comprised less than 5% of the total number of saprophytic bacteria. This proportion remained relatively unchanged after 3 months of contamination in six stations (1, 2, 3, 4, 7, 9) but reached values $\geqslant100\%$ at the end of the experiment in the three other stations (5, 6, 8).

The remaining oil concentrations evaluated at the end of each experiment are shown in Figs. 5 and 6. The evaluated concentrations differ strongly between the different stations but the same general pattern was observed for the short- and long-term experiments. Stations 5 and 6 had more than 20-fold higher residual oil concentrations than the seven other sites. There were relatively few quantitative differences between the results observed during the two experimental durations. After 3 months of contamination the remaining oil concentrations were significantly correlated with the corresponding numbers of hydrocarbon-degrading bacteria ($r=0.93$, $p<0.0001$).

The biodegradation indexes recorded after 3 months of contamination are reported in Table 2. They were significantly different from the initial values for only three stations: 5, 6 and 8. However, the three indexes used were significantly correlated with corresponding numbers of hydrocarbon-degrading bacteria ($r>0.83$, $p<0.004$) and remaining oil concentrations ($r>0.80$, $p<0.007$).

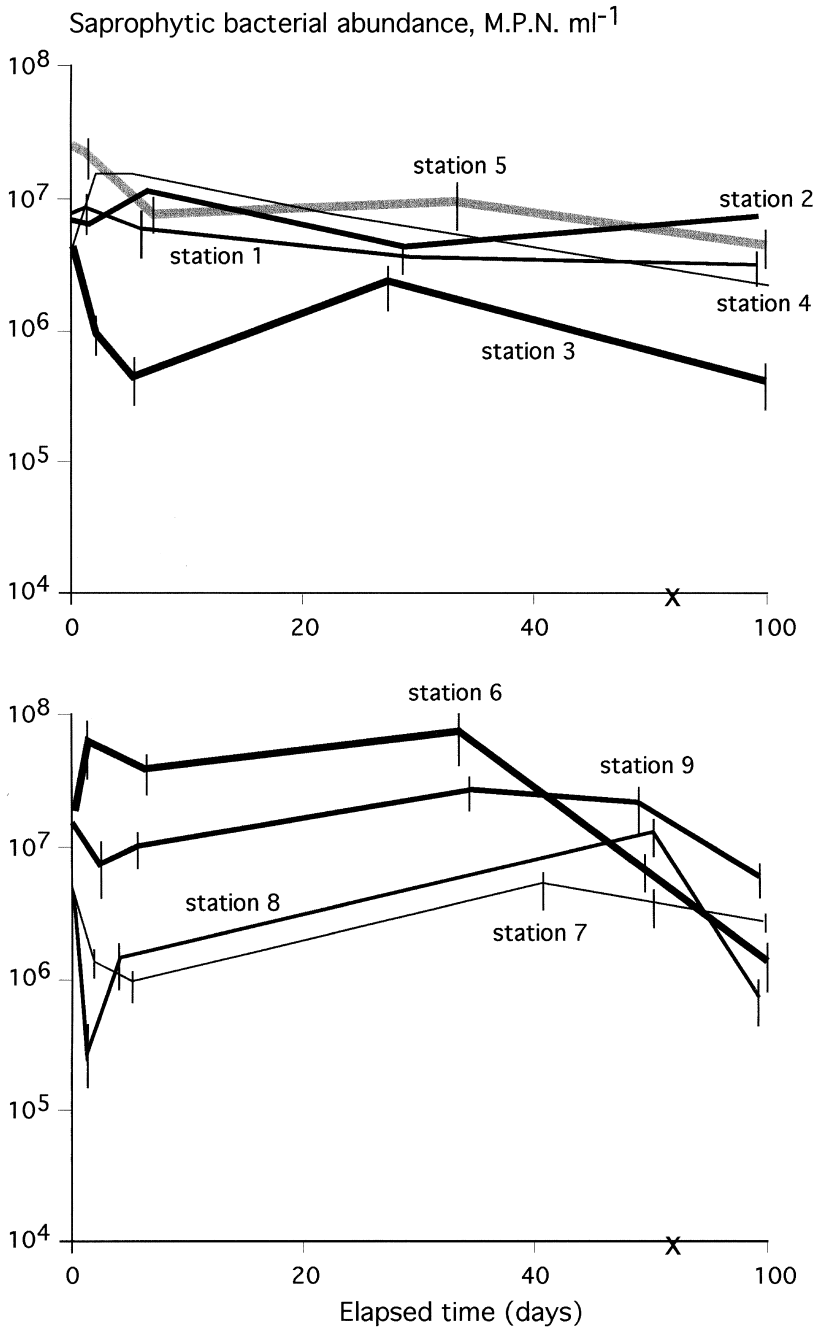


Fig. 3. Changes in saprophytic bacterial abundance (MPN $ml^{-1}$) during the course of the experiments at the nine contaminated sites. Bars indicate standard deviations (in order to improve clarity, some of them, stations 2 and 4, have been omitted).

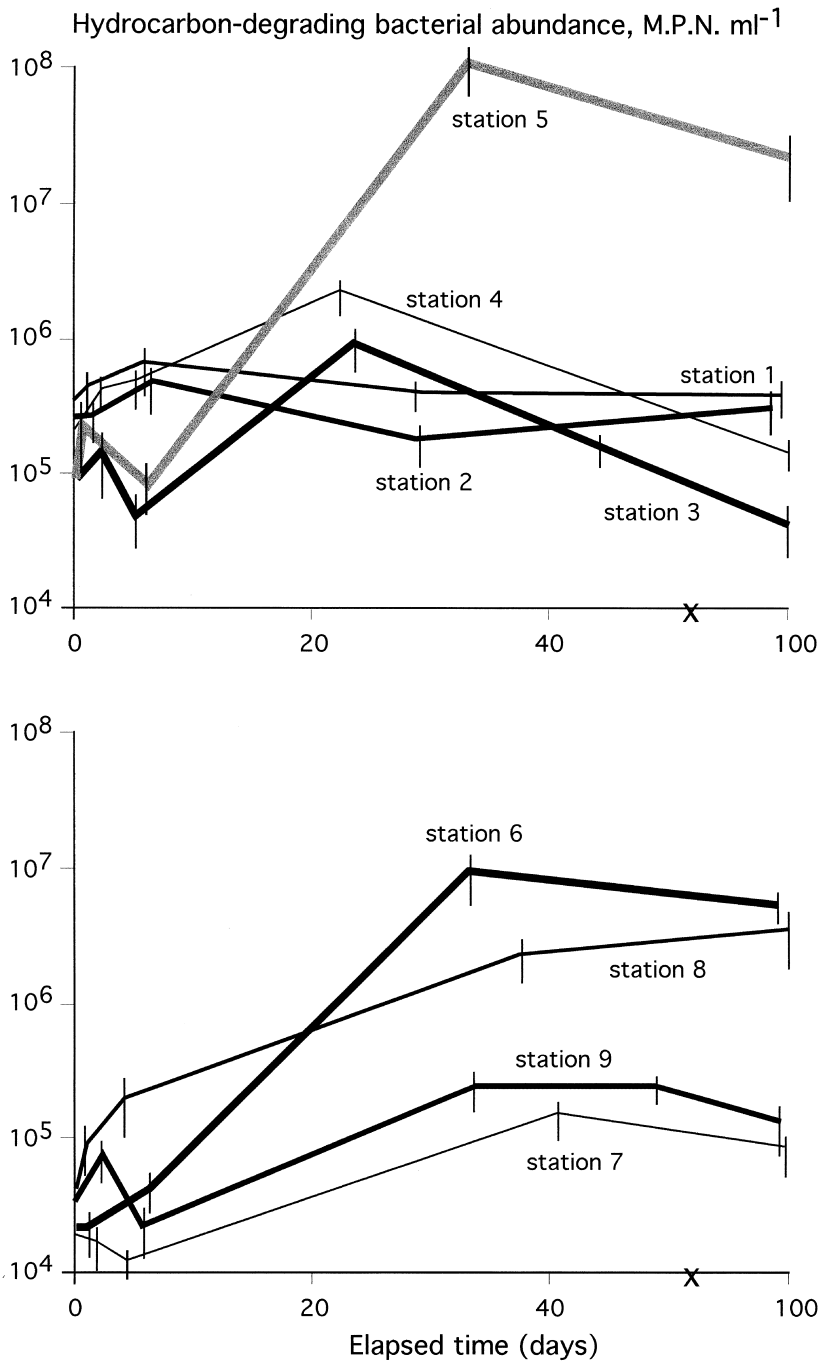


Fig. 4. Changes in hydrocarbon-degrading bacterial abundance (MPN $ml^{-1}$) during the course of experiments in the nine contaminated sites. Bars indicate standard deviations.

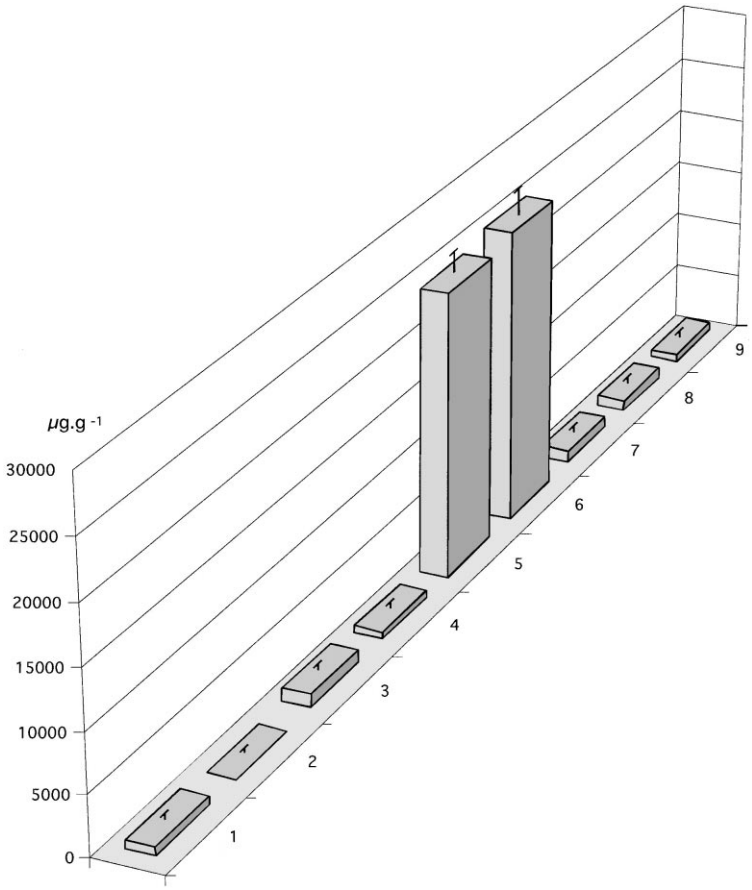


Fig. 5. Oil remaining at the nine sites after the short-term contamination (5 days). Bars indicate standard errors.

## 4. Discussion

Clear decreases of three biodegradation indexes occurred during the 90-day experiments conducted in Couvreux harbour and Sandy cove (Stations 5 and 6, Table 2). In contrast, there were only few changes in biodegradation indexes during the course of the other experiments. Furthermore the rapid decreases in crude oil concentrations observed in beaches 1–4 and 7–9 were too short in time to be attributed to biodegradation. In designing bioremediation experiments on a sandy beach, there is the complication of sand transportation into, out of, and along a beach by several mechanisms; e.g. long-shore currents, tidal action, wind action, bioturbation and storms. Any movement of sand might result in oiled sand leaving and fresh sand entering the plot. If the whole beach were contaminated, these movements of sand would not result in any major experimental problems since both the sand entering and leaving the study station would be contaminated. However, when only

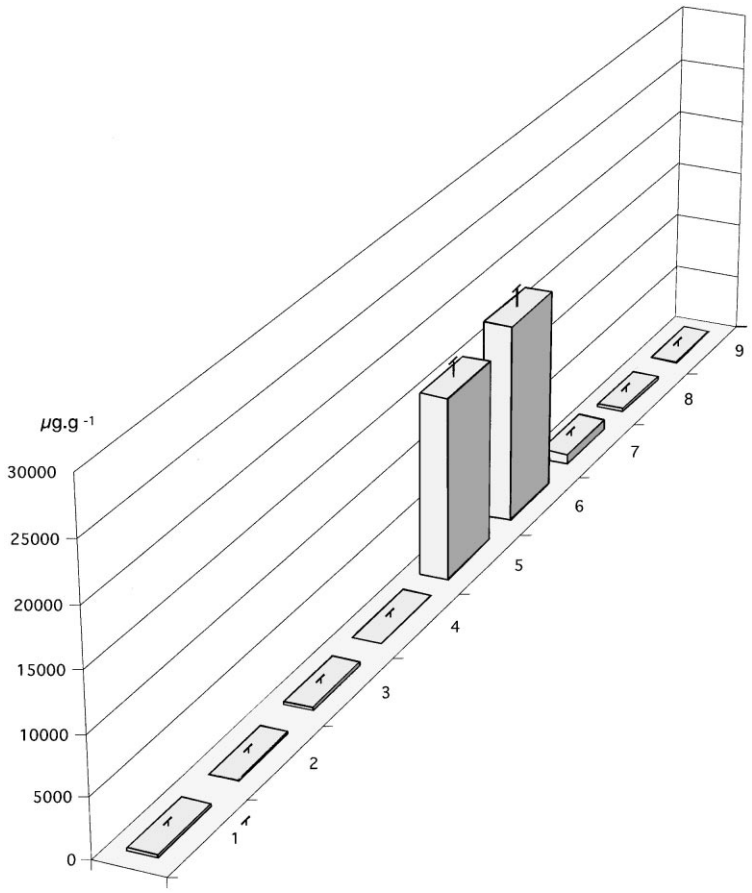


Fig. 6. Oil remaining at the nine sites after the long-term contamination (90 days). Bars indicate standard errors.

the study site is contaminated, any movement of sand will result in artificially accelerated dilution of the contaminated sand. Furthermore, sorption of hydrocarbons would likely affect their biodegradation via effects on their 'bioavailability' (Mohn, 1997). Bioavailability is a general term describing numerous factors capable of limiting biodegradation which are poorly understood in complex environments. These factors, which include dissolved concentration, surface area and emulsification, have been well summarised by Alexander (1994). They depend greatly of the hydrodynamic conditions of the contaminated beaches. It has been shown that the stonger the hydrodynamics, the faster the oil is degraded (Gundlach & Hayes, 1978; Sergy & Blackall, 1987).

The ubiquitous distribution of oil-degrading bacteria has already been reported in a wide variety of niches (Floodgate, 1984; MacCormack & Fraile, 1997; Wang, Lau & Button, 1996; for reviews see Atlas, 1981; Leahy & Colwell, 1990). In the studied pristine sediments, levels ranged from $10^4$ to $5.0 \times 10^5$ bacteria $ml^{-1}$, values

Table 2
Changes in biodegradation indexes during the course of the experiments[a]

| Biodegradation index | Day 0 | Day 30 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Station 1 | Station 2 | Station 2 | Station 4 | Station 5 | Station 6 | Station 7 | Station 8 | Station 9 |
| C17/prystane | 12.1 ± 0.6 | 11.9 ± 1.2 | 11.7 ± 1.4 | 12.0 ± 1.3 | 11.5 ± 1.5 | 7.6 ± 1.1 | 7.2 ± 1.0 | 11.8 ± 1.2 | 9.5 ± 0.8 | 11.6 ± 1.1 |
| C18/phytane | 4.1 ± 0.2 | 3.9 ± 0.5 | 4.0 ± 0.6 | 4.1 ± 0.7 | 3.8 ± 0.6 | 2.9 ± 0.3 | 2.2 ± 0.2 | 3.9 ± 0.5 | 3.4 ± 0.4 | 4.0 ± 0.6 |
| LMW/HMW | 3.5 ± 0.2 | 3.5 ± 0.4 | 3.4 ± 0.3 | 3.5 ± 0.5 | 3.3 ± 0.6 | 2.8 ± 0.4 | 2.4 ± 0.3 | 3.5 ± 0.6 | 3.0 ± 0.4 | 3.3 ± 0.5 |

[a] LMW/HMW = low molecular weight alkane {C12–C18}/high molecular weight alkane{C24–C32}. LMW, low molecular weight; HMW, high molecular weight.

comparable with those reported by Venkateswaran and Harayama (1995) in Japanese sediment. Before contamination, hydrocarbon-degrading micro-organisms comprised less than 5% of the total number of saprophytic bacteria. These results were consistent with those of Wright, Weaver and Webb (1997) who reported that hydrocarbon-degrading micro-organisms comprised 1–10% of the total number of heterotrophic bacteria in marine bacterial communities. The difference in original richness in hydrocarbon-degrading bacteria of the different studied locations reached one order of magnitude. The presence of an initially high number of oil-degrading bacteria has been previously reported as an indication of a previous history of oil contamination or a chronic contamination by oil (Atlas, 1991). However, the difference of nutrient richness (organic matter and mineral nutrients, data not shown) may also explain the observed differences in counts of oil-degrading bacteria. In any case, due to more than 50 years of human activity in the direct vicinity of the station 4 (derelict whaling station at Port Jeanne d'Arc) former accidental contamination at this site cannot be totally excluded.

Introduction of oil into the previously oil-free sediments results in enrichments for hydrocarbon-degrading micro-organisms by several orders of magnitude within a few days at some of the beaches studied. Such a very large enhancement of specific micro-organisms has been previously reported (Gunkel, 1967; Wagner-Döbler et al., 1998). Over a 3-year period following the *Exxon Valdes* oil spill in Alaska and under climatic conditions comparable to those of Kerguelen Island, Braddock et al. (1995) found significantly higher numbers of hydrocarbon-degrading micro-organisms at sites within the path of the oil slick (from $3.6\times10^3$ to $5.5\times10^5$ bacteria $ml^{-1}$) than at reference sites ($<10^2$ bacteria $ml^{-1}$). However, the quantitative importance of the hydrocarbon-degrading bacterial enrichment differs greatly amongst the sub-Antarctic studied beaches. This observation confirms the work of Sugai, Lindstrom and Braddock (1997) on shorelines of Prince William Sound (Alaska). For three field seasons they examined the mineralisation potential of hydrocarbon-degrading bacteria after the *Exxon Valdez* oil spill and found that environmental factors influenced the ability of microbial populations to mineralise polycyclic aromatic and aliphatic compounds.

Crude oil contains thousands of hydrocarbons and related compounds, with its major constituents being classified into saturated and aromatic fractions. The saturated fraction contains straight-chain and branched alkanes and cycloalkanes, while the aromatic fraction contains both mononuclear and polynuclear aromatic hydrocarbons possessing alkyl side chains (Clutter, Petrakis, Stenger & Jensen, 1972). Some of these petroleum contaminants could exert toxic effects on the active microbial community. The dissolved phase essentially contains naphthalene and other light PAHs which are known to be among the most toxic hydrocarbons to bacteria (Hodson, Azam & Lee, 1977; Siron & Pelletier, 1994). Although it has been demonstrated that microbial communities can affect chemical pollutants, the presence of toxicants can also affect microbial community structure (Delille & Siron, 1993; Long, Aelion, Dobbins & Pfaender, 1995). These chemicals can alter the community structure through selection of pollutant degraders or through acute toxicity to micro-organisms. Readily biodegradable pollutants can increase population

densities by promoting growth through providing carbon and energy to micro-organisms in otherwise oligotrophic environments. Thus, the marked differences observed in the remaining concentrations of oil at the end of the experiments would easily explain the observed differences in bacterial numbers. The greater the oil contamination the more numerous were the hydrocarbon-degrading bacteria (stations 5 and 6). Previous chronic exposure to petroleum hydrocarbons has also been proposed as a partial explanation for variability in bacterial response to petroleum hydrocarbons (Griffiths, McNamara, Caldwell & Morita, 1981). Perhaps of even more importance are the factors that determine the carrying capacity for oil-degrading bacteria in a marine beach environment (Venosa et al., 1996). The physical environment of the bacterial assemblage seems to play a major role in the biodegradation capacities. Presumably, the coarser grained and more porous sediments (at sites 5, 6 and 8) retain more oil compared to the finer sediments where the oil is probably only present on the surface, and is subsequently resuspended and removed from the cores.

In conclusion, the results obtained under the same general climatic conditions and with the same experimental design differ greatly from one beach to another. Care must be taken in extrapolating the results of any experimental study to more general environmental conditions. For optimisation of biodegradation, it will be necessary to understand the factors that control microbial activity at a specific site. This finding suggests that, in the event of a catastrophic oil spill impacting a shoreline, the first task should be to evaluate the natural degradation capacity of the various parts of the affected site (wind exposure, along-shore currents, natural nutrient concentration, background oil-degrading bacterial assemblages, etc.) to determine the best strategy of bioremediation to use in each of these eventually impacted sites.

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