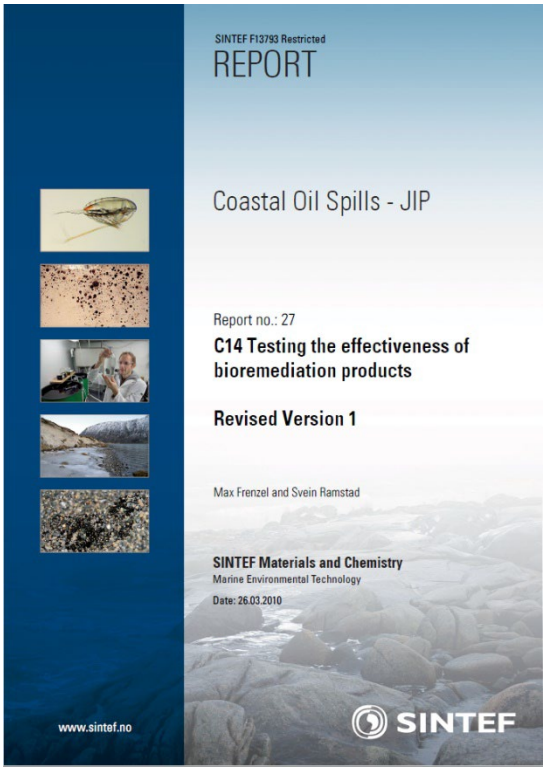
SINTEF




# Report

## Testing the effectiveness of bioremediation products

**Coastal Oil Spills – JIP – report no.: 27**


## Author(s):

Max Frenzel and Svein Ramstad



## Report No:

OC2023 A-026 - Unrestricted


## Client(s) (pos partner):

Det norske oljeselskap ASA, Eni Norge AS, A/S Norske Shell, Statoil Petroleum AS

SINTEF Ocean AS
Postal address:
Postboks 4762 Torgarden
7465 Trondheim, Norway
Switchboard: +47 46415000

Enterprise /VAT No:
NO 937 357 370 MVA

# Report

# Testing the effectiveness of bioremediation products

Coastal Oil Spills – JIP – report no.: 27

**KEYWORDS**
Environment,
Oil
Shoreline
Bioremediation
Effectiveness
Miljø
Olje
Strand
Bioremediering
Effektivitet

**VERSION**
2

**DATE**
2023-03-23

**AUTHOR(S)**
Max Frenzel and Svein Ramstad

**CLIENT(S)**
Det norske oljeselskap ASA, Eni Norge AS, A/S Norske Shell, Statoil Petroleum AS

**CLIENT'S REFERENCE**

**PROJECT NO.**
801064

**NO. OF PAGES**
3 + 36

**SUMMARY**
Downgrading of SINTEF report SINTEF F13793, Originally issued on 2010-03-26 in accordance with the 5 year exclusive rights agreement in the JIP Project Contract.

The report was downgraded by SINTEF Ocean AS, department of Climate and Environment.

The original report is enclosed

**PREPARED BY**
Max Frenzel and Svein Ramstad

**DOWNGRADED BY**
Liv-Guri Faksness

SIGNATURE
Liv-Guri Faksness
Liv-Guri Faksness (24. mar. 2023 09:05 GMT+1)

**DOWNGRADING APPROVED BY**
Trond Rørvik Størseth

SIGNATURE
Trond R. Størseth (27. mar. 2023 13:45 GMT+2)

**REPORT NO.**
OC2023 A-026

**ISBN**
978-82-7174-453-3

**CLASSIFICATION**
Unrestricted

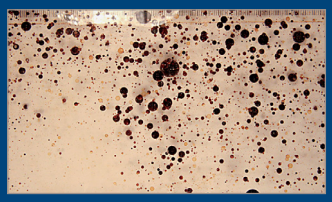
**CLASSIFICATION THIS PAGE**
Unrestricted

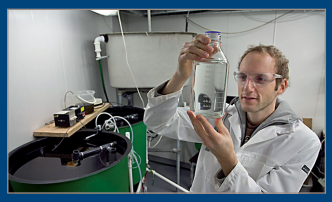
# Document history

| VERSION | DATE | VERSION DESCRIPTION |
|---|---|---|
| Restricted | 2010-03-26 | Revised version 1 |
| Open | 2023-03-23 | Downgraded JIP report |

SINTEF F13793 Restricte(

# REPORT





# Coastal Oil Spills - JIP

Report no.: 27

## C14 Testing the effectiveness of bioremediation products

Max Frenzel and Svein Ramstad

**SINTEF Materials and Chemistry**

Marine Environmental Technology

Date: 21.12.2009

www.sintef.no

SINTEF

# SINTEF REPORT

SINTEF

**SINTEF Materials and Chemistry**

Address: NO-7465 Trondheim, NORWAY
Location: Brattørkaia 17C, 4. etg.
Telephone: +47 4000 3730
Fax: +47 930 70730

Enterprise No.: NO 948 007 029 MVA

TITLE

**C14 Testing the effectiveness of bioremediation products**

**Coastal Oil Spill JIP Report 27**

AUTHOR(S)

Max Frenzel and Svein Ramstad

CLIENT(S)

Det norske oljeselskap ASA, Eni Norge AS, A/S Norske Shell and Statoil Petroleum AS

| REPORT NO. | CLASSIFICATION | CLIENTS REF. | |
|---|---|---|---|
| SINTEF F13793 | Restricted | Edgar Furuhold, Ole Hansen, Gina Ytteborg and Ingeborg Rønning | |
| CLASS. THIS PAGE | ISBN | PROJECT NO. | NO. OF PAGES/APPENDICES |
| Restricted | | 801064 | 33 |
| ELECTRONIC FILE CODE | | PROJECT MANAGER (NAME, SIGN.) | CHECKED BY (NAME, SIGN.) |
| | | Svein Ramstad | Odd Gunnar Brakstad |
| FILE CODE | DATE | APPROVED BY (NAME, POSITION, SIGN.) | |
| | 2009-12-21 | Tore Aunaas, Research Director | |

ABSTRACT

Standardised testing is required to determine the efficacy of products used to stimulate bioremediation of oil stranded on shorelines. This report outlines the experiments testing the efficacy of bioremediation products using two experimental systems: static flask and column systems. In the flask systems thin oil films were immobilised on hydrophobic Fluortex fabrics in seawater, and biodegradation investigated as the effects of different treatments. The column experiments were microcosm experiments involving oiled substrate continual tidal cycling of fresh seawater.

The flask experiments were found to be reproducible within experiments; however, between experiments problems were encountered. This is thought to be due either to heterogeneity of the seawater bacterial population, to preferential use of organic compounds within the products as a carbon source over the oil compounds themselves, or to initial toxic effects of products, resulting in prolonged lag-periods before onset of biodegradation.



| KEYWORDS | ENGLISH | NORWEGIAN |
|---|---|---|
| GROUP 1 | Environment | Miljø |
| GROUP 2 | Oil | Olje |
| SELECTED BY AUTHOR | Shoreline | Strand |
| | Bioremediation | Bioremediering |
| | Effectiveness | Effektivitet |

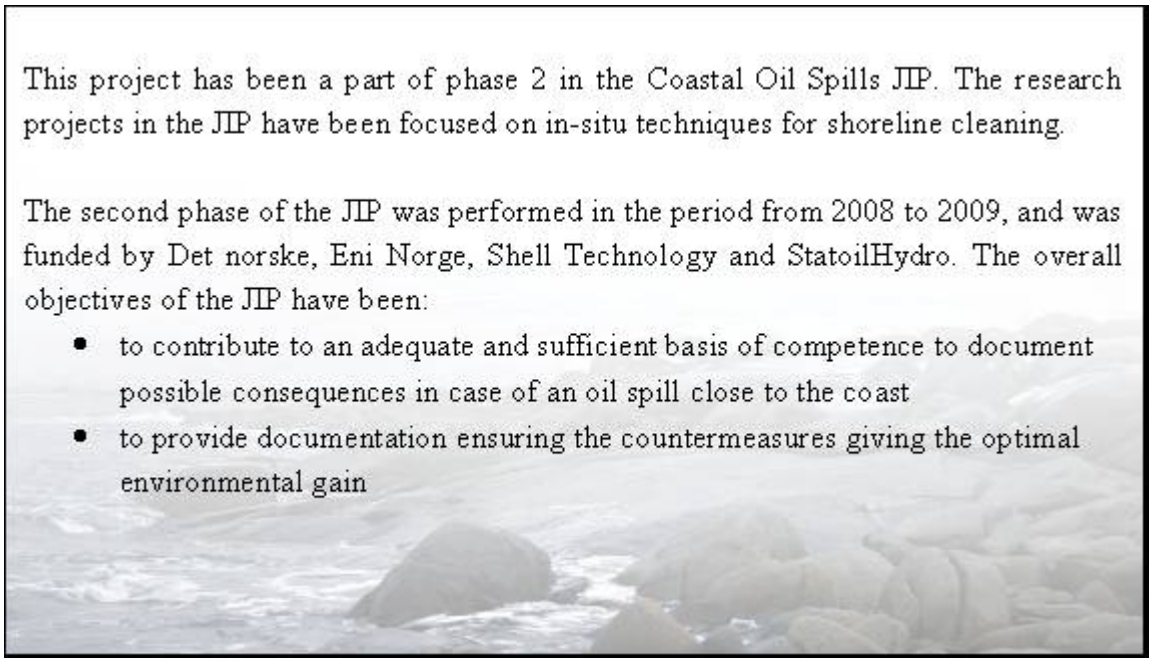
# TABLE OF CONTENTS



# LIST OF FIGURES

## LIST OF TABLES

# 1 Introduction




Oil that is not contained or dispersed at sea will usually land on a shoreline as a reasonably contiguous slick as occurring during the *Exxon Valdez*, *Sea Empress* and *Amoco Cadiz* spills. The oil can appear as tar balls as was shown during the Ixtoc-1 well blow out (Prince and Atlas, 2005). Depending upon the substrate of the beach, different cleaning methods are employed. Physical removal of the bulk oil is important in order to prevent the formation of 'pavements'. Oiled sand can be removed from the shoreline and placed in landfill. Cobbled or pebbled beaches cannot be removed so easily and are thus washed using non-dispersing surfactants. This may allow retention of the oil by booms and recovery. It has become widely acknowledged that bioremediation is not a technique that can be used to mitigate oil spills, rather it is a finishing technique that gives a 'final polish' to an ecosystem after the bulk oil has been removed by other clean-up techniques (manual or mechanical removal, washing).

Bioremediation products generally act through biostimulation—the addition of nutrients to accelerate the natural biodegradation process through environmental modification (Prince and Atlas, 2005)—or bioaugmentation—the addition of specific competent strains or consortia of microorganisms to improve the capacity of a contaminated matrix to remove pollution. In practise many bioaugmentation products also include a nutrient component to stimulate the growth of the microorganisms contained within the product.

Biostimulation products generally fall into one of three different categories: water soluble, slow release and oleophilic. As their name suggests water soluble products dissolve in the water column delivering the nutrients directly to the microorganisms; however, the disadvantage of

these products is that the nutrients may be rapidly washed out through tidal action. Slow release products are in the form of pellets and are encased within a water-impermeable coating. This allows the nutrients inside to leach out slowly, providing a continual supply to the hydrocarbon-degrading bacteria. The third type, oleophilic products, are liquid emulsions containing an external hydrophilic phase, and an internal aqueous phase, usually containing the nutrient and a surfactant to stabilise the microemulsion. The external phase allows the product to readily stick to oil, hence the term 'oleophilic'. Inipol EAP 22 is an example of such a product; it was used extensively during the *Exxon Valdez* cleanup operation (Bragg *et al.*, 1992).

In order for bioremediation products to be deemed successful enhanced oil metabolism must be noted over time after the product is applied when compared to non-treated controls. Testing is therefore required to determine the efficacy of such products before they are widely applied in the environment. To this end standard testing procedures are required to ensure that claims of efficacy can be independently verified.

Both the U.K. and U.S. regulation includes standard bioremediation product test procedures that incorporate a shake flask system. The U.K. system, as described by Swannel *et al*., (1997), is based on determining the extent of oil biodegradation in Erlenmeyer flasks treated with the product, compared to untreated controls. Products are tested on a standard inoculum of oil-degrading bacteria; *Micrococcus luteus* NCIMB 13267, *Pseudomonas fluorescens* NCIMB 9046, *Pseudomonas putida* NCIMB 9571, *Pseudomonas nautical*, and *Alteromonas* sp.; a standard amount of 250°C+ Forties crude oil; and a standard amount of nutrient. A positive control is included to ensure that the system is operating. If evidence of enhanced metabolism is observed in the flask experiments then the product is tested further in microcosm tests. A products is deemed to pass if it significantly enhances oil biodegradation above that of untreated controls ($p<0.05$).

In contrast, the U.S. test system is based on the premise that oil degrading microorganisms naturally present in seawater can biodegrade a large portion of crude oil within 28 days when supplemented with simple nutrients (National Environmental Technology Applications Corporation, 1993). In order for a bioremediation product to be considered effective it must demonstrate statistically greater biodegradation of the oil compared to a control of natural seawater supplemented with simple mineral nutrients. The difference must be statistically significant for both alkanes and aromatics as measured by GC-MS. The U.S. test system does not include microcosm and field monitoring studies, due to the costs involved in running such tests.

The tests described herein utilise two test systems previously established at SINTEF Marine Environmental Technology: a static flask system and a larger column system that includes tidal variation.

# 2 Test systems established at SINTEF Marine Environmental Technology

## 2.1 Flask System

SINTEF Marine Environmental Technology (MET) has established a flask system that assesses the biodegradation and dissolution of oil in seawater. The system consists of immobilised oil on a solid phase (hydrophobic fabrics) that is submerged in natural seawater (Figure 2-1). Biodegradation experiments are then conducted at the desired temperature and appropriate length of time. Degradation of hydrocarbons can be measured by extraction and analysis by gas chromatographic methods, while polar compounds emerging in the water phase as results of oxidative processes (biodegradation) may be analysed by liquid chromatography methods. Additionally, if required, changes in microbial communities may be analysed by PCR-amplification of the 16S rRNA gene by primers common for all bacteria and denaturing gradient gel electrophoresis (DGGE).

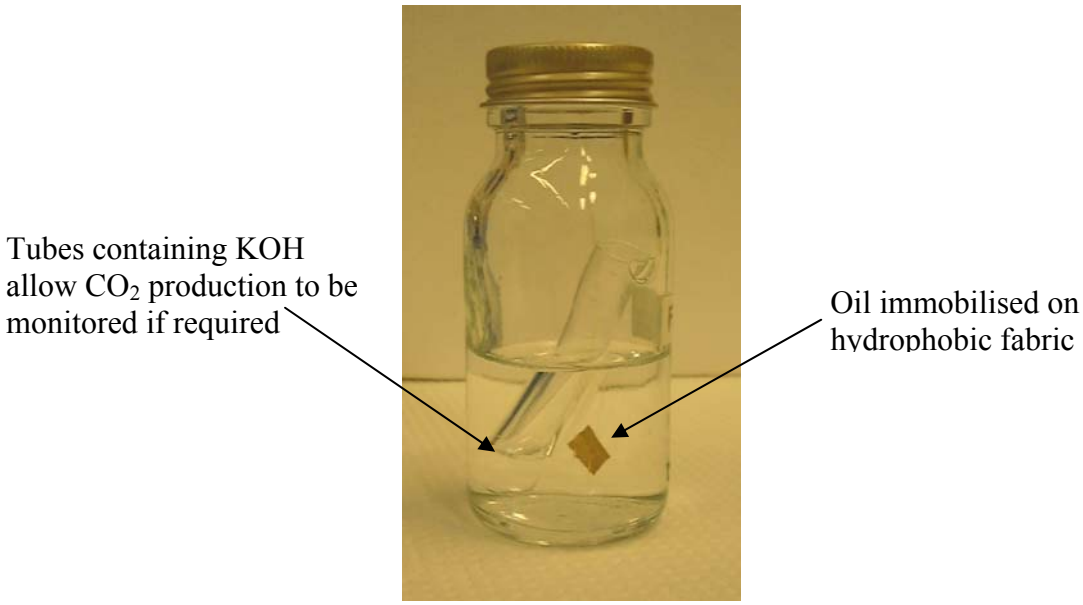



*Figure 2-1 The flask system used at SINTEF MET to investigate the biodegradation of thin oil films in seawater*

## 2.2 Column system

SINTEF also has a column system allowing simulation of tidal variation on shoreline sediment. The system has a continuous supply of seawater and can be operated under temperature-controlled

conditions. It consists of 16 columns, where a series of four columns are operated individually. A reservoir with seawater is connected to the column system via the in- and output valves located at the base of the columns, allowing tidal simulation. A computer program is used to simulate the tidal variation by controlling the number of tidal periods and cycles. An overview of the system is presented in Figure 2-2.

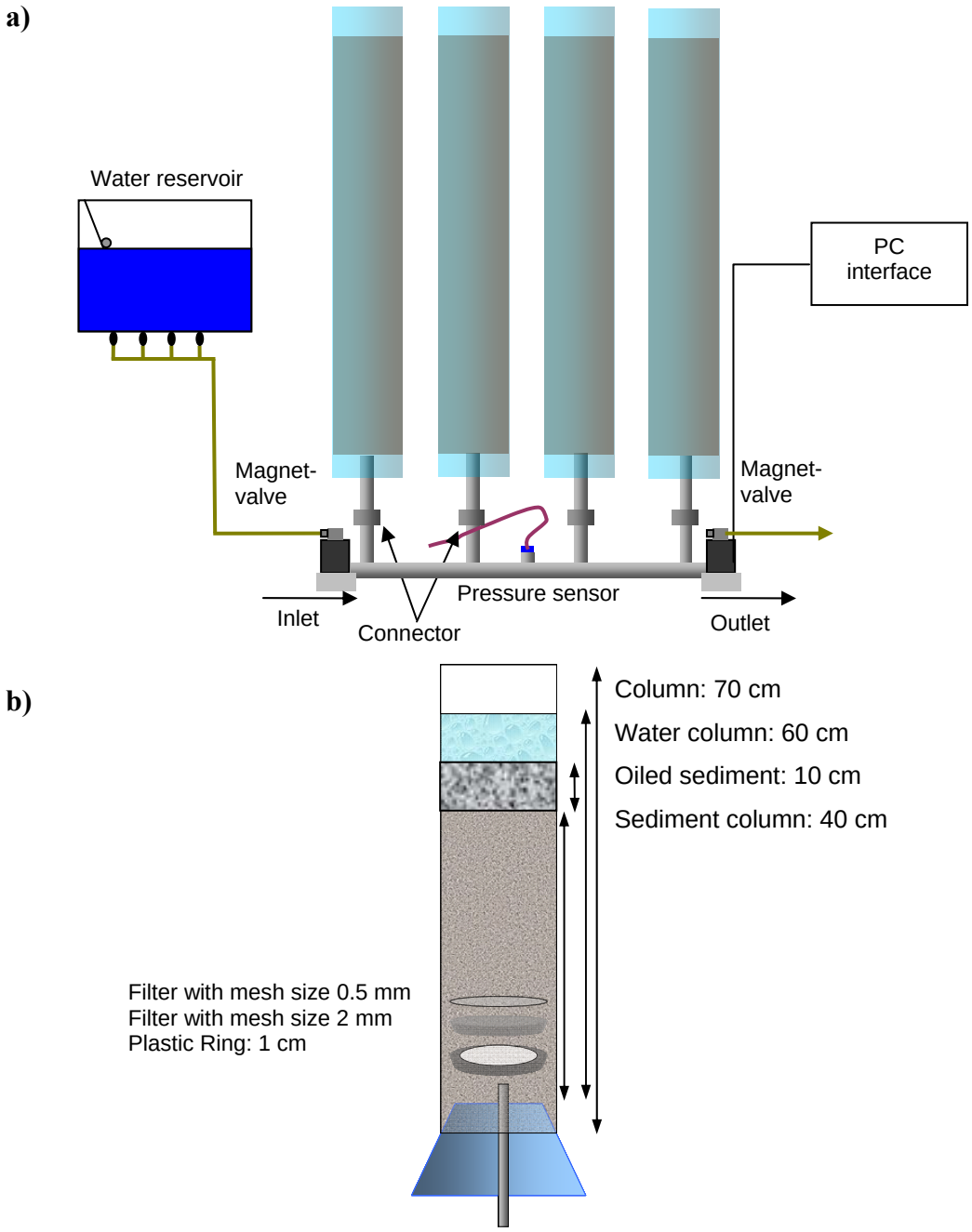



*Figure 2-2 a) The column system setup used at SINTEF. Overview of a single sediment column indicating the size of the column itself, enclosed sediment column and water column. Filter sizes are also shown.*

### 2.2.1 Column design

The columns have a length of 70 cm and an inner diameter of 10 cm; they are made of plexiglass for observation of oil behaviour. The columns are filled with sediment up to 50 cm above the bottom, and a vertical flux of simulated tidal water varies between 10 and 60 cm. To prevent sediment infiltrating into the pipes, two filters with different mesh size are placed in the bottom of each column. Figure 2-2b shows the setup of one column. The use of the data system, SimCol, built up by modules from Labview, allows the flux of seawater to be operated automatically. The height of the sediment- and water column, tidal period and the number of tidal cycles are programmed into the software. The water fluctuation takes the form of a sinus curve, which is similar to natural tidal cycles.

# 3 Materials and Methods

## 3.1 Flask system experiments

### 3.1.1 Oil used in experiments

Fresh Statfjord B crude oil (batch number 97-0264) was used for the test procedures.

### 3.1.2 Generation of oil film and immobilisation on fabric

The oil was immobilised on hydrophobic Fluorotex™ fabrics (Sefar Inc., Thal, Switzerland) for addition to the test flasks in the following manner. 500 ml of autoclaved, deionised water was added to each of seven 500 ml beakers. The Statfjord- B oil was incubated at 30°C for 30 min to dissolve any wax present. 50 µl of the oil was then added to the surface of the deionised water in the beakers to generate a thin oil film (appr. 10µm thickness).

DCM-rinsed Fluorotex™ fabric squares (1 × 1 cm) were carefully added to the oil surface in the beakers for 30 min to allow adsorption of the oil. Each square was then removed and washed in sterile water and secured to individual lengths of fishing line as described previously.

### 3.1.3 Media and bioremediation products

#### 3.1.3.a Product information

A number of products were chosen to include oleophilic, slow release, and water soluble products; and to encompass products specifically designed to treat environmental contaminants as well as commercially available fertilisers. An overview of the products is given in Table 3-1; where available information on the constituents and composition of the product is provided.

*Table 3-1 Overview of the products used in this study.*

| Name | Product Type | Additional information |
|---|---|---|
| S200 | Oleophilic | 7.9% N and 0.6% P, micro emulsion of a saturated urea solution in oleic acid as a carrier, oleophilic phoshate ester, viscosity reducer (Jimenez *et al.*, 2006) |
| Biocrack | Oleophilic | Contains a 'bio-surfactant/emulsifier' and a 'non-ionic surfactant based on vegetable raw materials' C:N:P 17:10:1 |
| Plantagen | Slow release | |
| Blåkorn | Slow release | 10-25 % Ammonium nitrate ($NH_4NO_3$) |
| Substral | Water soluble | |
| Bushnell–Haas | Nutrient Control | Composed of the following (g $L^{-1}$): $MgSO_4$ (0,2); $CaCl_2$ (0,02); $KH_2PO_4$ (1,0); $(NH_4)_2HPO_4$ (1,0); $KNO_3$ (1,0); $FeCl_3$ (0,05) |

### 3.1.3.b Preliminary study

Seawater was obtained from a depth of 90 m from a non-polluted Norwegian fjord (Trondheimsfjord; 63°26’ N, 10°26’ E). The water was collected from a continuous pipeline seawater supply system, and was filtered (50 µm) to remove coarse particles.

Seawater (90 ml) was dispensed into 100 ml autoclaved infusion bottles with butyl rubber septa. Bioremediation products were added to each bottle in the following manner: S200, 200 µl of 1/8 dilution S200 to each serum bottle (0,2 g $L^{-1}$); Blåkorn nitrophoska, three medium sized grains of the fertiliser to each serum bottle (2 g $L^{-1}$); Biocrack, 2 µl to each serum bottle; Plantagen, fertiliser grains ground in a pestle and mortar and 0,18 g added to each bottle (2 g $L^{-1}$); Substral, 85 µl added to 500 ml seawater, 90 ml dispensed into each flask. A sterile control series was included by adding $HgCl_2$ (0,1 g $L^{-1}$) to the seawater, whilst a positive control series of Bushnell–Haas seawater medium was made by adding Bushnell-Haas powder (3,27 g $L^{-1}$; Difco Laboratories, Detroit, Mi, U.S.A.) to seawater.

Individual adsorbents with immobilised oil were submerged into the water of each bottle with the aid of the fishing lines, and the bottles capped. Bottles with immobilized oil in sterilized seawater (50 mg/l $HgCl_2$) were used as sterile controls. Biodegradation experiments were conducted at

13 °C in the dark for 21 days. Adsorbents were removed for chemical analysis after 0, 7, 14, and 21 day incubation.

*Table 3-2 Overview of products and sampling days used in the preliminary study*

| | Sampling day | | | | |
|---|---|---|---|---|---|
| **Test/Product name** | **0** | **7** | **14** | **21** | **Total** |
| **Product 1: S200 (Oleophilic) 1/8 dil. 0,2 g $L^{-1}$** | 1 | 1 | 1 | 1 | **4** |
| **Product 2: Blåkorn nitrophoska (Slow release)** | 1 | 1 | 1 | 1 | **4** |
| **Product 3: Biocrack (Oleophilic) 0,2 g $L^{-1}$** | 1 | 1 | 1 | 1 | **4** |
| **Product 4: Plantagen slow rel. 0,2 g $L^{-1}$** | 1 | 1 | 1 | 1 | **4** |
| **Product 5: Plantagen slow rel. 2 g $L^{-1}$** | 1 | 1 | 1 | 1 | **4** |
| **Product 6: Substral 0,2 g $L^{-1}$** | 1 | 1 | 1 | 1 | **4** |
| **Positive Control: Bushnell–Haas medium** | 1 | 1 | 1 | 1 | **4** |
| **Unfertilised control: Seawater** | 1 | 1 | 1 | 1 | **4** |
| **Sterile control (+$HgCl_2$)** | 1 | 1 | 1 | 1 | **4** |
| **Total** | **9** | **9** | **9** | **9** | **36** |

### 3.1.3.c Detailed study

A detailed study was conducted using a restricted number of products. Seawater was obtained as described above. 72 bottles were set up as above to allow parallel experiments with sampling in triplicate after 0, 3, 5 and 7 days incubation. An overview is given in Table 3-3.

*Table 3-3 Overview of products and sampling days used in final test*

| | Sampling day | | | | |
|---|---|---|---|---|---|
| **Test/Product name** | **0** | **3** | **5** | **7** | **Total** |
| **Product 1: S200 (Oleophilic) 1/8 dil. 0,2 g $L^{-1}$** | 3 | 3 | 3 | 3 | **12** |
| **Product 2: Blåkorn nitrophoska (Slow release)** | 3 | 3 | 3 | 3 | **12** |
| **Product 3: Biocrack (Oleophilic) 0,2 g $L^{-1}$** | 3 | 3 | 3 | 3 | **12** |
| **Product 4: Plantagen slow rel. 0,2 g $L^{-1}$** | 3 | 3 | 3 | 3 | **12** |
| **Positive Control: Bushnell–Haas medium** | 3 | 3 | 3 | 3 | **12** |
| **Unfertilised control: Seawater** | 3 | 3 | 3 | 3 | **12** |
| **Sterile control (+$HgCl_2$)** | 3 | 3 | 3 | 3 | **12** |
| **Total** | **21** | **21** | **21** | **21** | **84** |

Seawater (90 ml) was dispensed into 100 ml autoclaved infusion bottles with butyl rubber septa. Bioremediation products were added to each bottle in the following manner: S200, 200 µl of 1/8 dilution S200 to each serum bottle (0,2 g $L^{-1}$); Blåkorn nitrophoska, three medium sized grains of the fertiliser to each serum bottle (2 g $L^{-1}$); Biocrack, 2 µl to each serum bottle; Plantagen, fertiliser grains ground in a pestle and mortar and 0,18 g added to each bottle (2 g $L^{-1}$). A sterile control series was included by adding $HgCl_2$ (0,1 g $L^{-1}$) to the seawater, whilst a positive control series of Bushnell Haas seawater medium was made by adding Bushnell–Haas powder (3,27 g $L^{-1}$; Difco) to seawater. Individual adsorbents with immobilised oil were submerged into the water of each bottle with the aid of the fishing lines, and the bottles capped. Biodegradation experiments were conducted at 13 °C in the dark for 7 days. Adsorbents were removed in triplicate for chemical analysis at days 0, 3, 5, and 7.

### 3.2 Chemical analysis

Adsorbents were placed in 30 ml DCM. The solvent was dried ($Na_2SO_4$), filtered (glass wool), and evaporated to volumes of 0.5–1.0 ml in 2 ml GC vials on a TurboVap 500 closed cell concentrator (Zymark Co., Hopkinton, Ma, U.S.A.).

### 3.3 Column experiments

#### 3.3.1 The column system

The sediment system consists of 16 columns, where a series of 4 columns are operated individually. A reservoir with temperate seawater is connected to the column system and the in- and output of seawater is placed at the bottom of the columns for tidal simulation. A computer program is used to simulate the tidal variation by controlling the number of tidal period and cycles. Figure 2-2 shows the set up of the column system with columns, pressure sensors for regulation of water level, valves, in- and outlets from the water reservoir, and monitoring equipment.

#### 3.3.2 Sediment

The grain size distribution of the sediments in a shoreline system is assumed to be an important parameter for the fate of the oil during stranding. Shoreline sediments contain fractions classified over a broad spectrum, mainly varying between sand and gravel. The sediment grains have a spherical form due to different weathering processes. During the experiments sediment with a

limited variation in grain size was preferred in order to achieve reproducible experimental conditions.

There are many classification scales for sediments, and the boundaries between the classification systems vary. The sediment used in this study was chosen on the basis of the Wentworth classification scale for sediments (www.wikipedia.no; Table 3-4). The sediment grain size distribution used in this study was 2–6 mm, corresponding from very course sand to very fine gravel.

*Table 3-4 Sediment fractions and properties*

| Size [mm] | Aggregate name | Porosity [Φ] (approx.) | Permeability [mD] (approx.) |
|---|---|---|---|
| 0,6–1,4 | Coarse sand | 1 to 0 | $10^5$ |
| 1,4–2,8 | Very coarse sand | 0 to -1 | $10^6$ |
| 2,8–6,3 | Very fine gravel | -1 to -2 | $10^7$ |
| 8–16 | Medium gravel | -3 to -4 | $10^8$ |

The porosity of a medium, such as sediment, describes the fraction of void space in that material; the larger the porosity, the larger the amount of void space for containment of liquids.

Permeability is a measure of the ability of a medium to transmit fluids. The permeability of sediments are dependent on the size of the pores. Sand will generally have a high porosity due to several small void spaces per cubic meter, but low permeability because the voids are too small for fluid transportation. Gravel gives a high permeability due to fewer, but larger, void spaces; the sediment will therefore have a lower porosity (Brattli, 1999).

The sediment used in this study was supplied by Trondheim Mørtelverk AS and originates from glacial- and river deposits in Sør-Trøndelag. The sediment is therefore natural and rounded. In addition the sediment was fractioned and washed to remove unwanted dust, making the sediment representative of shoreline sediment.

### 3.3.3 Oil used in the column system

The oil used in the column experiments was a 250°C+ topped fraction. This was used to simulate the weathering that an oil has undergone (and will undergo) during a spill and subsequent stranding on a beach.

### 3.3.4 Experimental procedures

The columns were filled with a 40 cm column of pre-washed sediment. The final 10 cm of the column was filled with oil-coated sand and bioremediation product to simulate the stranded oil on the surface of the beach, and treatment with a bioremediation agent. The oiled sand was prepared in the following manner: using a cement mixer approximately 10L of the same sediment was blended for approximately 1 min with 200 ml of the test oil (250°C+ fraction). For each column and treatment 750 ml of the oiled sediment was removed and mixed with the appropriate bioremediation agent in a plastic box. The treated, oiled sediment was then added as a 10 cm thick layer to the top of the sediment column, bringing the total height of the column to 50 cm.

### 3.3.5 Control and monitoring system – SimCol

With the use of the data system, SimCol, built up by modules from Labview, the flux of seawater could be operated automatically. The height of the sediment- and water column, tidal period and the number of tidal cycles were inserted into the data system. The water fluctuation takes the form of a sinus curve, which is similar to natural tidal cycles. Figure 3-1 shows the graphical appearance of SimCol.

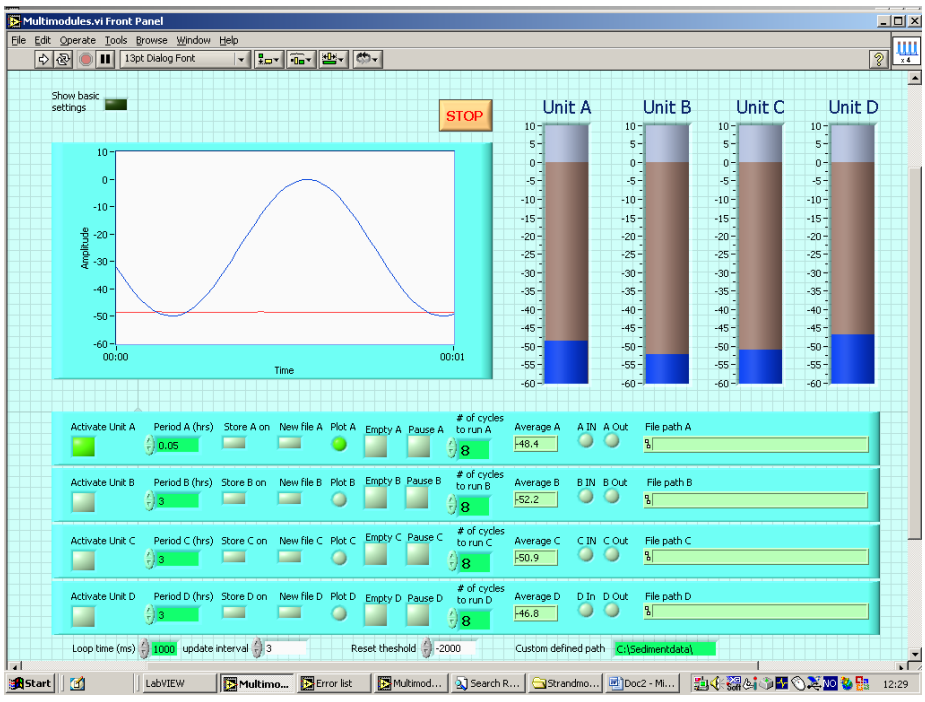



*Figure 3-1 Graphical appearance of SimCol*

### 3.3.6 Sediment sampling and analysis

The columns were placed in a temperature-controlled room at 13°C. Experiments were run for 28 days; after this time samples of the top layer of oiled sediment were taken for analysis. Sediment plugs approximately 2 cm long were removed in triplicate and extracted using DCM. Extracts were analysed using GC-FID.

## 3.4 *Skeletomema costatum* toxicity tests

The test procedure is described in the ISO Guideline ISO/DIS 10253 "Water quality – Marine algae growth inhibition test *Skeletonema costatum* and *Phaeodactylum tricornutum*", 1991. Briefly, the clone *Skeletonema costatum* Bac 1 was used as test culture. The culture was kept as stock culture in our laboratory, and the algal culture was inoculated to each prepared test solution and to algal medium without test substance (controls) to give final initial densities of $3\times10^3$ cells/ml. All test tubes were incubated with agitation under constant light intensity (60-120 $\mu E/sec/m^2$) at a temperature of 20± 2°C for 72 hours. *In vivo* chlorophyll fluorescence was measured daily in a fluorometer. Growth rates of each culture were determined by linear regression and as the area under the growth curves (biomass increase). Chemical inhibition of the algal *in vivo* photosynthesis was estimated at different concentrations. The concentrations (mg/L) inhibiting algal photosynthesis by 50 % ($EC_{50}$), by 10 % ($EC_{10}$), and by 90 % ($EC_{90}$) relative to the control cultures were determined with the 95% confidence interval. Calculations were performed with the computer program Toxedo (Water Quality Institute, Hørsholm, Denmark).

# 4 Results and discussion

## 4.1 Flask studies

### 4.1.1 Preliminary test.

A preliminary test was carried out with different bioremediation products for 14 days, with samples extracted and analysed after 0, 7 and 14 days incubation at 13°C. After this time the Fluoretx fabric squares were removed from the test vessels and the remaining oil extracted and analysed by GC-FID. Oil biodegradation was quantified by comparing *n*-$C_{18}$/phytane ratios among samples within the series and by quantification of the Total Hydrocarbon Content (THC). Figure 4-1 shows the *n*-$C_{18}$/phytane ratio of the preliminary test samples after 0, 7 and 14 days incubation at 13°C: a temperature relevant for coastal Norwegian surface seawater in the summer season.

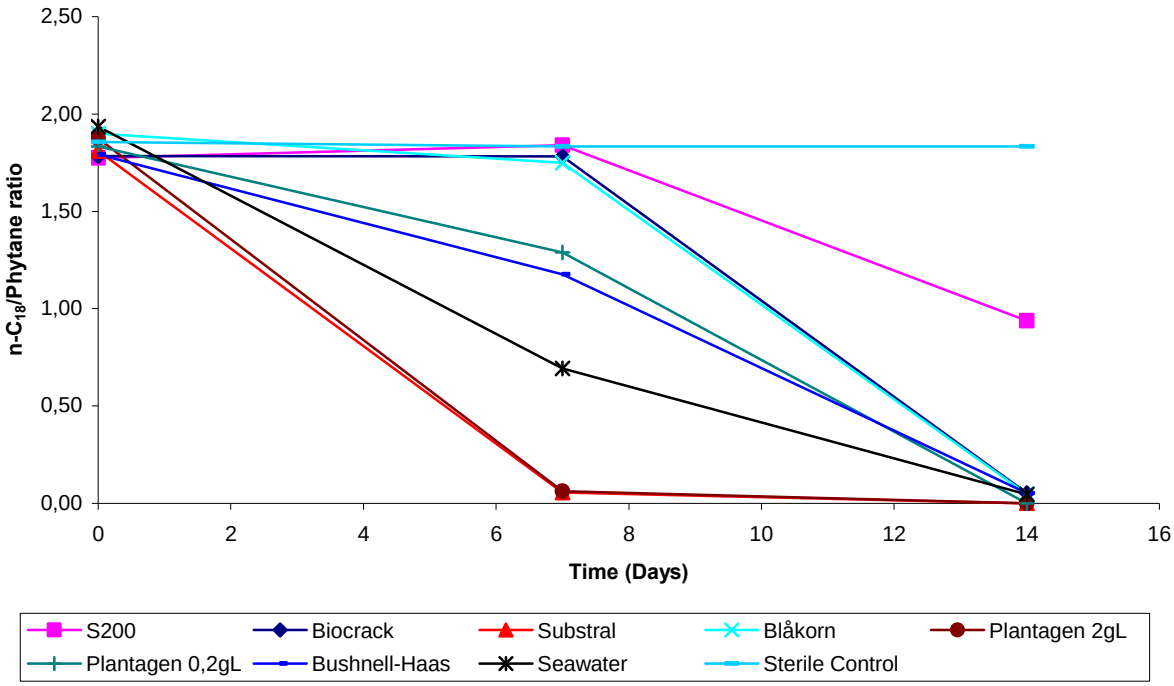



*Figure 4-1 Oil degradation of the remaining oil recovered from the Fluortex fabrics after incubation with seawater in combination with various bioremediation products.*

All test products resulted in a decrease in the *n*-$C_{18}$/phytane ratio from the initial ratio (Figure 4-2), indicative of oil biodegradation (Figure 4-1). After seven days incubation at 13°C the *n*-$C_{18}$/phytane ratio of both the Substral (Figure 4-1) and Plantagen (2 g $L^{-1}$) treatments was reduced

to ≤ 0.05. Interestingly the seawater control exhibited the next largest reduction in *n*-$C_{18}$/phytane ratio to 0.69 (Figure 4-4 and 4-1); larger than the positive nutrient control Bushnell-Haas medium. Plantagen at a concentration of 0.2 g $L^{-1}$ resulted in a reduction of the ratio to 1.29 after seven days. The Blåkorn treated flasks exhibited a small reduction in the ratio from 1.90 to 1.75 between day 0 and day 7, whilst the ratios for S200, Biocrack and the sterile control remained unchanged after one week of incubation. After 14 days all products except S200 had reduced the ratio to ≤ 0.05 while the S200 treated sample had a ratio of 0.94 (Figure 4-5 and 4-1).

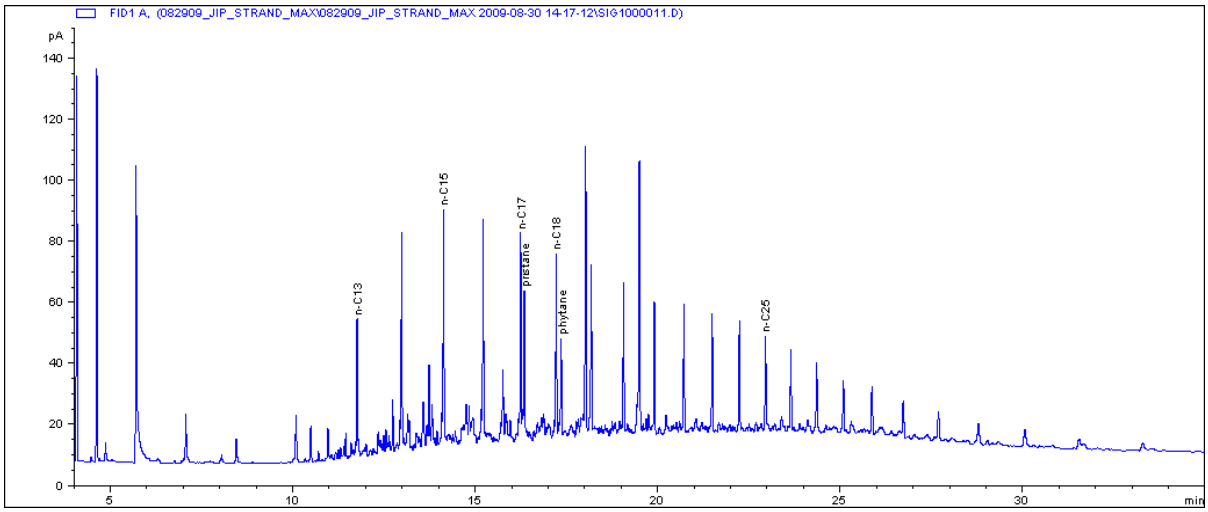



*Figure 4-2 Total Ion Chromatogram (TIC) of initial oil recovered from the Fluortex fabrics incubated in seawater alone*

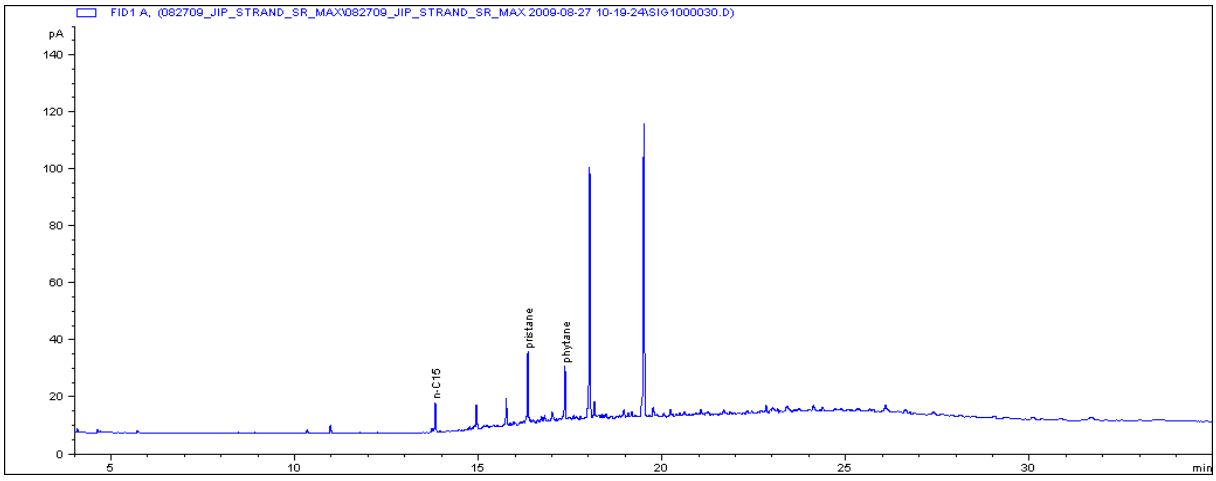



*Figure 4-3 TIC of recovered immobilised oil film from substral inoculated sample after 7 days incubation*

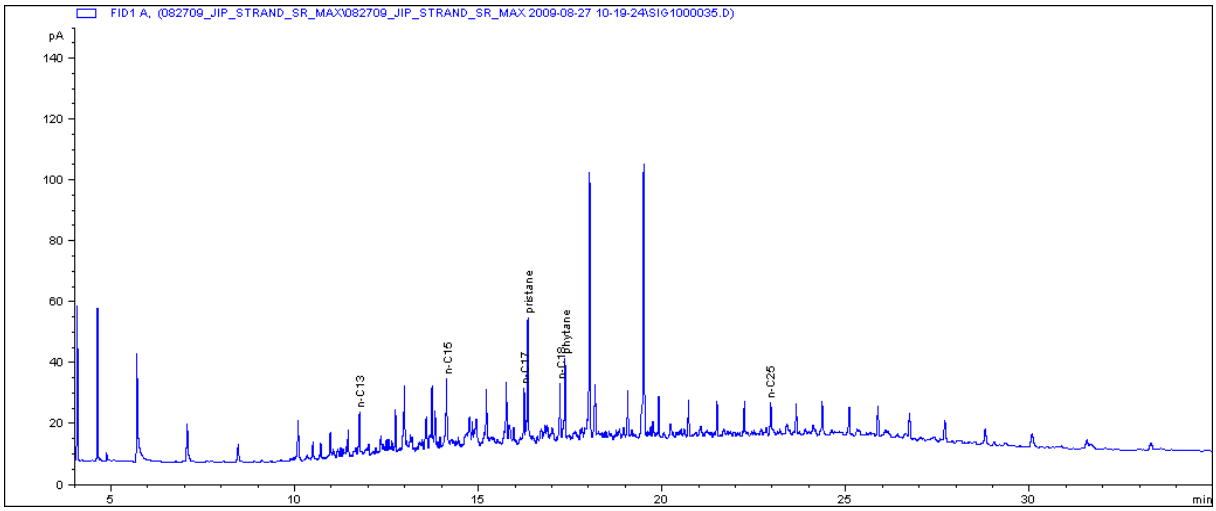



*Figure 4-4 TIC of recovered immobilised oil film from seawater control sample after 7 days incubation. $nC_{18}$/phytan peaks at approximately17-17.5 minutes retention time*

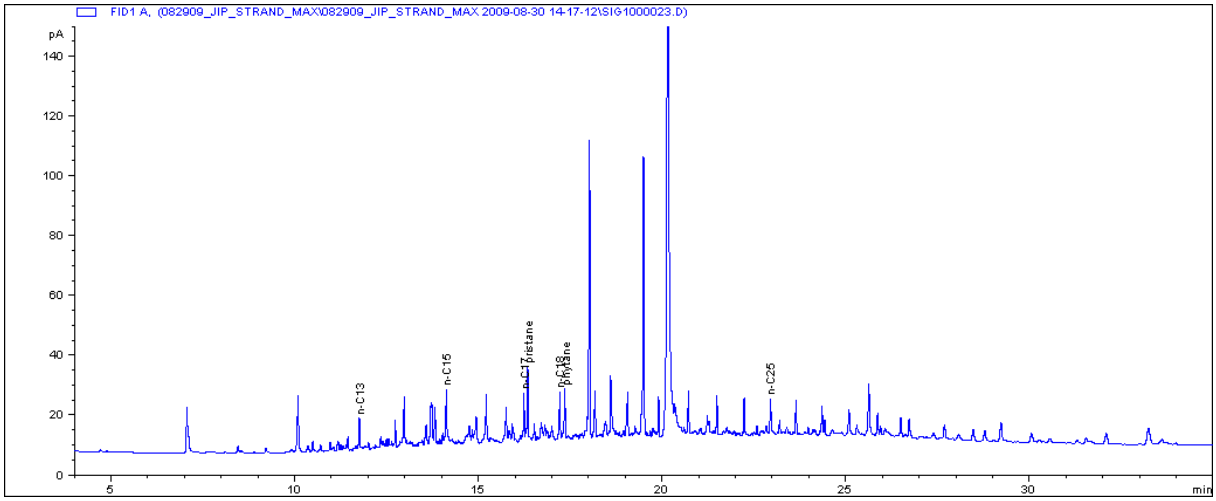



*Figure 4-5 TIC of recovered immobilised oil film from S200 inoculated sample after 14 days incubation*

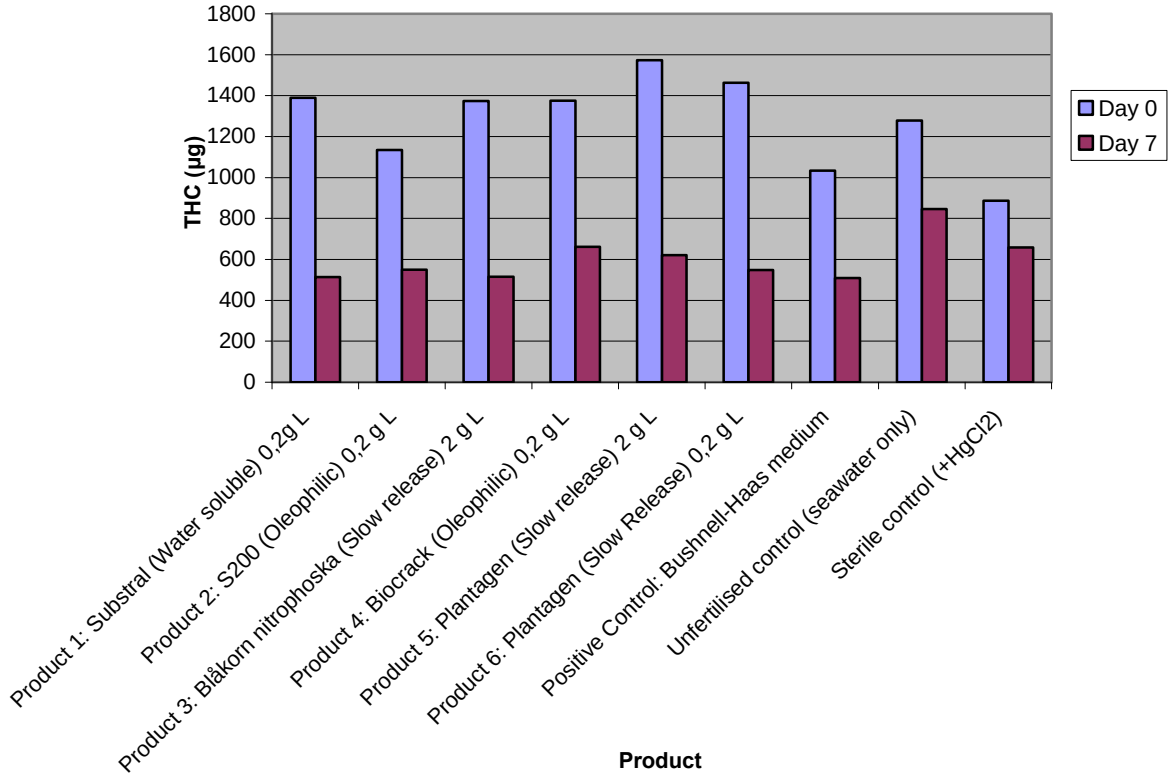



*Figure 4-6 THC ($C_{10}$–$C_{40}$) recovered from Fluortex fabrics coated with Statfjord B oil films initially (blue bars) and after 7 days incubation in seawater with various bioremediation products (maroon bars) during the preliminary experiment.*

The initial recovered THC values (Figure 4-6) varied within a range of 886 to 1573 886 µg. After seven days incubation a reduction was observed in all treatments, including the sterile control, albeit a minor reduction. This decrease in the sterile control value likely represented losses of the more soluble oil compounds (e.g. lower molecular weight aromatic compounds) from the oil film to the water phase through dissolution.

Complete biotransformation of n-$C_{18}$ was observed after 14 days incubation at 13°C in several test flasks(Figure 4-1), indicating that an incubation period of this length is too long. The ideal time frame should allow differences between the efficacies of the products to be distinguished in order to allow a comparison.

Based on these results, a test period of 14 days appeared to be too long: significant biodegradation of the oil could be observed after seven days incubation. This length of time also allowed differences to be observed between the different treatments: some had reduced the *n*-$C_{18}$/phytane

ratio to zero whilst others have had no discernable effect compared to the control or result in intermediate ratios (Figure 4-1).

### 4.1.2 Detailed study

Following the preliminary study a longer, more detailed study was established to test the efficacy of the bioremediation products. Products included were S200, Blåkorn, Biocrack and Plantagen. Bottles were incubated for 0, 3, 5 and 7 days at 13°C with oil films immobilised on Teflon fabrics as previously described. The oil film was extracted from the Teflon squares and analysed by GC-FID. Biodegradation was quantified by examining the changes in *n*-$C_{17}$/pristane, *n*-$C_{18}$/phytane and pristane/phytane ratios, as well as THC ($C_{10}$–$C_{40}$) over the incubation period. Figure 4-3 shows the *n*-$C_{17}$/pristane ratios of the extracted oil over the course of the experiment, which sha\ows great similarities to the n$C_{18}$/phytan in Figure 4-8, which is mainly used to discuss oil degradation.

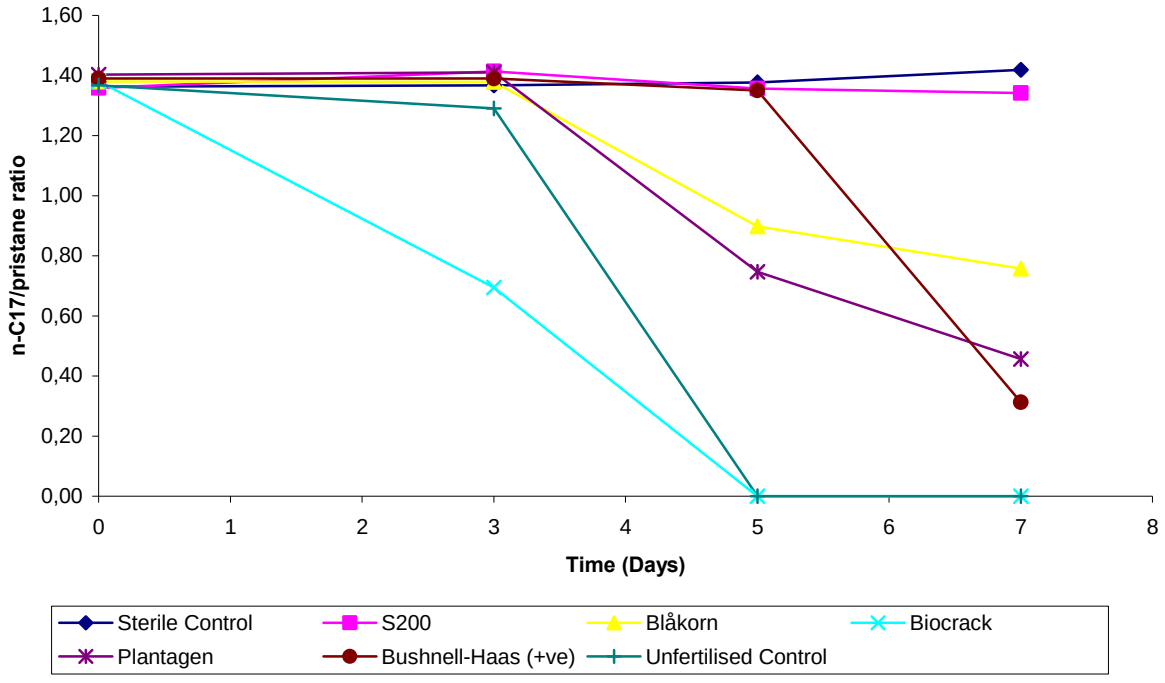



*Figure 4-7 $C_{17}$/pristane ratios recovered from the extracted samples measured by GC-FID*

In all treatments the initial *n*-$C_{17}$/pristane ratio is 1.4 ± 0.04. After three days incubation two treatments showed a reduction in the *n*-$C_{17}$/pristane ratio: those incubated with Blåkorn and the unfertilised control treatment, with values reduced to 0.69 and 1.29 respectively. After five days incubation the ratio of *n*-$C_{17}$/pristane in both treatments was reduced to zero, indicating extensive biodegradation of *n*-$C_{17}$. The Plantagen and Blåkorn products showed a reduction in ratio values

to 0.75 and 0.9 respectively, whilst the ratios of the S200 and Bushnell-Haas treated samples remained unchanged. After seven days incubation biodegradation was evident in all samples except the sterile control and those treated with S200, indicative of a lack of significant biodegradation in these samples.

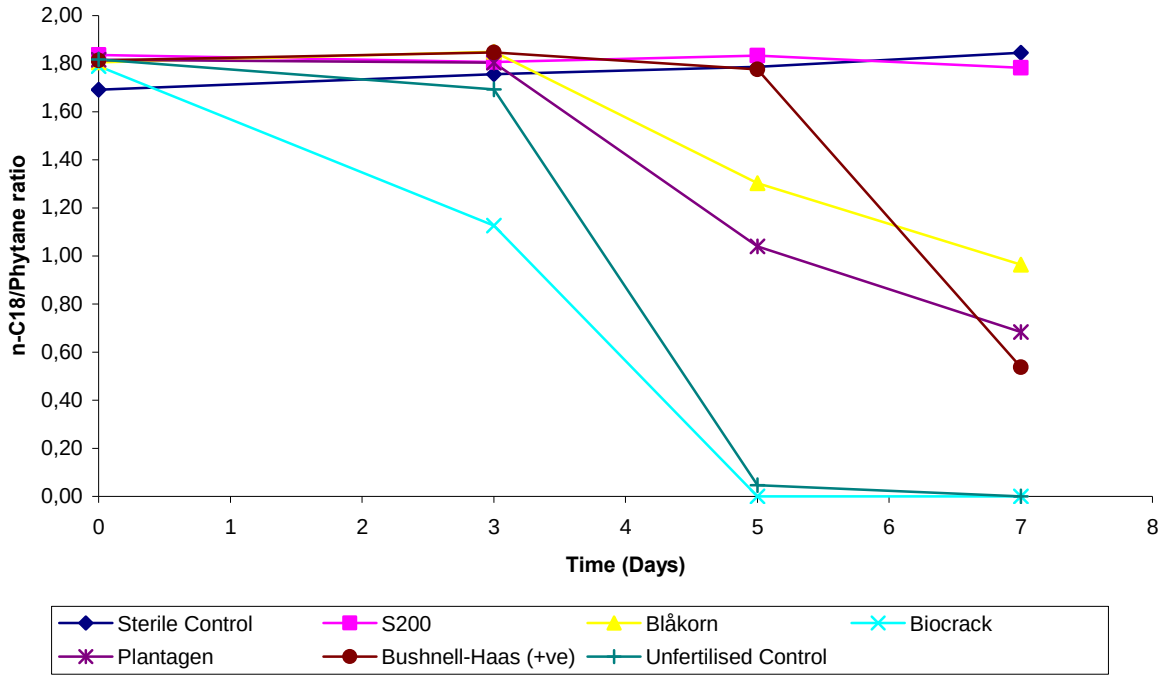



*Figure 4-8 $C_{18}$/phytane ratios recovered from the extracted samples measured by GC-FID*

Figure 4-8 shows the *n*-$C_{18}$/phytane ratio of the oil recovered from the samples. The curve shapes are very similar to those observed for the *n*-$C_{17}$/pristane ratios (Figure 4-7). The initial ratio values of all samples except the sterilised control were in the range 1.81 ± 0.03. The average ratio of the sterilised control was 1.69. After three days incubation a reduction in the *n*-$C_{18}$/phytane ratio was measured in the samples treated with Biocrack; no change was measured in the *n*-$C_{18}$/phytane ratio of the samples subjected to the other treatments. After five days incubation the ratio of *n*-$C_{18}$/phytane was reduced to zero in both the Biocrack and unfertilised controls, indicating extensive biodegradation of *n*-$C_{18}$. Reductions in the ratio were also observed in the samples incubated with Blåkorn and Plantagen; however, no reduction in the *n*-$C_{18}$/phytane ratio was measured in the sterile control, S200- or Bushnell–Haas-treated samples. After 7 days incubation the *n*-$C_{18}$/phytane ratios Bushnell–Haas, Plantagen and Blåkorn samples were reduced to 0.54, 0.68 and 0.96 respectively. No reduction in the *n*-$C_{18}$/phytane ratio was observed in the sterile control or Bushnell–Haas-treated samples.

Examination of the pristane/phytane (pr/ph) ratio in the sample can also be used to assess the extent of biodegradation that the oil has undergone and, due to the increased recalcitrance of phytane, gives an indication of whether the biodegradation has affected all resistant components equally (Figure 4-9).

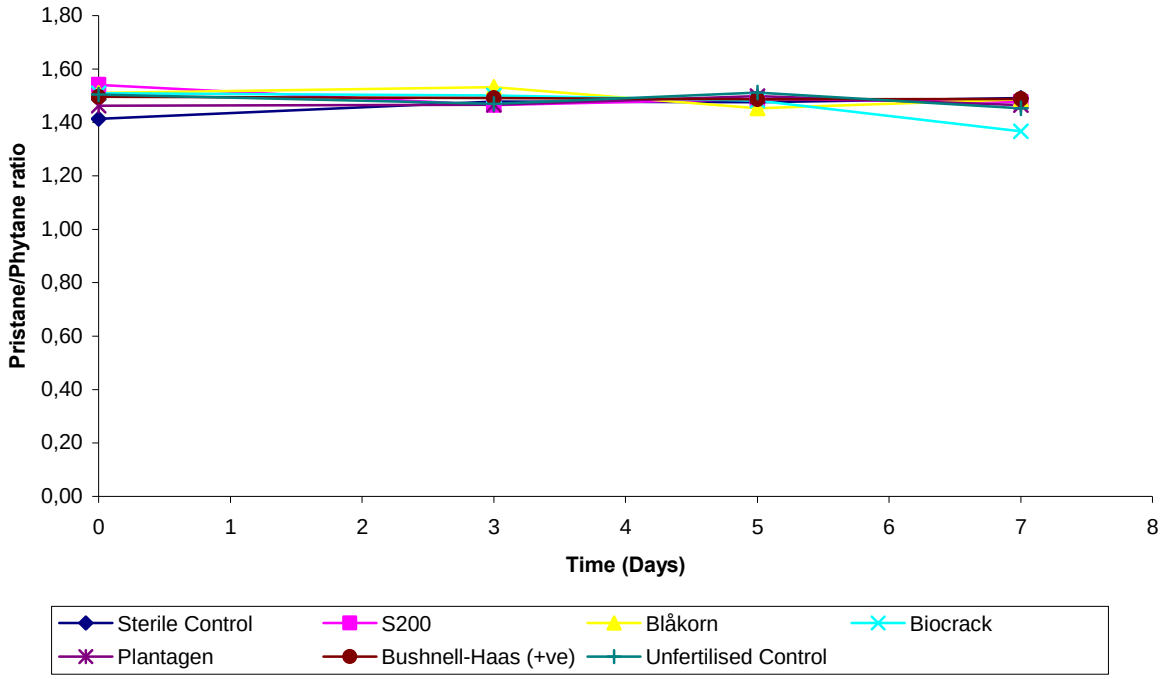



*Figure 4-9 Pristane/phytane ratios for the biodegraded oil film extracts measured by GC-FID.*

The pr/ph ratios of all samples are stable throughout the course of the experiment within the range of 1,5 ± 0,1. The stability of the ratio indicates that biodegradation of both compounds proceeds at a similar rate (or not at all).

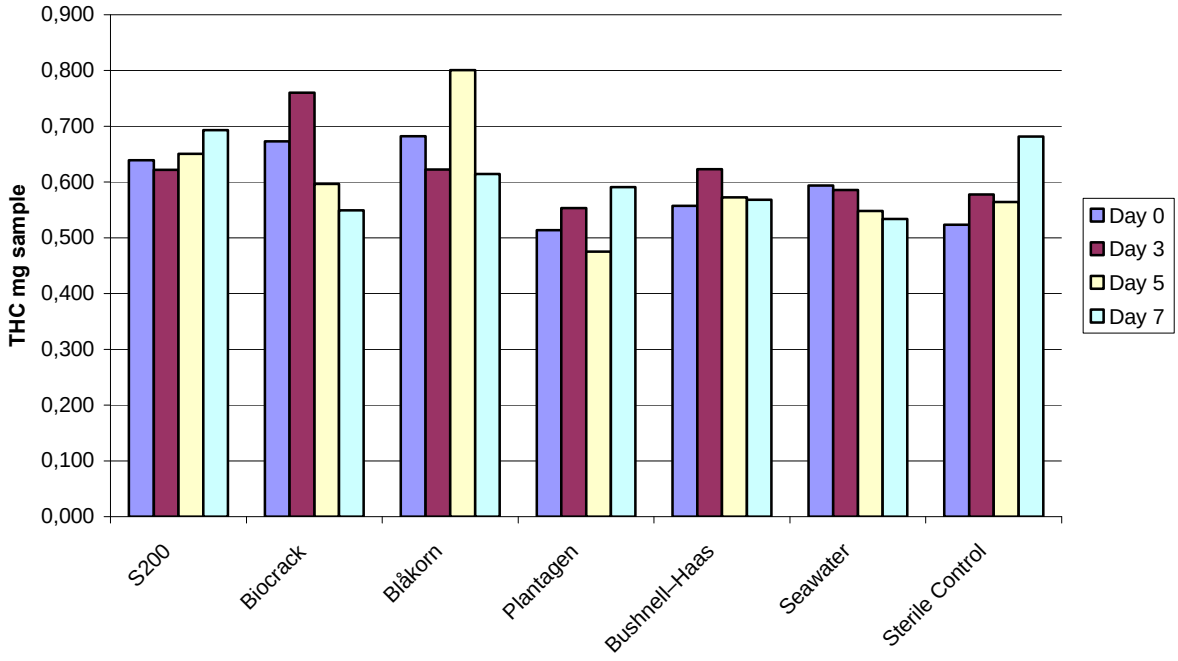



*Figure 4-10 THC ($C_{10}$–$C_{40}$) extracted from the Teflon® fabrics at each time point during incubation with the bioremediation products (n = 3)*

Figure 4-10 shows the THC extracted from the fabrics over the course of the experiments. Flasks treated with Biocrack exhibited the fastest oil biodegradation rate (as measured by *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios). Interestingly, the next most effective treatment in the study was seawater alone, which exhibited faster biodegradation of the oil than the remaining products in seawater. This may be attributed to:

1. Heterogeneity of the bacterial populations within each test flask.
2. Organic compounds within the bioremediation product acting as a preferred substrate for the bacteria within the flask.

The seawater used in this study was piped from 90m below the surface of a non-polluted Norwegian fjord. The water is filtered and standard practise is to run the tap for 5 min before collection to ensure that it does not contain water that has been sitting in the pipes for long periods. The water is also collected in large plastic carboys allowing free mixing during collection. Heterogeneity between the bacterial populations within different flasks should therefore be minimised. This could be assessed by using molecular genetic techniques such as

DGGE to fingerprint the community within each flask; alternatively, a standard mix of strains could be used.

Alternatively, the bacteria may be using compounds within the bioremediation products as a source of carbon and energy before metabolising the oil components, leading to an increased rate of biodegradation in the seawater-only flasks compared to those that contain bioremediation products. Oleophilic fertilisers contain organic compounds such as surfactants and emulsifiers that bacteria may use as sources of carbon. Similarly, slow release fertilisers are encased within organic matrices that allow the nutrients to leach out over time. Possibly these organic components may be utilised by the bacteria within the seawater in the place of the oil compounds. In addition, naturally occurring organic compounds within the seawater may compound this problem. One possible solution to this would be to allow the seawater to stand for aging in containers for 5–7 days before use; this would allow any residual organic compounds in the seawater to be metabolised before use in the biodegradation tests. A third explanation could be linked to the toxicity of the products; With a toxicity of 15 mg/l while the test concentrations were 200 mg/L the high concentration used in the experiments could affect the bacteria, resulting in a prolonged lag-period. The toxicity of some of the products may therefore be one additional reason for the reduced biodegradability when compared to the control (see Figure 3-7 and 4-9).

### 4.2 Column system studies

The columns were packed with beach sediment, the upper 10 cm of which had been pre-mixed with oil and a bioremediation product (Figure 2-2b). The columns were connected to a seawater reservoir and experienced a tidal variation in water height. The experiments were run at 13°C for 28 days. After this time cores of the oiled sediment were removed for chemical analysis.

The initial experiment looked at the reduction in THC after 28 days, compared to an unfertilised control, when 10 g (or 10 ml for liquid products) of each product type (water soluble, agricultural fertiliser, slow release and oleophilic) was mixed with the sediment. Figure 4-11 presents the recovered THC values from each of the treatments.

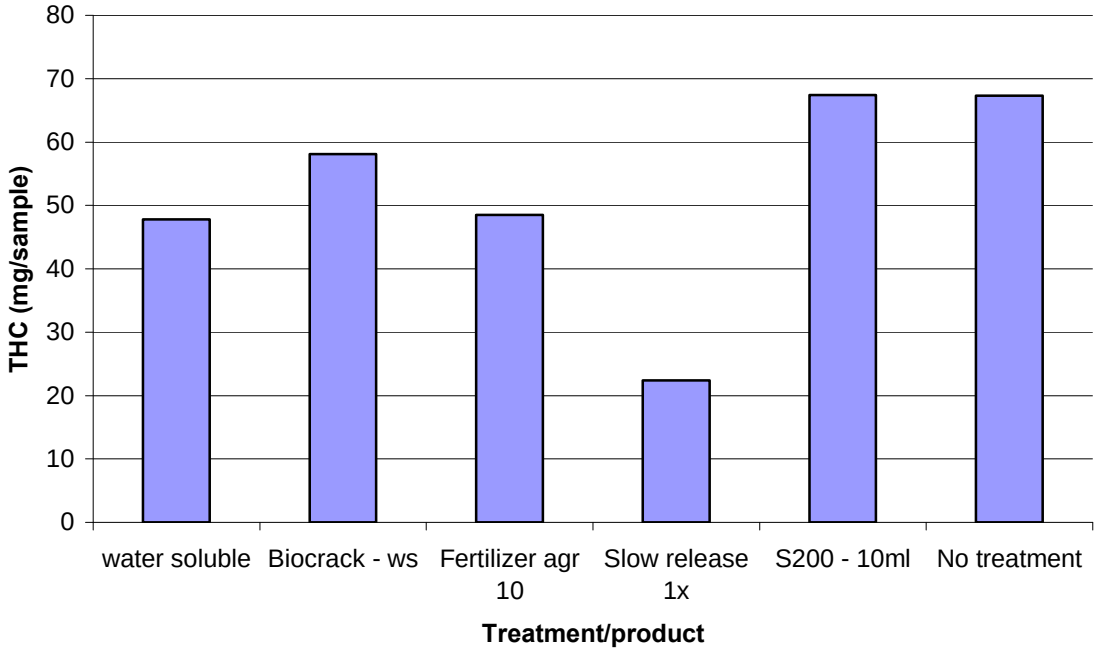



*Figure 4-11 Initial column study results indicating the quantity of oil remaining after 28 days incubation with bioremediation products*

A difference in THC values were observed between all treatments and the control except with the oleophilic product. The most effective treatment in reducing the THC of the sediment samples was application of the slow release fertilizer (10 g), which resulted in a reduction to 33% that of the control amount. The oleophilic fertiliser S200 performed no better in reducing the THC burden in the sediment than the control (without treatment) whilst the water soluble, biocrack and slow release fertilisers resulted in reduction to 71%, 86% and 72% that of the control amount respectively.

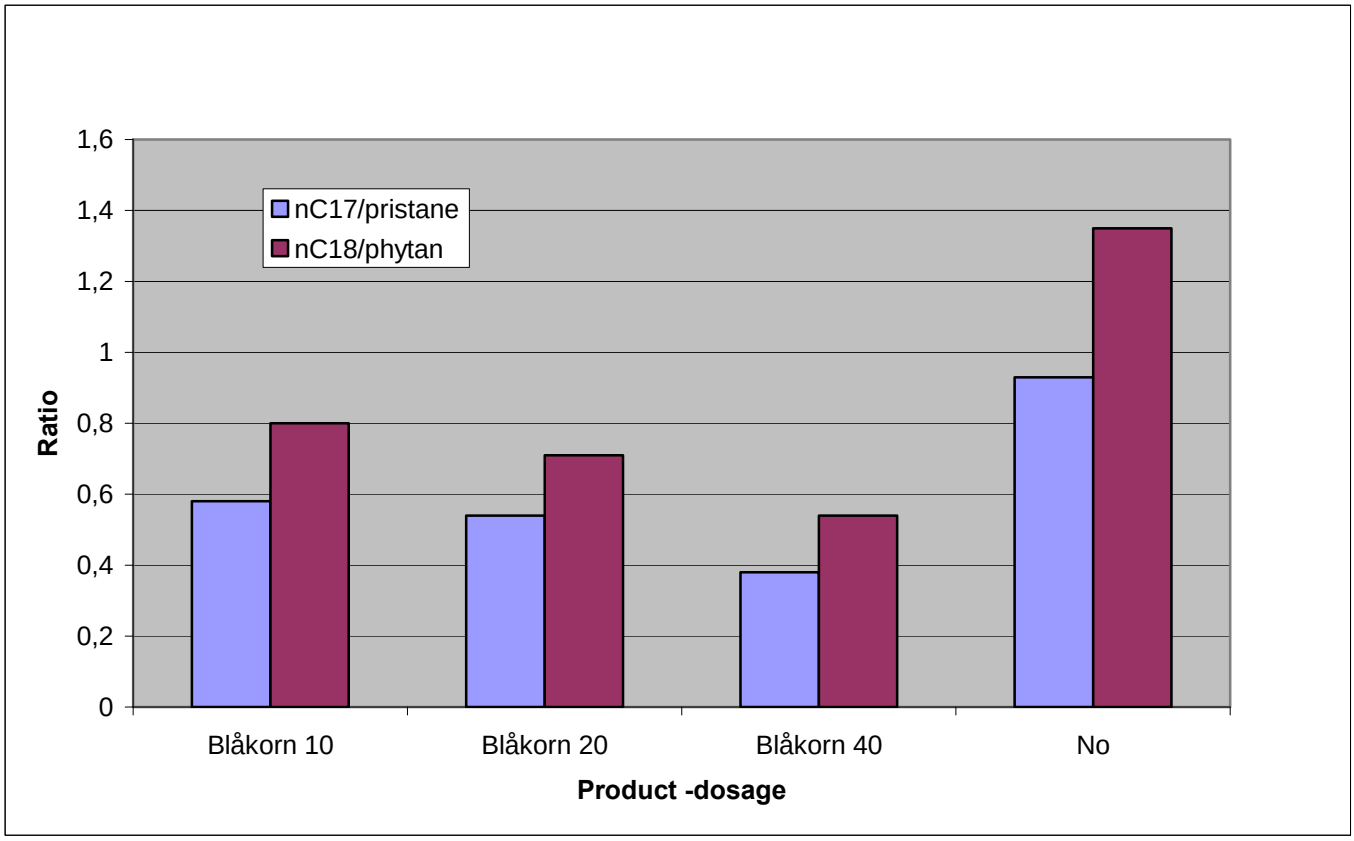



*Figure 4-12 Effect of increasing product dose (agricultural fertiliser - Blåkorn); g) on the recovered* n-$C_{17}$*/pristane and* n-$C_{18}$*/phytane ratios from the sediment columns after two weeks of simulated tidal cycles at 13°C*

Figure 4-12 shows the effect of increasing product dose of the agricultural fertiliser on the recovered *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios. Without fertiliser application *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios of 0.93 and 1.35 were recorded respectively. Application of 10 g of fertiliser resulted in reduction of the *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios to 0.58 and 0.8 respectively whilst application of 40 g reduced the ratios to 0.38 and 0.54 respectively. Application of increased amounts of fertiliser does appear to be more effective in this case; however, doubling the amount of fertiliser added does not double the amount of oil biodegraded. The extra benefits obtained (as observed with this system) may not outweigh the cost involved in adding additional fertiliser. Also it is possible that the application of 10 g of fertiliser will reduce the biodegradation level to that observed with 40g of fertiliser, it may just take a longer time period.

A similar trend was observed with increasing application of the oleophilic fertiliser (Figure 4-13); however, although the ratios were also reduced, the effects were smaller compared to the agricultural fertiliser.

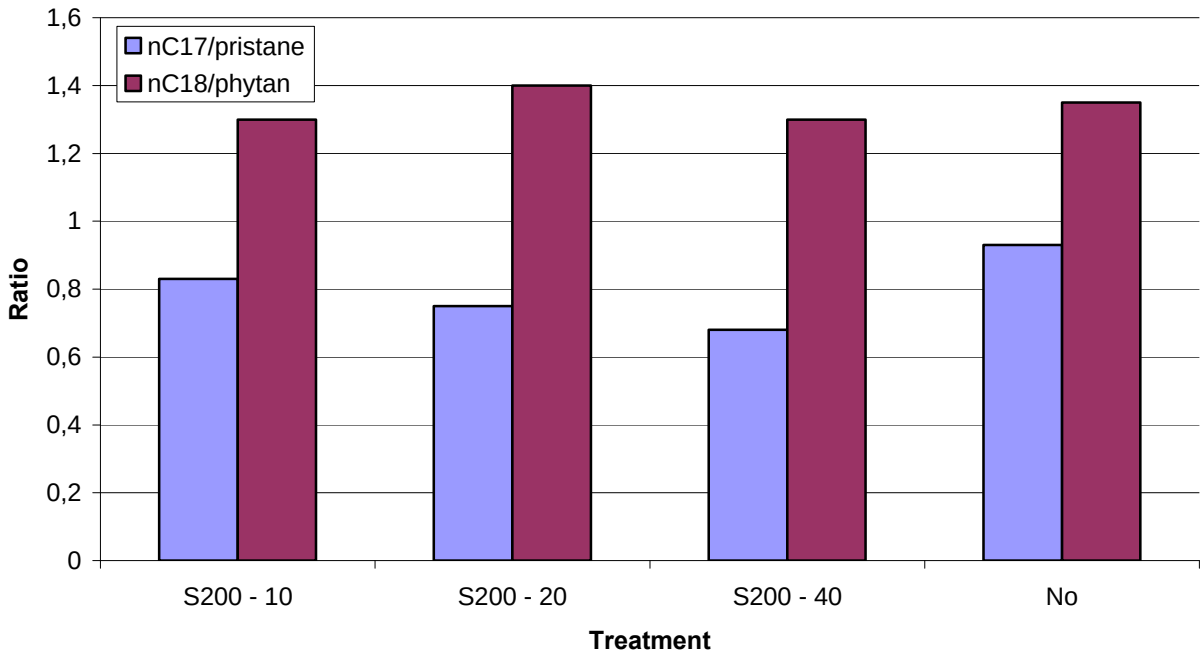



*Figure 4-13 Effect of increasing product dose (oleophilic fertiliser; ml) on the recovered* n-*$C_{17}$/pristane and* n-*$C_{18}$/phytane ratios from the sediment columns after two weeks of simulated tidal cycles at 13°C*

Application of 10 ml of the oleophilic fertiliser reduced the *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios to 0.83 and 1.3 respectively compared to 0.93 and 1.35 recorded for the control treatment. An increased application volume of 40 ml reduced the ratios of *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane to 0.68 and 1.3. Again, once the cost is taken into account, it seems unlikely that the increased application of 40 ml has any benefits over application of 10 ml.

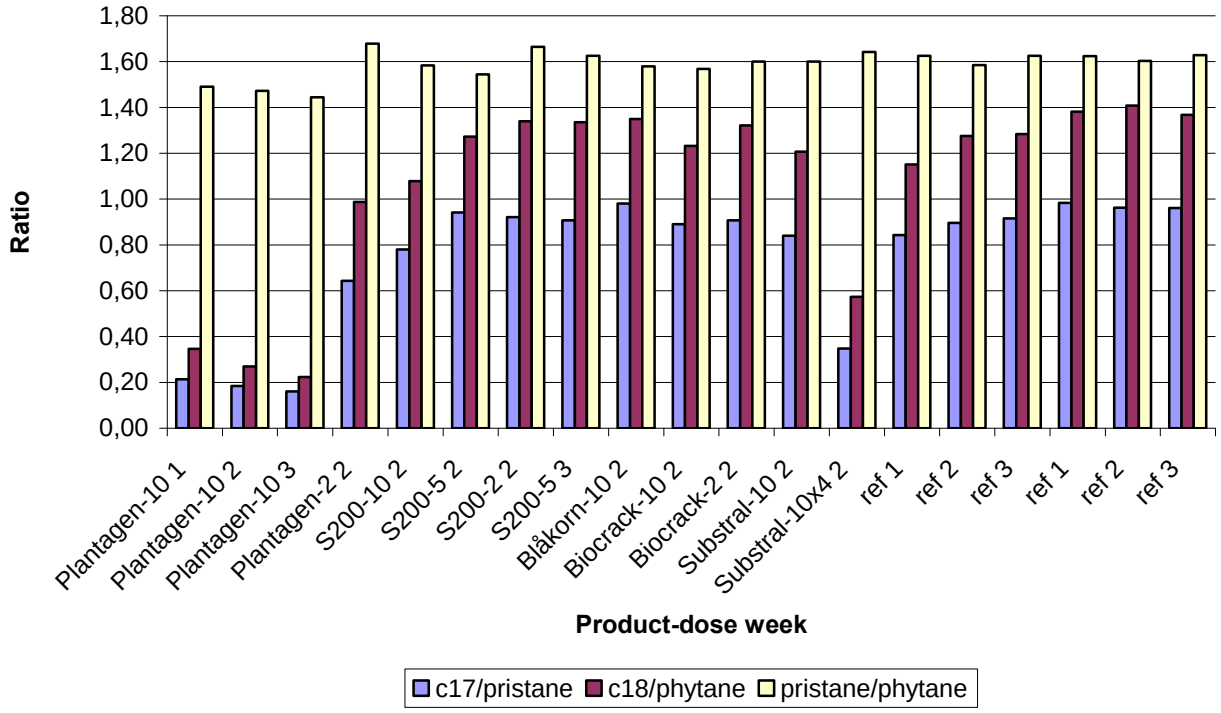



*Figure 4-14 Ratios of* n-$C_{17}$*/pristane,* n-$C_{18}$*/phytane and pristane/phytane in the remaining oil extracted from the column system after different treatment regimes. The treatments are labelled in the format product-dose week: Plantagen-10 1 therefore refers to incubation with 10 g of Plantagen sampled after one week's incubation.*

A testing period of three weeks allowed differences in the efficacy of the products to be observed. The slow-release fertiliser Plantagen (P-10 1-3; Figure 4-14) exhibited a large reduction in the *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios after one week of incubation; indeed, this fertiliser (P-10 1–3 and P-2 2) proved to be the most effective in the column system: reducing the *n*-$C_{17}$/pristane and *n*-$C_{18}$/phytane ratios to 0.16 and 0.22 after three weeks.

This test also included varying the amount of product applied, the frequency of application, and the duration. No significant difference was observed between the application of 5 ml of S200 and application of 2 ml of S200.

Repeated application in the case of the water- soluble fertiliser appears to be more effective than a single dose, as has been noted by other authors (Swannell *et al.*, 1995; Prince and Atlas, 2005). After two weeks incubation with a single 10 ml dose (Sub-10 2; Figure 4-14) of Sub the

*n*-$C_{17}$/pristane and *n*-C18/phytane ratios were reduced to 0.84 and 1.21 respectively; four doses of 10 ml at 3 day intervals (Sub-10×4 2) resulted in the ratio values being reduced to 0.35 and 0.57.

### 4.3 Toxicity of bioremediation products to *Skeletonema costatum*

A 72 hour acute toxicity test was setup in order to establish whether the bioremediation products used were toxic to a pelagic marine alga *Skeletonema costatum* The $EC_x$ results calculated from the exposure are presented in Table 4-1.

*Table 4-1 $EC_x$ toxicity values to Skeletonema costatum (72 h exposure)*

| Product | $EC_{10}$ | 95 % C.I. | $EC_{50}$ | 95 % C.I. | $EC_{90}$ | 95 % C.I. |
|---|---|---|---|---|---|---|
| **Biocrack** | 35.397 | 17.142 - 46.203 | 80.213 | 69.373 - 98.366 | 181.770 | 131.712 - 446.386 |
| **S200** | 9.057 | 8.656 - 9.433 | 15.224 | 14.873 - 15.575 | 25.592 | 24.74 - 26.563 |
| **Fullgjødsel** | 99.087 | 84.253 - 112.621 | 708.605 | 631.365 - 819.613 | 5067.474 | 3646.889 - 7738.447 |

The least toxic bioremediation agent tested was Fullgjødsel, an agricultural fertiliser, which had an $EC_{50}$ value of 708.6 mg $L^{-1}$. The most toxic agent to *S. costatum* was S200 ($EC_{50}$ 15.2 mg $L^{-1}$). Toxicity data for this product is available for two other species: a fish species, the waxen silverside *Menidia beryllina* (Cope, 1866) and an estuarine crustacean, the mysid shrimp *Mysidopsis bahia,* (Molenock, 1969). $EC_{50}$ values of 39.5- and 21.2 mg $L^{-1}$ were reported for these species respectively in 96 h exposure studies with S200. Biocrack exhibited an $EC_{50}$ value of 80.2 mg $L^{-1}$.

# 5 Conclusions and Recommendations

Both the flask and column systems have pros and cons. The flask system allows evaluation of the products based on a small amount of product and immobilised oil. This in turn allows significant differences between the efficacies of each product to be observed in only seven days. However, as can be seen when comparing Figure 4-1 and Figure 4-8—and as discussed above—due to the small amounts involved the system may be sensitive to heterogeneity in the seawater used, as well as organic matter contained within the products themselves. The system does not require any specialist equipment to operate and is of low cost.

The column system requires a longer period of testing and incurs a greater cost; however, due to the constant influx and efflux of seawater in the columns this system is also more realistic than the flask test. This constant mixing also overcomes any problems that may result from a heterogeneous bacterial population within the flask system. If required this system also allows sampling at different depths within the sediment column to investigate the biodegradation and loss of stranded oil below the sediment surface. The pros and cons of each test system are summarised in ***Table 5-1*** (Flask system) and Table 5-2 (column system).

Based on the pros and cons of each system we find that the column system gives the most consistent and reproducible data. It also provides the most realistic real-world simulation of the two systems. The flask system suffers from a lack of reproducibility between experiments; though the reproducibility between replicates of the same experiment appears acceptable.

*Table 5-1 Pros and cons of the flask system*

| | **Pro** | **Cons** |
|---|---|---|
| **Equipment** | Simple<br>Many units, replicates, screening<br>Kinetics | |
| **Oil type/properties/quantity** | | Fresh oil (150°C+) required to form oil films with uniform thickness |
| **Oil/water ratio** | Thin oil film (10µm) | Very low; limiting factor? Oil availability? Oil degradability |
| **Products/Agents** | Water soluble<br>Microbial | Oleophilic - surfactants<br>Granules |
| **Agent/oil ratio** | | Very high; 20:1 – 10:1 |
| **Time** | Short; 3–7 days | |
| **Realistic?** | | Closed system |
| **Sampling** | Solid surface and water phase | |
| **Analysis** | | Low oil quantities (10µg) |
| **Reproducibility** | Standard –simple<br>Good reproducibility parallels, | Low reproducibility between series |
| **Cost** | Low | |

*Table 5-2 Pros and cons of the column system*

| | **Pro** | **Cons** |
|---|---|---|
| **Equipment** | | Specialized<br>Limited experimental units |
| **Oil type/properties/quantity** | Any type of oil<br>Weathering degrees | |
| **Oil/water ratio** | Low 1% (v/v), 50-100µm | |
| **Products/Agents** | Oleophilic<br>Granulates/particles | Water soluble—washed out Microbial products—effluent collection and treatment |
| **Agent/oil ratio** | Realistic+ 1:2-1:5 (w/w) | |
| **Time** | | Moderate; 14-28 days (bioremediation is a long term strategy) |
| **Realistic?** | Sediment,<br>Open system; seawater exchange and supply,<br>Tidal variation – low energy exposure | |
| **Sampling** | Sampling requires sacrifice of the column.<br>Mobilisation/transport | Water phase |
| **Analysis** | Larger oil quantities<br>Sediment extraction – composition and total quantity | |
| **Reproducibility** | Parallels and between experimental series | |
| **Cost** | | Moderate? |

# 6 References

Bragg, J. R., R. C. Prince, J. B. Wilkinson and R. M. Atlas (1992). *Bioremediation for Shoreline Cleanup Following the 1989 Alaskan Oil Spill.* Exxon Company, U.S.A.

Brattli, B. (1999). Fysisk og kjemisk hydrogeology. *Andre utgave*.

Jimenez, N., M. Vinas, J. Sabate, S. Diez, J. M. Bayona, A. M. Solanas and J. Albaiges (2006). The Prestige Oil Spill. 2. Enhanced Biodegradation of a Heavy Fuel Oil under Field Conditions by the Use of an Oleophilic Fertilizer. *Environmental Science & Technology* **40**(8): 2578-2585.

Prince, R. C. and R. M. Atlas (2005). Bioremediation of Marine Oil Spills. *Bioremediation Applied Microbial Solutions for Real-World Environmental Cleanup*. R. M. Atlas and P. J. Washington D.C., ASM Press**:** 269-292.

Swannell, R. P. J., B. C. Croft, A. L. Grant and K. Lee (1995). Evaluation of bioremediation agents in beach microcosms. *Spill Science & Technology Bulletin* **2**(2-3): 151-159.