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# Crude oil bioremediation in sub-Antarctic intertidal sediments: chemistry and toxicity of oiled residues

E. Pelletier[a,*], D. Delille[b], B. Delille[b,c]

[a]*Institut des Sciences de la Mer de Rimouski (ISMER), Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Canada G5L 3A1*
[b]*Laboratoire Arago, Université Paris VI, UMR-CNRS 7621, 66650 Banyuls sur mer, France*
[c]*Université de Liège, Unité d'océanographie chimique, Mécanique des fluides géophysiques, Institut de Physique, B5, B-4000 Sart Tilman, Belgium*

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**Abstract**

The effectiveness of fertilizers for crude oil bioremediation in sub-Antarctic intertidal sediments was tested over a one-year period in a series of ten (10) experimental enclosures. Chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine environment where hydrocarbon degrading bacteria (HDB) had not been stimulated by previous accidental spills or human activities. The low temperature of seawater (3–4 °C) had no obvious effects on the HDB community and the bioremediation process. Over 90% of *n*-alkanes were degraded in the first six months and most light aromatics (2–3 rings) disappeared during the first year of observation. The toxicity of oiled residues (Microtox® SP) was significantly reduced in the first 6 months of the process, but it increased again in the last months of the experiment. One of the fertilizers containing fishbone compost enriched with urea, inorganic phosphorus and a lipidic surfactant reduced significantly the toxicity of oil residues in the last 3 months of the experiment. Interstitial waters collected below the oil slicks during the remediation showed no toxicity, and even stimulated *Vibrio fischeri*. When comparing all fertilizers to the control plots, a good correlation ($r^2 = 0.82$) was found between the growth rate of HDB and the degradation rate of *n*-alkanes in the first 90 days of the experiment only indicating that fertilizers were efficient for at least 3 months but their beneficial effects were lost after 6 months.


*Keywords:* Crude oil; Bioremediation; Antarctica; Sediments; Fertilizers; Effectiveness; Toxicity; Hydrocarbon degrading bacteria; Surfactants; Dry fish compost

* Corresponding author. Tel.: +1-418-723-1986; fax: +1-418-724-1842.
*E-mail address:* emilien_pelletier@uqar.qc.ca (E. Pelletier).

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## 1. Introduction

Since fertilisation was demonstrated as a valuable tool for oil bioremediation during cleanup operations of the *Exxon Valdez* spill (Bragg, Prince, Horber, & Atlas, 1994), a number of investigators have explored physical, biological and chemical factors that could make successful conditions for field application of fertilizers (Hoff, 1993; Prince, 1993; Sugai, Lindstrom, & Broisblock, 1997). Primary factors affecting hydrocarbon bioremediation in marine sediments as well as in soils are: (1) the presence of hydrocarbon degrading bacteria (HDB); (2) the optimal environmental conditions stimulating bacterial activity; (3) the chemical composition of spilled petroleum and its relative toxicity, and, (4) the bioavailabilty of hydrocarbons to HDB.

The characterisation of bacterial communities particularly efficient to grow in presence of hydrocarbons have been the subject of decades of academic and industrial research (Zobell, 1946; Atlas, 1977; Foght, Fedorak, & Westtake, 1990; Ladousse & Tramier, 1991). Results were applied to the development of commercial fertilizers which have all in common the addition of nutrients to oily residues and the induction of optimal conditions for HDB. Meanwhile, microbial techniques to count specific HDB and estimate hydrocarbon mineralization potential have been developed and applied to a number of laboratory and field studies (Mills, Breuil, & Colwell, 1978; Lindstrom et al., 1991; Delille & Siron, 1993). HDB have been found active in most marine environments including in the Arctic ocean (Sveum & Ladousse, 1989), but it has been suggested that some sub-Antarctic remote locations could have indigenous HDB densities so low that biodegradation rate could remain extremely slow in spite of less severe weather conditions encountered in the Antarctic Peninsula (Kennicutt II, 1990).

The availability of added nutrients and their stimulating effects seem to be linked to their chemical speciation and their ability to stay at the remediation site. Although inorganic salts ($NaNO_3$ and $KNO_3$) were used in some laboratory and field experiments with conflicting results (Wrenn, Haynes, Venosa, Kadhodayon, Suidan, 1994; Mearns et al., 1995), the addition of reduced nitrogen was more successful and was usually supplied in commercial fertilizers as ammonium salts (i.e $NH_4NO_3$ or $NH_4Cl$) or as urea $(NH_2)_2CO$ (Prince et al., 1994a; Hueseman, 1995; Wrabel & Peckol, 2000) . Some organic fertilizers containing fishbone meal were also used in experimental plots with limited success (Lee, Siron, & Tremblay, 1995). Authors observed an increase of the toxicity following periodic additions of the fish compost which was attributed to anoxia and also to the formation of toxic metabolites from a too rapid degradation of the fertilizer itself instead of the treated oil. In contrast, Santas, Korda, Tenente, Buchholbz and Santas (2001) reported a successful bioremediation of crude oil by a fish compost in mesocosm assays simulating Mediterranean winter conditions with 70% alkane degradation in 30 days. The effectiveness of organic fertilizers, particularly those using inexpensive dried fishbone meal, needs to be reassessed under different field conditions, particularly at lower concentrations and under colder climates where the natural biodegradation is usually considered as a very slow process.

The chemical composition of spilled oil as a factor affecting bioremediation has received a sustained attention from analysts but the huge complexity of natural hydrocarbon mixtures prevented from the definition of a clear relationship between the presence and concentration of some particular chemicals and success or failure of the remediation process (Krahn et al., 1992; Fayad & Overton, 1995; Salanitro et al., 1997). The success of in situ bioremediation for a given type of hydrocarbons might be dictated by optimal environmental conditions which must be in place at the beginning of the process. Among these conditions are certainly the bulk toxicity of the spilled oil. Determining the toxicity of freshly spilled oil and weathered residues towards the naturally occurring microbial community is still an unsolved problem. The best practical approach is still the use of a non specific microbial biotest. Bioluminenscent marine bacterium *Vibrio fischeri* currently used in Microtox® assays seems an appropriate candidate as luminescence is related to respiration and provides a good indicator of the general toxicity of a large number of chemicals (Environment Canada, 1993) including polycyclic aromatic hydrocarbons (PAHs) and their photodegradation products (McConkey Duxbury, Dixon, & Greengerg, 1997). Microtox® Solid-Phase assay (Microtox SP) was found sensitive to toxic components of crude oil and was used successfully to monitor oil residues toxicity during bioremediation (Lee et al., 1995), but appeared less sensitive or too variable in some other circumstances (Mearns et al., 1995; Salanitro et al., 1997).

The objective of this work was to evaluate the effectiveness of various organic fertilisation treatments and to assess factors affecting oil bioremediation under field conditions encountered in a remote sub-Antarctic environment. Chemical biodegradation indexes were determined by gas chromatography in oiled sands collected over a one-year period and were compared to HDB production. Toxicity of oil residues and interstitial water was determined by Microtox assays and correlated to chemical and microbial parameters. Furthermore, the location of the experimental site in *Kerguelen Archipelago* (sub-Antarctic Ocean) was carefully chosen to minimise possible effects of past and present human activities which could have contributed to adapt local bacterial communities to hydrocarbon spillage.

## 2. Materials and methods

### 2.1. Environmental setting

The experiment was settled in the *Anse sablonneuse* (49° 19′S, 69° 42.5′E), a remote sandy beach of the main island of the *Kerguelen Archipelago* located at the northern limit of the Antarctic Ocean. Details of the experimental setting are described elsewhere (Delille, Delille, & Pelletier, 2002). A series of 10 enclosures of 1 $m^2$ was settled in the intertidal zone at the mid-tide mark. Each enclosure was made of four wood boards (1 m long×0.6 m width) fitted together by steel right angles and deeply inserted into the beach sand. Enclosures were covered with a stainless steel grid (2 mm holes) allowing the free circulation of tidal seawater but avoiding losses of oiled sediments and contacts with sea birds and marine mammals. Without disturbing the

sand surface, 2.85 l of Arabian light crude oil (topped at 150 °C) (abbreviated as BAL hereafter) were added uniformly to each enclosure over a surface of about 0.60 $m^2$ leaving a 12-cm clean strip between the enclosure wall and the oiled surface. The chemical characteristics of crude oil BAL are summarised in Table 1.The reproducibility of the three replicates was excellent with a standard error better than ±5% on a weight basis.

Four different treatments were applied in duplicate to enclosures and two plots where kept as controls without fertilisation. The fertilizers were three dry fish composts (F1, F2, and F3) and the commercial liquid Inipol EAP 22® (INIPOL hereafter). Each fertilizer was added twice to the surface of oiled sand at a 5% proportion to the oil (w/v). Chemical composition of the fertilizers are summarised in Table 2. The experiment started on February 12, 1997, and the sampling lasted on December 20, 1997. The first fertilisation occurred 10 days (day 10) after the spill and the second 21 days later (day 31). During the course of the experiment the average daily temperature of seawater decreased slowly from 9.0 to 2.6 °C up to early October and then started to increase up to 7.9 °C with the return of the sub-Antarctic summer in December (see Delille, Bassères, & Pelletier, 2002 for details). Air temperature at Port-aux-Français (Kerguelen scientific station) averaged 6.6 °C during the summer and 2.8 °C during the winter.

### 2.2. Chemical analysis

Oiled samples (10 g) were extracted twice with dichloromethane and extracts were eluted on silica gel column (20 cm) with dichloromethane:hexane to separate

Table 1
Chemical characteristics of Arabian light crude oil (BAL) used in the *Anse sablonneuse* experiment. Results are means of three replicates. DCM = dichloromethane

| Characteristics | Values |
|---|---|
| Specific gravity (g/$cm^3$) | 0.89 |
| Weight loss by evaporation at 20 °C over a 48h period (w/w) | 24% |
| Saturated hydrocarbons (fraction 1 eluted in hexane) | 45.5% |
| Aromatic hydrocarbons [fraction 2 eluted in hexane/DCM (1:1)] | 41.2% |
| Polar compounds (fraction 3 eluted in DCM only) | 3.4% |
| High molecular weight aromatics (asphalthenes) insoluble in hexane | 9.5% |
| Solid residues insoluble in DCM (ash) | 0.4% |

Table 2
Chemical composition of fertilizers used in the *Anse sablonneuse* experiment

| Fertilizers | %C | %N | %P | Composition |
|---|---|---|---|---|
| F1 | 51.3 | 11.7 | 0.7 | Basic dry fish compost without additives |
| F2 | 25.7 | 18.5 | 3.2 | Fish compost + urea + phosphate + cationic surfactant |
| F3 | 31.3 | 17.0 | 3.3 | Fish compost + urea + phosphate + lipidic surfactant |
| INIPOL | 62.0 | 7.4 | 0.7 | Urea in oleic acid + organic phosphate + 2-butoxyethanol |

saturated hydrocarbons (fraction 1), aromatics (fraction 2) and polar compounds (fraction 3) following standard procedures. Fractions were analysed by gas chromatography/flame ionisation detection (Varian 3400 GC/FID system) using a 30 m×0.32 mm(i.d.) glass capillary column (DB-5MS from Supelco®) and helium as carrier gas. The gas chromatography with mass spectrometry (GC/MS) analyses were carried out using a Finnigan Ion Trap Detector coupled to a Varian 3400 GC. The oven for both GC/FID and GC/MS was programmed from 80 to 300 °C at a rate of 5 °C/min with a final plateau of 15 min. The peaks were identified by comparing sample peaks with retention times of the EPA 610 PAH mixture from Supelco® and were confirmed by mass spectrometry when required.

### 2.3. *Microtox® assays*

The toxicity of oiled sands was evaluated by the response of the luminescent bacteria *Vibrio fischeri* (Qureshi et al., 1998) using the Microtox Analyser 500 and the solid-phase test kit (AZUR Environmental, Carlsbad, CA). The assays were conducted as described by the Microbics user manual by incubating suspended sediment concentrations in thermoregulated bath at 15 °C for 20 min. The sediment dilution that inhibited 50% (EC50) of the light output relative to oil-free sample collected at the external sampling site was calculated for each oiled sample and expressed as a percent (%) of the pristine sample. Each EC50 value is calculated from a dilution curve with 5 independent determinations.

The toxicity of interstitial waters (filtered on 0.45 μm GF/F filters) was evaluated using the Microtox Analyser 500 and the basic test protocol for liquid samples (Microbics user manual). As none of the 47 interstitial water samples inhibited the light emission of *V. fisheri* below the 100% of reference solution and some of them stimulated the activity of bacteria, $EC_{50}$ can not be calculated.

Hydrocarbon-degrading bacteria (HDB) were counted by the Most Probable Number method (MPN) as described in Delille et al. (2002). Statistical results described in this paper have been obtained with a 1-way ANOVA test (pairwise multiple comparison using Student–Newman–Keuls method) using the commercial software SigmaStat®.

## 3. Results and discussion

### 3.1. *Chemical indexes*

Hopanes are molecular fossils of bacterial hopanoids and ubiquitous constituents of crude oil now commonly used as markers for fossil hydrocarbons and oil residues (Volkman, Holdsworth, Neill, & Baver,1992; Prince et al., 1994b). Hopanes were not detected in any sand samples collected at the external sampling site during the course of the experiment indicating the absence of any recent or present oil contamination. The presence of land-derived hydrocarbons from detritic organic matter and vascular plants was indicated by the dominance of C25, C27, C29 and

C31 *n*-alkanes (biowaxes) which represented more than 30% of n-alkanes in all reference samples. Linear C21-polyenes and C25-trienes, indicators of marine phytoplankton production (Wakeham, 1990; Green & Nichols, 1995) were detected in reference samples collected at the end of the summer (day 90) and during the winter (day 177). Phytane was barely present in all reference samples, but pristane was not detected.

Using C30-hopane (base peak ion m/z 191) as the persistent marker for alkane biodegradation and *n*C18-alkane as the corresponding biodegradable marker, C18/hopane ratios were calculated for all oiled samples. The calculation of the index from the total gas chromatographic detectable hydrocarbons (TGCDHC/hopane) as proposed by Prince et al. (1994b) was found inadequate in this particular case as natural biowaxes interfered strongly at low oil concentrations observed in the second half of the experiment. The C18/hopane ratio was high is fresh unweathered oil but decreased rapidly as the reduction of C18 took place in the first 2–3 weeks after the spill. The behaviour of this ratio in the following weeks is illustrated in Fig. 1 indicating at day 90 a better efficiency of F1, F2 and F3 treatments, a moderate success for INIPOL and a much slower process in unfertilized BAL enclosures. In spite of these differences, all ratios reached values below 3.1 in day 177 indicating advanced degradation in all plots including untreated ones. At day 311, *n*-C18 was

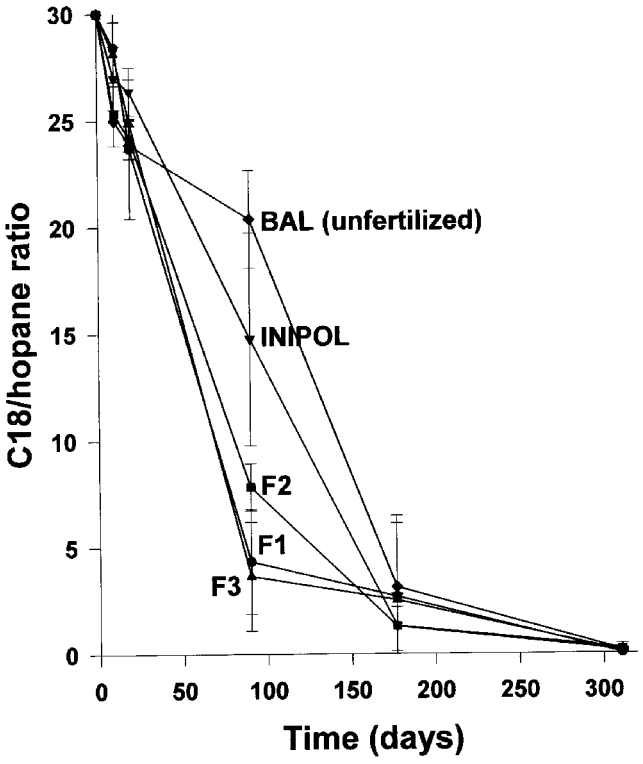


Fig. 1. Change in C18/hopane ratios in response to the oil bioremediation process.

absent or barely present in all samples (C18/hopane $\cong$ 0) and alkanes fingerprints were dominated by natural biowaxes. The apparent degradation rates calculated for different sampling periods from C18/hopane ratios and given in% per day or in % par period considered (Table 3) provide a practical classification of the fertilizers efficiency, particularly well defined between days 19 and 90 (F3 $\geqslant$ F1 > F2 > INIPOL $\gg$ BAL) where the rate enhancement of F3 was over six-fold compared to unfertilized BAL plots. The winter period between days 90 and 177 shows the reverse situation (BAL > INIPOL $\gg$ F2 $\geqslant$ F1 $\cong$ F3) where oil in untreated plots was degraded even faster than INIPOL treatment. Actually, INIPOL may have reduced the weathering process in the first 19 days of the experiment as the rate of degradation of the INIPOL treatment (0.64% $day^{-1}$) was significantly lower ($P > 0.05$) than the mean rate of other enclosures ($1.03 \pm 0.09\%$ $day^{-1}$).

The initial quick reduction of the C18/hopane ratio in all enclosures might tentatively be attributed to evaporation (photo-degradation being minimal due to the experimental design shading the oiled sand surface) rather than biodegradation as an important fraction of BAL (over 20%) was volatile at 20 °C and the first fertilisation occurred only 10 days after the spill. The C18/hopane index was not sensitive enough to assess this early weathering process and a better index was defined. As isoprenoids (*n*-alkanes with one branched methyl group) are quite resistant to biodegradation in the very early stages of the process (the correlation between *n*C12/iso-C12 and *n*C18/hopane for all treatments was linear in the first 90 days with $r^2 = 0.989$), the ratio *n*C12/iso-C12 can be used as an early indicator of weathering process. As *n*C12 and iso-C12 have very close boiling points ($\cong$ 214–216 °C) and should evaporate approximately at the same rate without affecting their ratio, any reduction of this ratio would be an indication of a preferential biodegradation of *n*C12 over iso-C12. On the other hand, assuming both iso-C12 and hopane are not biodegraded (at least in the first weeks of the process), any reduction of the ratio iso-C12/hopane would be an indication of the loss of iso-C12 by evaporation. Physical losses of oil by abrasion or migration out of the enclosures will not affect this ratio. The initial *n*C12/iso-C12 ratio in unweathered BAL was 7.3.

Table 3
Relative degradation rates calculated as the change of C18/hopane ratios within given periods of time. Values are reported in% of change per day and per time period (in brackets). All results are means of duplicate enclosures

| Period (d) | F1 | F2 | F3 | INIPOL | BAL (unfertilized) |
|---|---|---|---|---|---|
| 0–19 | 1.11 (21.1) | 1.02 (19.4) | 0.9 (17.2) | 0.64 (12.2) | 1.1 (20.9) |
| 19–90 | 0.91 (64.8) | 0.66 (46.9) | 1 (71.1) | 0.55 (39) | 0.16 (11.4) |
| 90–177 | 0.06 (5.2) | 0.34 (29.6) | 0.04 (3.5) | 0.53 (46.1) | 0.67 (58.3) |
| 177–311 | 0.07 (9.4) | 0.03 (4) | 0.06 (8.1) | 0.02 (2.7) | 0.07 (9.4) |

The mean *n*C12/iso-C12 ratios for all treatments including untreated BAL plots were $7.1 \pm 0.2$ and $6.7 \pm 0.3$ at days 10 and 19, respectively, indicating a slight but significant change ($P < 0.1$) of this ratio in this short period of time and confirming that biodegradation was already well engaged only 9 days after the first addition of fertilizers at day 10. During the same period, the mean iso-C12/hopane ratio was stable at $6.4 \pm 0.5$ indicating that evaporation has not significantly contributed to the early weathering process of alkanes.

Fayad and Overton (1995) reported an inhibiting effect of added nutrients on the degradation rate of emulsified crude oil under short-term laboratory conditions (1–6 days at ambient temperature) and using unspecified water-soluble nutrients. Our results, obtained from a long-term field experiment at low temperature, provide new evidences supporting the hypothesis that the naturally occurring HDB assemblage is mainly formed by microbial species that can growth on both saturated hydrocarbons and aromatics, but are subject to differential metabolic regulation (Prince, 1993). The availability of organic nutrients in the present case seems to largely favour alkanes as the preferred carbon source while $C_n$-3-rings aromatics (phenanthrenes) are neglected. The relative proportion of phenanthrenes remained stable at 25–30% of total aromatics in all treated plots in the first 200 days and then started to decrease in the last 3 months.

In their study of the bioremediation of the *Exxon Valdez* oil spill, Prince and co-workers (Prince et al., 1994a) observed that the relative rate of biodegradation, calculated as the change of the oil component/hopane ratio with time, was first controlled by the amount of nitrogen delivered per unit of oil. In the present study the same amount of fertilizer (in weight) was added to each treated enclosure, but fertilizers F2 and F3 contained more organic nitrogen and inorganic phosphorus in their composition than F1 and INIPOL (Table 2). Total amount of nitrogen added to plots during the experiment was 33, 53, 48, and 21 g (as N) for F1, F2, F3, and INIPOL, respectively. It should be noted that INIPOL had a much higher content in carbon than others which can explain the slower action of that fertilizer as the bacterial community (including HDB) was first busy to degrade oleic material from the fertilizer and apparently neglected alkanes and aromatics from the oil. F1 and F3 were the most efficient fertilizers in the first 3 months of the bioremediation, but F2 also enriched with urea as F3 was significantly behind ($P < 0.05$).

The presence of a lipidic neutral surfactant (structure not revealed by the manufacturer) in F3 might be responsible for its faster action and its enhanced efficiency. Non-ionic surfactants with a high affinity for oil (low to moderate HLB) are efficient to disperse crude oil in seawater (Brochu, Pelletier, Caron, & Desnoyers, 1986) and thus increasing the oil/water interface easily colonised by bacterial community. Cationic surfactants such as aryl sulfosuccinates are usually much less efficient to form stable oil-in-water emulsion (Brochu et al., 1986) which could explain the poorer results obtained with F2 which has the highest amounts of N and P added. The enrichment in organic nitrogen and inorganic phophorus might be a governing factor for the fertilizers efficiency but other factors such as the low bioavailability of the carbon content, and the presence of an appropriate and efficient surfactant should contribute to the success of a fertilization formula. Dry fish compost without

additives (F1) contains proteins and lipids from residual flesh and skins, calcium and essential minerals from bones and appeared as an efficient fertilizer by itself and an excellent solid carrier for bioremediation additives (nutrients and surfactants). Dry fish composts tend to mix and attach to the oil slick surface providing a large solid interface for bacterial colonisation and making nutrients immediately available.

### 3.2. Toxicity of oiled sands

Toxicity data of oiled samples as determined by Microtox SP as a function of the degradation time are summarised in Table 4. The toxicity decreases when EC50 (%) increases. Microtox SP revealed to be particularly sensitive to the Arabian Light crude oil used in this experiment as all enclosures showed EC50 below 6% in the first 19 days. The toxicity generally decreased in the following 3 to 6 months and then started to increase again. As all enclosures, except F3, presented a similar general pattern, mean EC50 values for all treatments including untreated plots (but excluding F3) were calculated for all sampling days and are compared to F3 in Fig. 2. When compared together for the whole experimental period, treatments are not significantly different form each others (ANOVA multiple comparaison, all pairewise SNK test, $P=0.67$) and F3 is not different from the mean of all other enclosures ($P=0.54$). However, the mean EC50 (all enclosures) at day 90 is significantly higher ($P>0.05$) than $EC_{50}$ at day 19 as well as the EC50 at day 249 is significantly lower ($P>0.05$) than mean EC50 at day 208. The apparent final increase of $EC_{50}$ at day 311 is not statistically different from day 249 ($t$-test, $P=0.278$). The EC50 in F3 enclosures was not statistically different from other treatments up to day 208, but then started to increase (toxicity decreased) in the last 3 months of the experiment and became highly different from others at day 311($P<0.001$).

Table 4
Microtox SP results on oiled samples from *Anse sablonneuse*. The EC50 are expressed in percent (%) of sediment that inhibits 50% of the light output relative to hydrocarbon-free samples taken at 100 m from the enclosures. Each value is the mean±S.D. of two determinations (two enclosures with the same treatment) except when indicated in parenthesis

| Time (day) | Treatments | | | | |
|---|---|---|---|---|---|
| | F1 | F2 | F3 | INIPOL | BAL (unfertilized) |
| 10 | 5.3±1.3 | 3.8±1.0 | 4.0 (1) | 4.6 (1) | 3.0 (1) |
| 19 | 4.1±1.7 | 4.3±1.0 | 2.3±0.4 | 2.2±0.3 | 3.5±1.2 |
| 90 | 12.5±2.0 | 10.0±1.9 | 9.3±3.6 | 7.7 (1) | 4.9±0.1 |
| 177 | 14.1±0.6 | 5.8 (1) | 8.4±0.9 | 6.2 (1) | 12.1 (1) |
| 208 | 10.2±1.6 | 8.5±0.8 | 9.6±0.3 | 10.0 (1) | 11.9±0.5 |
| 249 | 8.8 (1) | 8.3 (1) | 9.7 (1) | 5.8 (1) | 4.6 (1) |
| 311 | 7.5±1.4 | 7.2 (1) | 15.1±2.0 | 8.8±2.1 | 8.7±2.0 |

When grouped together, $EC_{50}$ values collected in the first 177 days are not linearly correlated to C18/hopane ratios indicating that alkanes are only one component of the observed toxicity (Fig. 3a). However, a closer look at day 19 data (Fig. 3b) shows that the toxicity was still maximum for most enclosures but a negative linear correlation between EC50 and C18/hopane is already present. This expected reduction of toxicity as C18 and other n-alkanes are biologically removed from the oiled residues is again illustrated at day 90 where the effectiveness of fertilizers F1, F2 and F3 on the toxicity reduction towards the more toxic untreated BAL is particularly well defined (Fig. 3c). Using the slopes from linear regressions of Fig. 3b and c, one can estimate that a change of one% of the toxicity unit ($EC_{50}$) required a reduction of the C18/hopane ratio of 1.3 and 2.9 units at days 19 and 90, respectively. This result indicates that the toxicity reduction rate slowed down with time whereas the rate of biodegradation was maintained or even increased in the first 3 months. The relative importance of alkanes in the whole toxicity effect to *Vibrio fisheri* decreased with time. At day 177 (Fig. 3d), the situation was drastically changed as all C18/hopane ratios were very low in all enclosures, but the lowest C18/hopane ratios (F2 and INIPOL) exhibited the highest toxicity. Examination of gas chromatograms (not shown) of sand from F2 and INIPOL enclosures at day 177 shows that the aliphatics fraction represented less than 10% of the total residual oil and contained mainly isoprenoids (nor-pristane, pristane, and phytane) and long chain *n*-alkanes (C27 to C35) in a smaller proportion. These residual alkanes quite similar to those found in the reference site are most probably not responsible for the increased toxicity of INIPOL and F2 enclosures. The increased toxicity of highly degraded

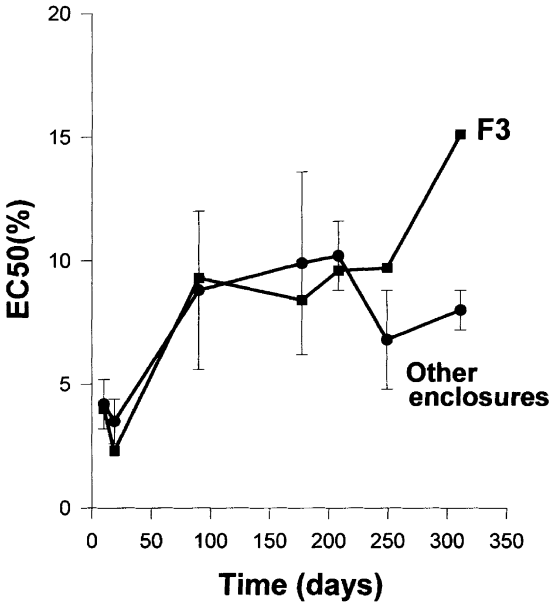


Fig. 2. Time course of oiled sediment toxicity (EC50) expressed as % of reference external site (100%). Means of all enclosures including BAL, but excluding F3 and means of F3 enclosures only.

residues in all enclosures (except F3) is confirmed by the significantly lower $EC_{50}$ at day 249 compared to day 208 (Fig. 2). It should be noted that F1 exhibited the lowest toxicity in days 90 and 177 then F3 became the less toxic treatment from day 249 (Table 4).

The bulk toxicity of oiled sands as monitored by Microtox SP might be seen as the result of the additive effects of two components: (1) the alphatic fraction with a low toxicity, but a high bioavailability and degradability; (2) the aromatic component with a higher toxicity potential, but a lower biodegradability under present bioremediation conditions. The aromatic fraction of most fresh crude oils contains a large proportion of substituted C1–C3 benzenes, C1–C3 naphthalenes, fluorenes and phenanthrenes. However, BAL was very low in one-ring aromatics as we used a fraction topped at 150 °C. The distribution of rings in the aromatic fraction is available for F1 and BAL plots and shows that after almost one year the three-ring compounds represented about 15–20% of the residual aromatics and heavier 4–5 rings counted for about 76% (Table 5). The contribution of light one-and two-ring compounds decreased steadily from the beginning to day 177 and then remained almost stable in the last four months for both treatments. Benzenes were not detected at day 90 and later one. Similarly, After only 11 days of weathering in Prince William Sound, Khran et al (1992) showed that Prudhoe Bay crude oil has lost all C1–C3 benzenes, about 50% of naphthalenes, but heavier compounds persisted without apparent changes.

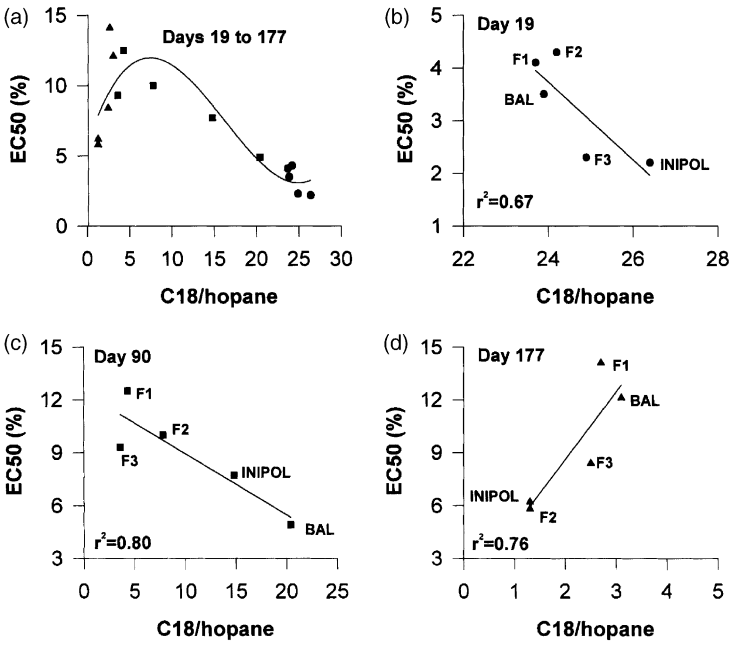


Fig. 3. Relationship observed between toxicity (EC50%) and C18/hopane ratios: (a) all data from day 19 to day 177; (b) day 19; (c) day 90; (d) day 177.

Barron, Podrabsky, and Kavanagh (1999) found that water accommodated fractions with a high naphthalene content were less toxic to mysid shrimps that those with a low % naphthalenes. Their tested oil with the lowest PAH and heterocycles concentrations had the greatest toxicity. In the present case, the same oil was used for all plots and the contribution of low molecular weight aromatics did not change drastically between treatments. Evaporation and dissolution should have been roughly the same for all plots. If the toxicity was essentially due to the aromatics, we should observe roughly the same EC50 for F1 and BAL, and also the other plots. Fig. 3c shows that amazing negative correlation between CI50 and alkane degradation with BAL and F1 at the opposite ends of the regression line. F1 and BAL are again close together at day 177 as it was at day 19. The only clear difference between BAL and F1 at day 90 in their C18/hopane ratio. To our knowledge, the toxicity of a mixture of hydrocarbons to *V. fischeri* is not documented but we can not brush away that some isoprenoids or cycling alkanes could have a toxic effect on Microtox test.

As we expected the release of some toxic water soluble metabolites from the bioremediation process particularly in presence of fertilizers (Lee et al., 1995), a series of 47 Microtox tests were conducted on interstitial waters sampled just below the oiled sands at low tide from day 10 to day 311. No toxicity was detected in these samples and most of them exhibited an enhancement of the bioluminescent intensity of *Vibrio fischeri* possibly due to soluble nutrients released by fertilizers or naturally present in seawater. This lack of a toxic response is most probably due to a constant dilution of toxic metabolites in seawater by tidal action on sandy substrates which prevented the accumulation of metabolites at a toxic level in interstitial waters. It might also indicate that most metabolites from hydrocarbon bioremediation are much less toxic than oiled residues making bioremediation a safe and suitable procedure under sub-Antarctic conditions as already observed for Arctic conditions (Prince et al., 1994b).

A number of authors reported that weathered oil residues were more toxic to aquatic organisms (fish embryos, shrimps and mussels) than previously expected (Carls, Rice, & Hose, 1999; Barron et al., 1999; Rowland, Donkin, Smith, & Wraige, 2001). However, recently published field toxicity results from the Exxon Valdez oil

Table 5
Relative distribution (%) of the number of rings in the aromatic fraction of oil residues recovered from F1 and BAL (unfertilized) plots during the course of the experiment

| Treatment | Aromatic rings | Time (days) | | | |
|---|---|---|---|---|---|
| | | 0 | 90 | 177 | 311 |
| BAL | 1–2 | 44 | 26 | 9 | 7 |
| | 3 | 25 | 18 | 15 | 16 |
| | 4–5 | 31 | 56 | 76 | 77 |
| F1 | 1–2 | 44 | 20 | 8 | 3 |
| | 3 | 25 | 28 | 32 | 21 |
| | 4–5 | 31 | 52 | 60 | 76 |

spill site (Page et al., 2002) showed that the long-term acute sediment toxicity, as determined by the mortality of the marine amphipod *Rhepoxynius abronius,* was not related to the increased fractions of high molecular weight PAHs in weathered oil. These apparently conflicting results are first related to differences in toxicity biomarkers and, second, to changes in toxicity of oiled residues with time.

Our results provide some clear indications that the toxicity of oiled residues changed in a non-linear way during the bioremediation process (Fig. 2). The reduction of toxicity in the first months of weathering seems to be linked to the loss of alkanes and light aromatics but the regain of toxicity appears much more difficult to explain. As seen from the non toxic interstitial waters collected below oil slicks, the accumulation of toxic metabolites seems not to be the answer. The toxicity of oil residues results from complex chemical interactions involving synergetic and antagonistic mechanisms not yet understood. In unfertilized BAL enclosures, the relative proposition of fraction 1 (saturated hydrocarbons), fraction 2 (aromatics), and fraction 3 (polar compounds) was 65%, 23% and 13%, respectively after 90 days. In F3 enclosures, this proportion was 79%, 14% and 6%, respectively, showing an apparent better degradation of fractions 2 and 3 in F3 treatments. This better result might be related to the ability of the fertilizer to make available some aromatics and polar compounds earlier in the degradation process and reduce their residual toxicity later one. The lack of a complete chemical characterisation of UCM of oil residues precludes to any exhaustive investigation of toxicity mechanisms at a molecular level. For this reason, toxicity tests using fish embryos, amphipods and bacteria can not be compared as toxic hydrocarbons can act differently from one species to another.

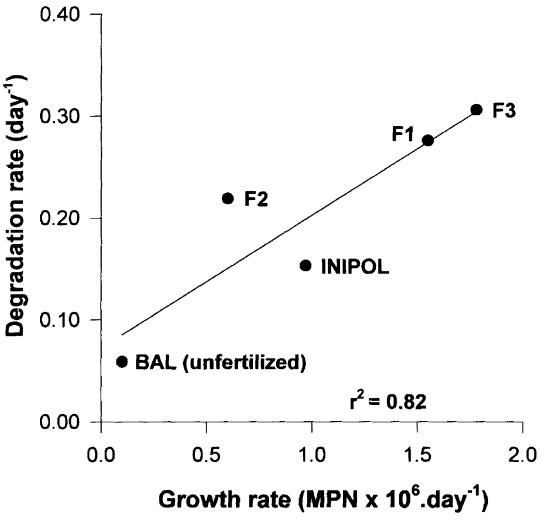


Fig. 4. Degradation rate of alkanes (δ C18/hopane per day) as a function of the mean growth rate of hydrocarbon-degradation bacteria (HDB per day) between sampling days 10 and 90.

The treatment with the fish compost F3 seems to have a different behaviour from others indicating an almost constant decrease of the toxicity up to the end of the experiment. Such a difference might be related to the capacity of F3 in changing the composition of oil residues and/or modifying the water/solid oiled residues interface in such a way that toxic compounds were less available to Microtox® bacteria.

### 3.3. Bacterial activity

Results on the total bacterial abundance and development of hydrocarbon-degrading bacteria (HDB) are discussed elsewhere (Delille, Delille, & Pelletier, 2002). In summary, HDB found in control intertidal sediments never exceeded 1% of the total saprophytic assemblage, but bounced to 45–50% of the total between days 177 and 208 before to return to an average value of 25% near the end of the experiment. The reduction of the relative proportion of HDB after the first seven months is more likely related to the lack of easily available food (*n*-alkanes) than to the toxicity of the substrate as the number of heterotrophic bacteria was still high even 2.5 years after the spill (Delille et al., 2002). The presence of HDB community in a remote location not previously exposed to fossil hydrocarbons is associated to the presence of biowaxes derived from vascular plants and other terrestrial carbon sources. The coincidence between high C18 mineralisation rate and the presence of high concentrations of C25 + C27 + C29 + C31 alkanes in some reference and oiled bays of the Prince William Sound (60°30′N; 147°05′W) suggested that terrestrial biowaxes pre-conditioned the microbial populations for an efficient degradation of oil-derived alkanes (Sugai et al., 1997). A recent work conducted in *Terre Adelie* (Delille, Bassères, & Dessommes, 1997) showed a significant response of the Antarctic sea-ice HDB community to the addition of oil and fertilizers to newly formed sea-ice. The contribution of HDB to the saprophytic communities passed from less than 0.1% to more than 95% in some sea-ice samples in 6 months in spite of the severe conditions of the Antarctic winter.

As HDB are using carbon from hydrocarbons to proliferate, it should be possible to establish a relationship between HDB and biodegradation markers. When the mean growth rate of HDB (δ HDB per day) and the apparent degradation rate (δ C18/hopane per day) are calculated between sampling days 10 and 90, a clear correlation appears between bacterial production and hydrocarbon consumption (Fig. 4). Although this positive correlation is expected it is rarely observed in field experiment because a number of environmental factors can obscure it. The correlation was not significant after day 90 because alkanes and light aromatics became less abundant in some enclosures (particularly F3 and F1) and the efficiency of fertilizers have decreased with time.

## 4. Conclusion

Assessing in situ bioremediation of oil is a complex task plagued by methodological limitations, the heterogeneity of substrates and uncontrolled environmental

events. This work shows the importance of integrating chemical, microbial and toxicological parameters to eventually fully understand and predict long-term field biodegradation processes. Our results show the efficiency of fishbone fertilizers in the first 3 months after the spill. In the following 3 months, all fertilized and unfertilized plots reached the same level of alkane biodegradation. To be useful, fertilizers have to be efficient in the first weeks after the spill by speeding up the natural biodegradation process. The use of fertilizers under severe weather conditions in polar regions seems to be beneficial as low temperatures tend to slow down bacterial growth. Fish composts enriched with nutrients and surfactants are promising efficient and low cost fertilizers which can be improved in the future. The real challenge for chemists and microbiologists working on oil bioremediation in high latitudes might be to formulate fertilizers which will reduce the lasting toxicity of oiled residues. The use of an appropriate surfactant (a neutral molecule with a low HLB) with a natural carrier (with a low available carbon content) seems to be part of the solution.

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