Assessment of Bioremediation Agent Efficiency: Development of a Test Protocol.

Preliminary result

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Abstract (ID 2017-410)

Bioremediation is considered as a soft green technique which can be deployed on an oil contaminated shoreline to treat the remaining oil after the initial clean-up operations have taken place. This technique relies on the ability of bacteria to use the hydrocarbon molecules as a

carbon source for growth. Nevertheless, specific environmental conditions are needed for ensuring effective biodegradation of oil, such as sufficient concentrations of nutrients and dissolved oxygen. To address these issues, the industry has developed commercial products, called bioremediation agents, which can be nutrient solutions (i.e. nitrogen, phosphorus and potassium) (biostimulation) or hydrocarbonoclastic bacterial solutions (bioaugmentation).

In France, Cedre is in charge, at a national level, of the validation of response products currently used to treat oil spill (e.g. sorbents, dispersants, cleaning agents): standardised protocols are routinely used to assess their efficiencies and select the most efficient products. So far, no such procedure exists for bioremediation agents. In 2014, Cedre developed a new protocol to test the efficiency of bioremediation agents at a pilot scale: a small portion of shoreline is simulated in small tanks filled with contaminated sand and placed on an oscillating table. The periodic movements of this table recreate a continuous and identical wave action in each tank. In order to simulate the dilution that occurs in the natural environment due to the tidal cycle, the test is conducted in a semi-open circuit, with natural seawater automatically renewed twice a day. For three months, oiled sediment was sampled and oil extracted to assess its biodegradation rate according to the experimental conditions: oil alone, oil with biostimulation agent, oil with bioaugmentation agent.

INTRODUCTION

In the event of an oil spill, the implementation of in situ bioremediation operations is generally recommended during the final clean-up phase. These operations overcome the need for techniques liable to cause more harm to an already fragile environment, such as soil removal or

excavation for instance. During bioremediation operations, populations of indigenous hydrocarbonoclastic bacteria develop in large numbers and, as the oil is broken down, gradually decrease in number to return to their initial level. This process results in the production of biomass, carbon dioxide, water and oil residues which are less toxic than the undegraded oil. Biostimulation and bioaugmentation techniques, although commonly used on contaminated soil in industrial areas, are not often applied in the natural environment in the event of oil spills.

Prior to any bioremediation treatment, certain key parameters must be taken into consideration in order to ensure this technique is successful, such as the biodegradability of the pollutant, the temperature of the environment, the dissolved oxygen and nitrogen concentrations, etc. (API, 2014). If the conditions prove suitable, the choice of technique – biostimulation or bioaugmentation – will be made following an evaluation of the bacterial quality of the environment, and more specifically of the hydrocarbonoclastic bacteria present (Nikolopoulou and Kalogerakis, 2011). The IMO (International Maritime Organization) guidelines (2004) on bioremediation include a flowchart intended for use by the response authorities to assist them in determining the strategy to be implemented and defining the use of bioremediation processes. The most relevant product must then be selected from the forty-odd products currently listed at the time of writing.

To date, with no standard currently in existence in France, Cedre has not yet established a list of bioremediation products having undergone efficiency tests, as it has done for sorbents and dispersants, for which the lists are available on Cedre's website. Efficiency assessment protocols do exist however in certain countries but given their heterogeneity, the comparison of results is currently problematic.

Yet Cedre has been known to perform efficiency tests on remediation productions, in particular at the manufacturer's request. The protocol used in these cases involves comparing the efficiency of the product tested on a single reference oil with that of a mixture of nutrients in the laboratory. The protocol is based on the AFNOR NFT 90-347 (1990) standard, initially developed to measure the inhibitive action of dispersants on the biodegradability of oil. These trials are carried out with seawater in closed microcosms and sediment from a site chronically exposed to oil is used as an inoculum. Biodegradation is assessed after 15 to 30 days, by analysing the concentrations of total hydrocarbons, n-alkanes and aromatic compounds. In the United States, the NCP Product Schedule issued by the Environmental Protection Agency (EPA) provides a list of products available on the market. In order for a product to be featured on this list, the manufacturer must submit the results of the efficiency tests carried out on the product by an accredited laboratory. The principle of the EPA efficiency test protocol is very similar to the protocol used by Cedre, presented above, with the exception of two major differences: no inoculum is used and a bacteria count is carried out at the end of the test. Other organisations (NETAC -National Environmental Technology Application Center in the United States, SINTEF in Norway, Environment Canada) also perform efficiency tests using similar protocols although, to the best of our knowledge, no lists of recommended products are publicly released.

To overcome this lack of list of approved remediation productions, Cedre decided to develop an efficiency assessment protocol for these products. The originality of this protocol lies in the consideration of tidal phenomena in the microcosms in order to simulate continuous dilution as it occurs in the natural environment.

MATERIAL AND METHODS

The Shoreline Test Bench

To support this study, Cedre acquired a new version of its Shoreline Test Bench (Figure 1) presented in previous articles (Jézéquel et al., 1999, 2012). The oscillating table concept remains unchanged. The new version of the test bench is composed of an oscillating table comprising 12 tanks (40 cm long, 20 cm wide, 30 cm high) equipped with a pneumatic cylinder to recreate different hydrodynamic conditions. The table's movements are set to agitate the surface of the sediment. The new feature of this version lies in the tidal simulation system which is now composed of a stationary tidal water distribution pipe and a mobile water collection pipe. The mobile pipe is mounted on a pneumatic cylinder supplied by a compressed air system via a solenoid valve. High tide occurs each day at 10 am and 10 pm with sand-filtered natural sea water (taken from the port of Brest). Low tide occurs each day at 4 am and 4 pm. With each low tide, the compressed air system closes causing the agitation to stop and the evacuation pipe to lower (the seawater flows out due to gravity). With each high tide, the compressed air system opens causing the oscillating table to start vibrating and the evacuation pipe to rise (preventing the water from flowing out of the tanks). The seawater inflow lasts 2 minutes, reaching a volume of 4 litres per tank. The whole system simulates the tidal flow twice a day with new seawater, without requiring a supply pump.

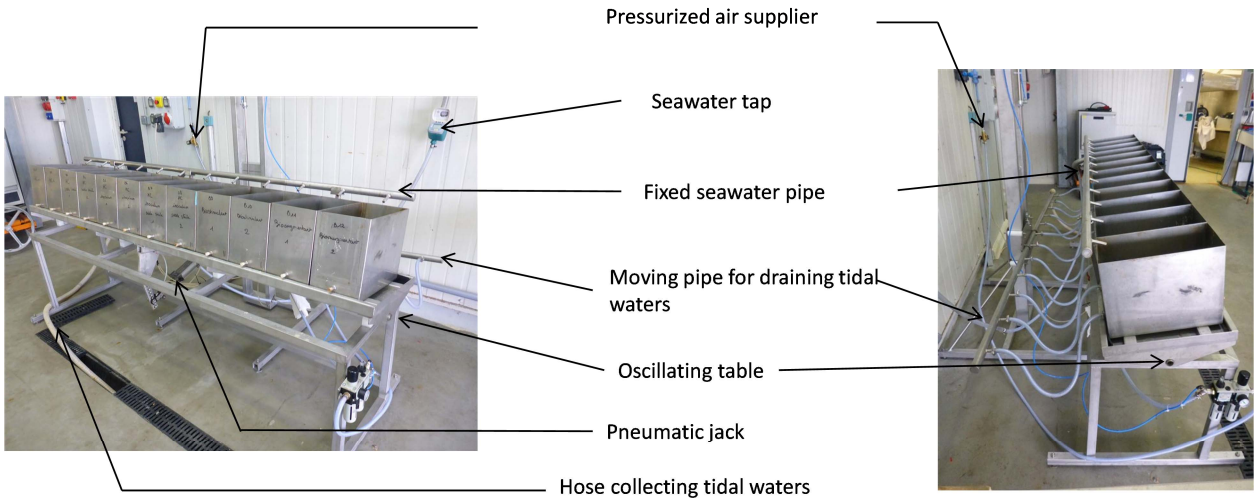

Figure 1. The Shoreline Test Bench.

Experimental matrix

In each of the tanks, 2.4 kg of sandy sediment contaminated with 5000 ppm of Arabian light topped at 110°C was deposited in each aquarium. This concentration of oil is generally taken to be the maximum concentration at which a bioremediation operation can be considered (IMO, 2004). For higher oil concentrations, the toxic effect of the oil can affect the bacterial flora and compromise the success of the clean-up operation. Bioremediation operations are therefore not recommended for concentrations in excess of this value. The sand particle size is presented in Figure 2. This sediment was obtained from a site located in northern Finistère (Brittany, France) and can be categorised as fine sand (AFNOR, 1997).

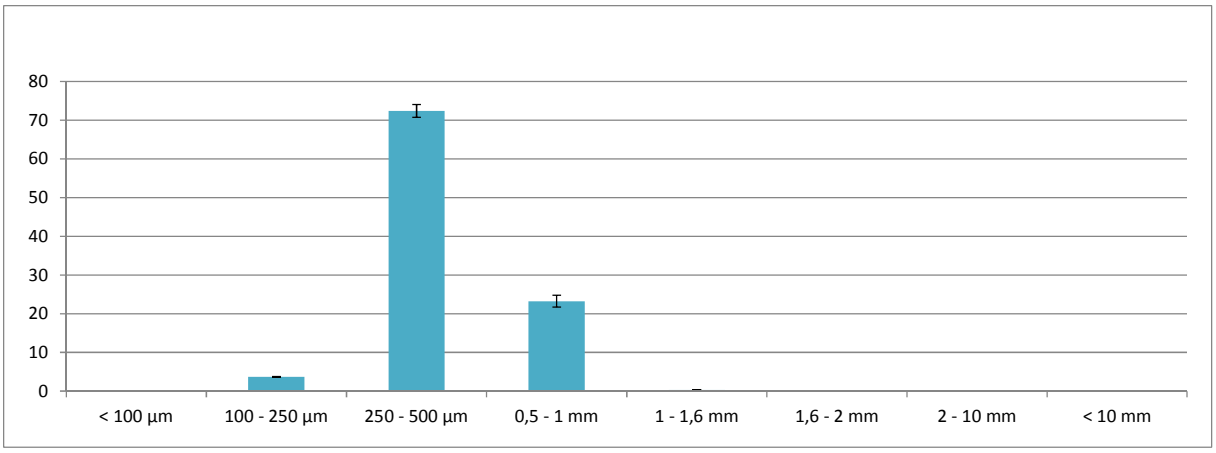

Figure 2. Sediment particle size.

The different experimental conditions detailed below were used to define the experimental matrix presented in Table 1. Each of these conditions was replicated in duplicate using 2 aquariums.

- Sand sterilisation: placed in oven at 100°C for 4 hours
- Bacterial inoculum: addition of 500 ml of an inoculum (see section on inoculum preparation)
- Bioremediation agent:
    - Biostimulation: commercial product sprayed onto sediment after 1 week using the concentration advised by the manufacturer.
    - Bioaugmentation: second application of the bacterial inoculum after 1 week.

This experimental matrix was defined in order to study the influence of the initial conditions on the results of the efficiency test and in particular i) to assess the need to sterilise the sediment due to the presence of overly active native flora and ii) to determine whether seeding of the sediment could accelerate the efficiency test.

Table 1. Details of the different experimental conditions

Experimental condition		Sterilisation	Bacterial inoculum	Bioremediation agent	Aim
1	Control				"Natural" evolution of the oil
2	Sterilised	X			Influence of the elimination of native bacterial flora
3	Inoculated		X		Efficiency of the inoculum
4	Sterilised and inoculated	X	X		Influence of the presence of native bacterial flora on the inoculum
5	Biostimulant		X	X	
6	Bioaugmentation agent		X	X	

Preparing the bacterial inoculum

As a general rule, oil biodegradation is a slow process especially in open environments such as that simulated by the experimental system. To reduce the time required to perform these efficiency tests, we decided to assess whether the seeding of the sand with hydrocarbonoclastic bacteria would accelerate the efficiency test.

To do so, a sample of sand was taken from a chronically oil-polluted site (Cedre's man-made beach). The bacteria present in this sediment were cultured (25°C) on Marine Agar (MA) with added crude Arabian light topped at 110°C to promote the development of hydrocarbonoclastic bacteria. To produce a large quantity of bacterial inoculum, strain identification and purification by successive isolation was an essential phase. The colonies obtained were subcultured on marine agar alone in order to isolate them and obtain pure strains. The isolation process had to be repeated several times to ensure the strains were pure. Once each strain had been isolated, it was then subcultured on Trypticase Soy Agar (TSA) slants to preserve it. Through bacteria identification analysis, 7 bacterial strains were differentiated (*Pseudomonas anguilliseptica, Burkholderia cepacia/Shewanella putrefaciens, Aeromonas hydrophila, Algoriphagus sp., Bacillus baekryungensis, Marinobacter sp., Bacillus jeotgali).*

Sediment sampling

Before each sample was taken, the sediment in each tank was homogenised and two 5 g samples were taken and stored at -20°C pending analysis.

Chemical analyses

Solvent extractions of the oil were performed using an ASE 350 (Accelerated Solvent Extraction, Dionex) (Table 2). The water content of each sample was assessed by weighing an aliquot of sediment before and after 24h at 50°C. Organic extracts were dried over Na_2SO_4 (activated at 400°C for 4 hours).

Table 3. Settings of the ASE 350.

Weight of sample (g)	2
Solvent	Methylene chloride
Temperature (°C)	100
Pressure (psi)	1700
Cycles	2
Heating (min)	5
Static (min)	5
Flush volume (%)	75
Purge (s)	60

Prior to GC/MS analyses, extracts were fractionated using a SPE (Solid Phase Extraction) cartridge (silica/cyanopropyl (SiO2/C3-CN; 1.0/0.5 g, 6 mL; Interchim, France) (Alzaga et al., 2004). Saturate and aromatic fractions were eluted simultaneously with 8 mL of methylene chloride/pentane (80:20, v/v) and concentrated to 2 mL.

Alkanes and aromatics were quantified using gas chromatography coupled with mass spectrometry (GC/MS). The GC/MS was an HP 6890N equipped with a split/splitless injector (Splitless time: 1 min, flow 50 mL/min) coupled to an HP 5973 Mass Selective Detector (MSD)

(Electronic Impact: 70 eV, voltage: 1200 V). The injector temperature was maintained at 300°C. The interface temperature was 300°C. The GC/MS temperature gradient was from 50°C (1 min) to 300°C (20 min) at 5°C/min. The carrier gas was helium at a constant flow of 1 ml/min. The capillary column used was an HP-5 MS: 30 m×0.25 mm ID×0.25 µm film thickness. *n*-Alkane semi-quantifications were performed using Single Ion Monitoring (SIM) mode with the most representative fragment (saturates) of each compound at a minimum of 1.4 cycles/s. 17α(H),21β(H)-hopane (m/z=191) was used as a conserved internal biomarker during analysis (Prince et al., 1994).

RESULTS

Microbial analyses

To confirm that the sediment had been seeded with the isolated bacterial mixture, sediment samples were taken from the "control" and "inoculated" tanks to carry out a bacterial count. Each sample (around 3 g) was agitated for an hour in 40 ml of seawater. 2 ml of the solution was then placed in a Petri dish. Three dilution steps (10^{-1}, 10^{-2}, 10^{-3}) were carried out for each sample. After 4 days of incubation at ambient temperature away from light, the dishes with between 30 and 130 colonies were selected for bacterial count. Figures 3 and 4 are examples of the results obtained for each of the 2 conditions. They show a higher bacterial concentration for the "inoculated" condition.

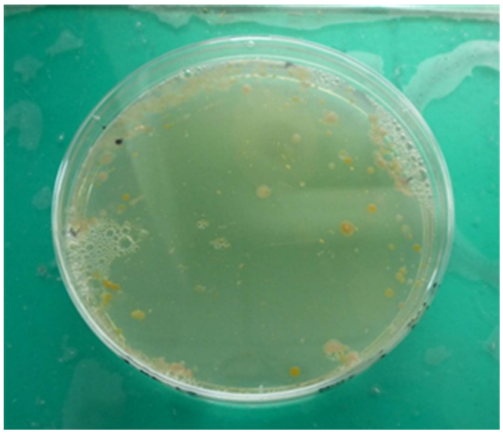

Figure 3: Petri dish for the "control" condition

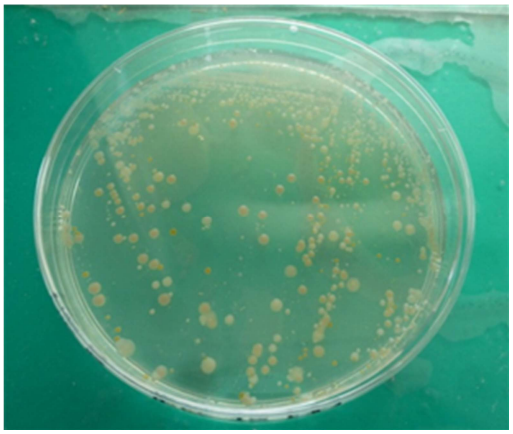

Figure 4: Petri dish for the "inoculated" condition

Chemical analyses

Figures 3 and 4 show the evolution of the values of the two biodegradation indices after 48 and 105 days for each of the experimental conditions. The two ratios applied indicate the initiation of the oil biodegradation process. The compounds n-C17 and pristane as well as n-C18 and phytane are chemical compounds with similar molecular weights and therefore have almost identical sensitivity to evaporation and dissolution processes. If the oil is broken down by evaporation and dissolution, the ratios will remain unchanged. Pristane and phytane are branched alkanes which, due to the presence of these alkyl groups, are less sensitive to the biodegradation process than *n*-C_{17} and *n*-C_{18} compounds. In the case of biodegradation, as linear alkanes are more easily broken down, the ratios decrease. In terms of the *n*-C_{17} to pristane ratio, after 48 days, biodegradation was observed for all the treatments although it was not significant for the "sterilised" condition. After 105 days, biodegradation had also occurred in all the tanks, with a significantly higher rate for the "biostimulant" condition.

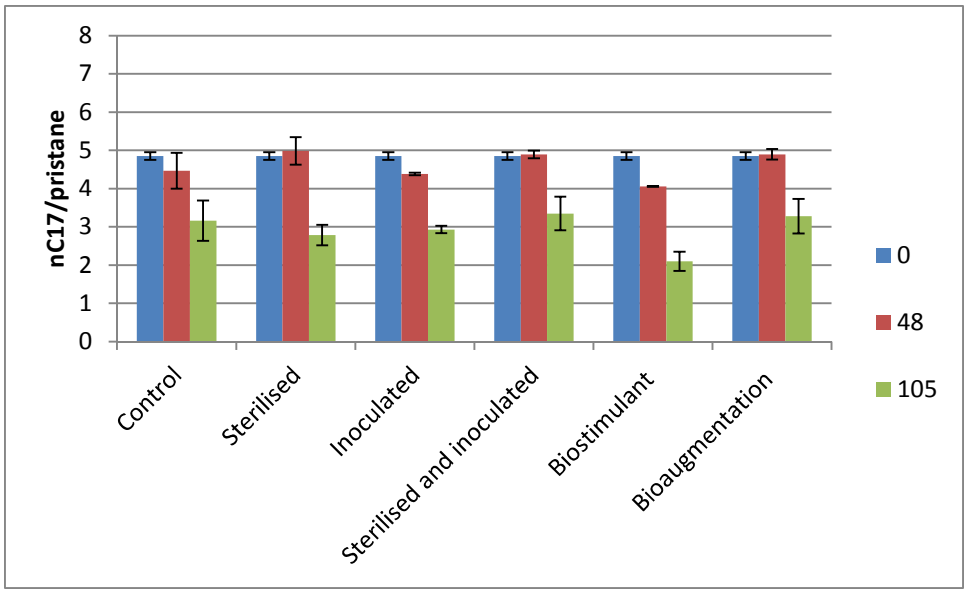

Figure 1. Evolution of the *n*-C_{17} to pristane ratio over time (days) for each experimental condition.

As for the *n*-C_{18} to phytane ratio, the previous observations were confirmed:

- biodegradation was observed from the first samples taken 48 days after the start of the experiment for all the treatments except the "sterilised" condition

- biodegradation was higher for the "biostimulant" condition for the last sample taken.

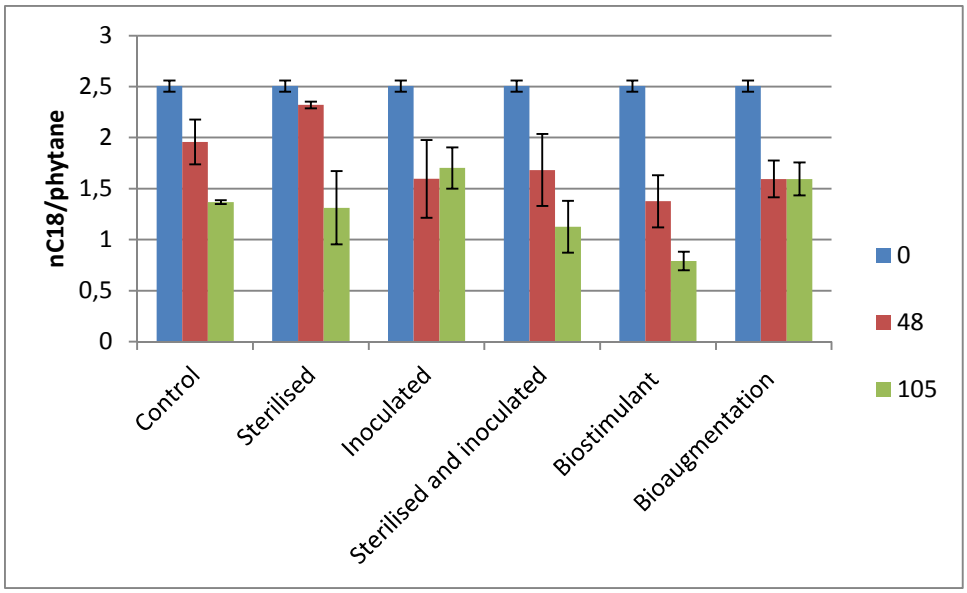

Figure 2. Evolution of the *n*-C_{18} to phytane ratio over time (days) for each experimental condition.

By considering the conditions with and without "inoculum" separately, we note that the addition of a bacterial population indeed accelerated the initiation of the oil biodegradation process, with an *n*-C_{18} to pristane ratio of 2.13 ± 0.25 after 48 days for conditions without inoculum and 1.56 ± 0.13 for conditions with inoculum. After 105 days, there was no significant difference between the 2 conditions.

Sterilisation did not appear to have an effect on the trials. The two biodegradation ratios did not differ significantly between the "sterile" and "non-sterile" conditions.

DISCUSSION

This experimental study focused on the development of a test protocol to compare the efficiency of bioremediation agents on oil-contaminated shoreline sediment. This experimentation was carried out using the Shoreline Test Bench, a tool which is able to simulate a constant, regular wave simultaneously in 12 tanks while renewing the seawater, hence recreating respectively the hydrodynamics and dilution phenomena associated with tides in the natural environment.

By the end of this experiment, it was apparent that:

1. sterilisation by eliminating native bacterial flora does not delay the biodegradation process. It is therefore deduced that this bacterial flora does not have hydrocarbonoclastic properties which could significantly interfere with the efficiency test.
2. an inoculum of oil-degrading bacteria was successfully added and logically led to a short-term acceleration of oil biodegradation. Nevertheless, there was no influence on the extent of biodegradation after 3 months in comparison to the non-inoculated conditions.
3. in terms of the efficiency of bioremediation agents, this test, carried out in an open-circuit system, showed that a bioaugmentation processes (repeated inoculation in our case) does not result in the acceleration of biodegradation. The water renewal and rapid dilution of the bioremediation agent impeded efficient inoculation of the sediment. Furthermore, it is recognised that there is systematic competition between indigenous and exogenous bacteria which renders the input of bacterial mixtures inefficient for this type of environment (Venosa et al., 1996, API, 2014). This is especially the case if the bioaugmentation agent is in liquid form, meaning that it is rapidly diluted with the first tide.

4. finally, as concerns the biostimulation agent, not only did this product accelerate the initiation of biodegradation in the sediment compared to the "control" conditions, it also resulted in the oil remaining at the end of the trial being degraded to a greater extent. Figure 5 shows an example of chromatograms for alkanes for the inoculated condition (positive control) (left) and the inoculated condition with a biostimulation agent (right): the lightest alkanes (left) appear to be less abundant, indicating a higher degree of degradation.

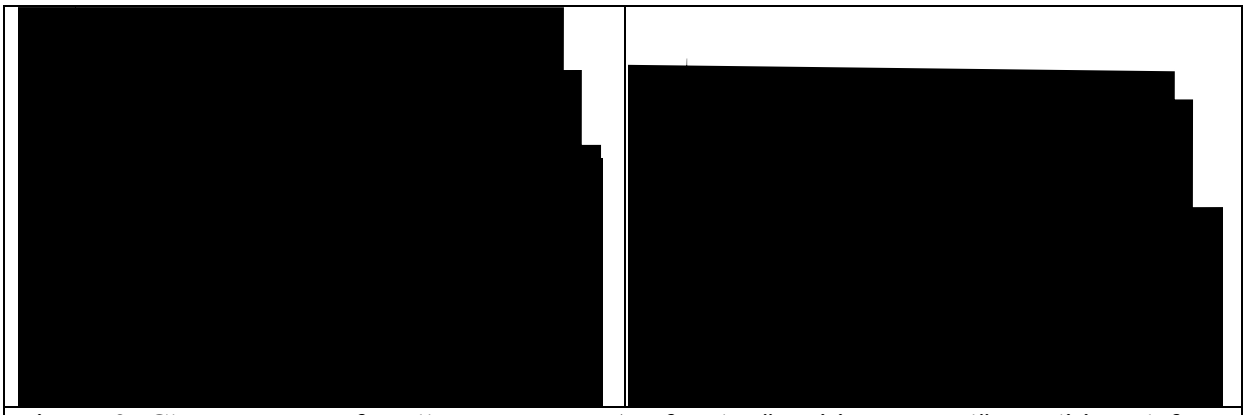

Figure 3. Chromatogram for alkanes (m/z = 57) for the "positive control" condition (left) and the "biostimulant" condition (right) after 105 days.

CONCLUSION

Following the end of this experiment, the experimental set-up is currently still operational and has demonstrated the efficiency of a bioremediation agent for treating sandy sediment contaminated with light crude oil.

Drawing upon the experience acquired through this study, a few adjustments remain necessary to optimise and finalise the protocol. More advanced topping of the oil, increasing its viscosity, would thus reduce the quantity of oil released from the sediment, observed in large quantities on the sides of the tanks from the beginning of the trial. The possibility of using finer sediment should also be assessed: in the natural environment, bioremediation operations are most often considered for sheltered sites, therefore less exposed to wave action, which are characterised by sandy/muddy sediment rather than simply sandy sediment.

In terms of the presentation of the results, the inclusion of all *n*-alkanes and PAHs and their expression in hopane units should be retained as this approach is already applied in other standardised tests. Similarly, the monitoring of bacterial flora at the end of the trial, at least quantitatively, provides an additional method of assessing the benefits of using a bioremediation agent.

Due to the need for this additional phase of system development and trial protocol definition, it was not possible to carry out "routine" trials in order to assess the repeatability of the results and to compare the efficiency of different agents. This phase remains to be implemented in order to definitively validate the protocol for assessing the efficiency of bioremediation agents.

ACKNOWLEDGEMENTS

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