PROTOCOL FOR EXPERIMENTAL ASSESSMENT OF BIOREMEDIATION AGENTS ON A PETROLEUM POLLUTED SHORELINE

by

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ABSTRACT

An experimental protocol is developed for monitoring the effectiveness of bioremediation agents in treating oil-contaminated coastal sediment. This methodology, the result of a technical seminar attended by the authors in March, 1993, is intended for use as a framework for the design of experimental field trials. Its use will enable independent organizations to conduct similar experiments in different environments. According to this protocol, a series of plots are set-up along a shoreline, and are treated either with oil and a bioremediation agent, only with oil for the control plots, or left untreated (to evaluate background oil levels). The bioremediation is monitored on a periodic basis for several months through measuring the changes in the quantity and chemical composition of the residual hydrocarbons of the oiled plots.

This protocol is considered to be a working document which will be improved by further experiments. In this aim, most of the authors join their efforts in order to conduct an experiment between October, 1993, through June, 1994, on a sheltered, coarse-sand beach on the French coast. A

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nutrient-adding technique (use of fertilizer) is the bioremediation stategy chosen to be evaluated. To determine bioremediation efficiency, changes in oil composition are measured by gas chromatography and mass spectrometry; additional monitoring of the bacterial numbers and activity was also performed .

1. INTRODUCTION

Phenomena of biodegradation can be of great help in restoring oiled coastal sites [1,2]. Consequently, during the last ten years, a lot of bioremediation techniques have been developed.

Various strategy have been proposed such as the addition of specific bacteria which are able to breakdown the hydrocarbons (bioaugmentation) [3,4], or the addition of nitrogen-phosphorous fertilizers stimulate the local micro-flora action (biostimulation) [5,6,7].

Up until now, this latter, fertilization treatments has proved to be effective, especially during the Exxon-Valdez oil spill [8]. A number of studies have been made concerning this biostimulation technique in order to evaluate and to optimimize bioremediation procedures [9,10].
In spite of all the work done, we are still a long way from understanding and mastering these techniques : it is especially difficult to predict the speed of degradation of a pollutant on a specific site. Furthermore, for a specific pollutant, we do not have the information required to chose the best bioremediation agent.
The results and conclusions of the various studies undertaken in the past are extremely difficult to compare and correlate since the individual research teams often use different experimental procedures and evaluation criteria.

In order to unify the test procedures, an international working group was set up with representatives from the following organisations :

→ CEDRE. France
→ Department of Fisheries and Oceans (DFO). Canada
→ Elf Aquitaine. France
→ French Naval Hydrographic and Oceanographic Service (SHOM). France
→ French Petroleum Institute (IFP). France
→ Marine Spill Response Corporation (MSRC). United States
→ National Natural History Museum in Paris (MNHN). France
→ Sintef. Norway
→ Warren Spring Laboratory (WSL). Great Britain

This working group, defined a standardized experimental protocol to evaluate the effectiveness of bioremediation agents on the basis of field experiments, and to provide a mean by which the results of one laboratory

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Once the protocol was defined, the members of the working group set up an experiment in France to validate the recommended protocol. In the future, it is expected that the working group members will set up similar experiments on their own, to verify the effectiveness of certain bioremediation techniques under different climates and coastal sites ; the long term aim of these studies is to produce a set of operational guidelines for the application of bioremediation agents in response to oil spill incidents.

2. THE EXPERIMENTAL PROTOCOL

The protocol is intended to experimentally monitor and evaluate the efficiency of bioremediation agents for treating petroleum contaminated coastal sediment. The protocol defines the experimental method, and aims at determining the rate and extent of biodegradation which can be achieved in the field through the addition of bioremediation agents. The protocol should be considered as a working document that will be complete or improve in light of future experiments.

2.1. Principle

The tests are carried out on a given coastal site, using plots of sediment.
These experimental plots should be treated with petroleum and bioremediation agents. To demonstrate the effect of such bioremediation agents, control contaminated plots, without fertilization, should be treated in an identical manner. Untreated plots are also established to monitor background oil levels.

Bioremediation is monitored for several months on a periodic basis by documenting the changes in the quantity and chemical composition of the residual hydrocarbons.

2.2. Experimental Conditions

The protocol stipulates the minimum number of plots to be set-up (at least 3 replicates for each test condition), their location (between the half and high tide level) and their size.

In particular each plot has a test zone (where the sampling will be performed) surrounded by a buffer zone (figure 1).

Within reason, the larger the plot the better. The minimum plot size (surface area) is defined as follows : the surface of the test zone has to be large enough To minimize the perturbation caused by the sediment sampling, the surface of the test zone must be at least 10 times larger than the total sampled surface area (number of samples x the surface area of the sampling core).

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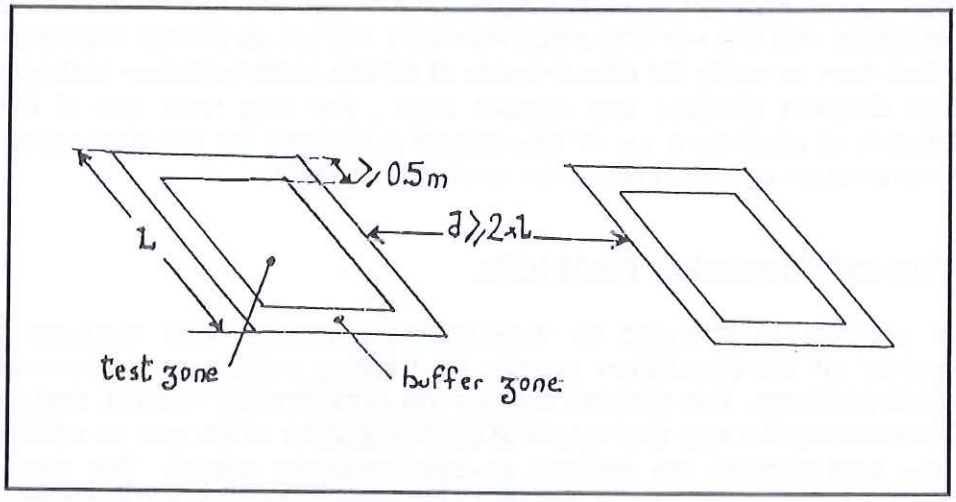

Figure 1 - Experimental design

The buffer zone, surrounding the test zone of each plot, must be at least 0.5m wide.

In the case of fine sediment, it is important to minimize the transport of treated sediments from the plots by waves and currents. In this aim, the protocol considers the possibility of covering the plots with a permeable membrane [11,12]. Care should also be taken not to disturb the natural physical and chemical gradients within the plot.

To avoid any cross contamination plots should be located as far apart as possible ; the distance should be at least twice the length or the longest plot dimension.

The protocol recommends the type of oil to be used (arabian light crude oil), its preparation, (topped at 150°C and emulsified with 25% of sea water),and the mode and conditions of its application, (8 to 15 days before the bioremediation process is started, during the low tide, without disturbing the sediment during the application).

2.3. Sampling

The protocol defines the criteria to be considered the site description, and the methods for sampling.

sediment :

- sampling depth : 0 to 5 cm for surface sediment ; subsurface sediment determined as the depth to which 75% of the oil can be found 7 days after application,
- sampling method : for each plot, at each sampling time, 10

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sediment samples taken then bulked to get one or several average samples for analyses,
- the sampling frequency and the method of conservation of the samples are also stated.

interstitial water :

- sampling is performed through perforated tubes pre-set in the plots
- sampling frequency, the collecting method and the conservation conditions are stated.

The document also recommends the different types of physical and chemical measurements and analyses to be undertaken (, total organic carbon, temperature, sediment granulometry, beach accretion.).

2.4. Measurements and Analyses

Chemical analyses make possible an evaluation of the degree of biodegradation which has been achieved : the analyses follow various changes in the relative oil composition relative to the residual bio-indicators present in the oil, and by reporting changes in the total quantity of oil present in a given sample.

Routine gas chromatography and gas chromatography-mass spectrometry analysis monitor the process of biorestoration.
According to the protocol, the concentrations of dissolved oxygen and nutrients (nitrogen and phosphorus) of the interstitial water should be followed as well as the total organic carbon content and the redox potential, within each plot.
Between the plots, accretion of beach should be taken into account.
Regular measurements of ambient sea water, beach and air temperature should be recorded during the study.
The protocol does not exclude additional measurements such as microbiological parameters to meet specific objectives.

3. EXPERIMENTAL FIELD TRIAL FOR PROTOCOL VALIDATION

In order to check the validity of the protocol, a field trial is performed according to the protocol recommendations on a sheltered coarse-sand beach on the west coast of France. In this experiment, the speed and the extent of the oil biodegradation is monitored on pre-oiled plots with and without addition of nutrient.
This experiment will allow the assessment of the biodegradation kinetics in natural conditions and without nutrient limitations.
For this experiment the work is shared between most of the members of the

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working group : while Cedre is managing the operations on the site, samples are sent to the different participants for analysis according to their laboratory facilities and expertise.
Different analytical methods (chemical as well as micro-bacteriological) are used in parallel, to enable the comparison at the end of the experiment.

3.1. Experimental Set-up

Five groups of 3 plots measuring 7.5 m2 (3 x 2.5 m) are made ; each group contains :

* one oiled plot with the addition of a nitrogen nutrient
* one oiled plot without nutrient addition
* one control plot, (without oil or nutrient).

The location of the 3 types of plots is chosen within each of the five groups. Each plot is enclosed in a frame, and covered with a fine mesh in order to ensure that the sediment and bioremediation agent remained in place, to counter the effects of tides and water currents (figure 2).

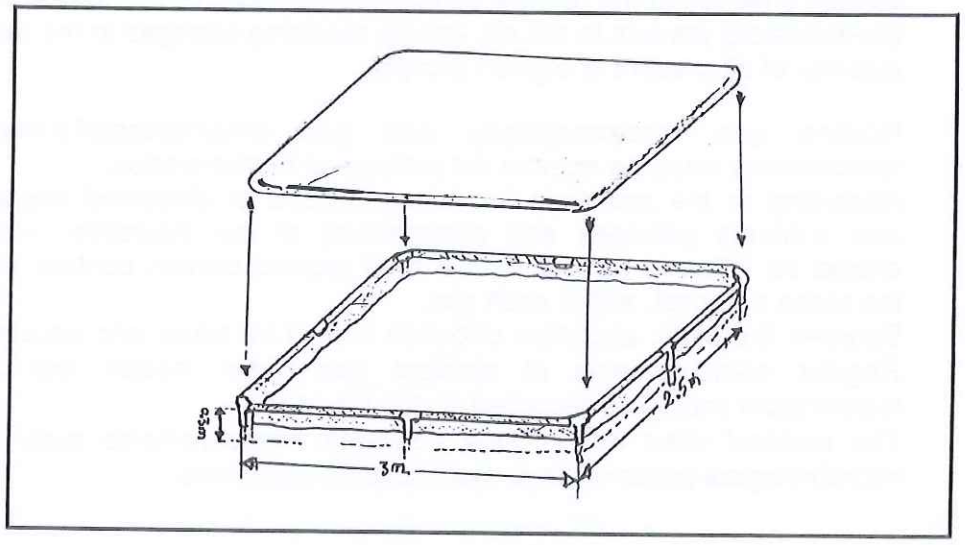

Figure 2 - A plot set-up

Within each plot, two perforated tubes are inserted to a depth of about 15 cm within the sediment in order to facilitate the sampling of interstitial water.

The plots are located in the mid-tide area 5 to 7 meters apart. Due to space considerations, the test area is set-up on two small beaches. Site A, down-stream, contained 3 groups of 3 plots, and Site B, approximatively 1000 m up-stream, is the location of two groups of 3

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plots (figure 3 : map).

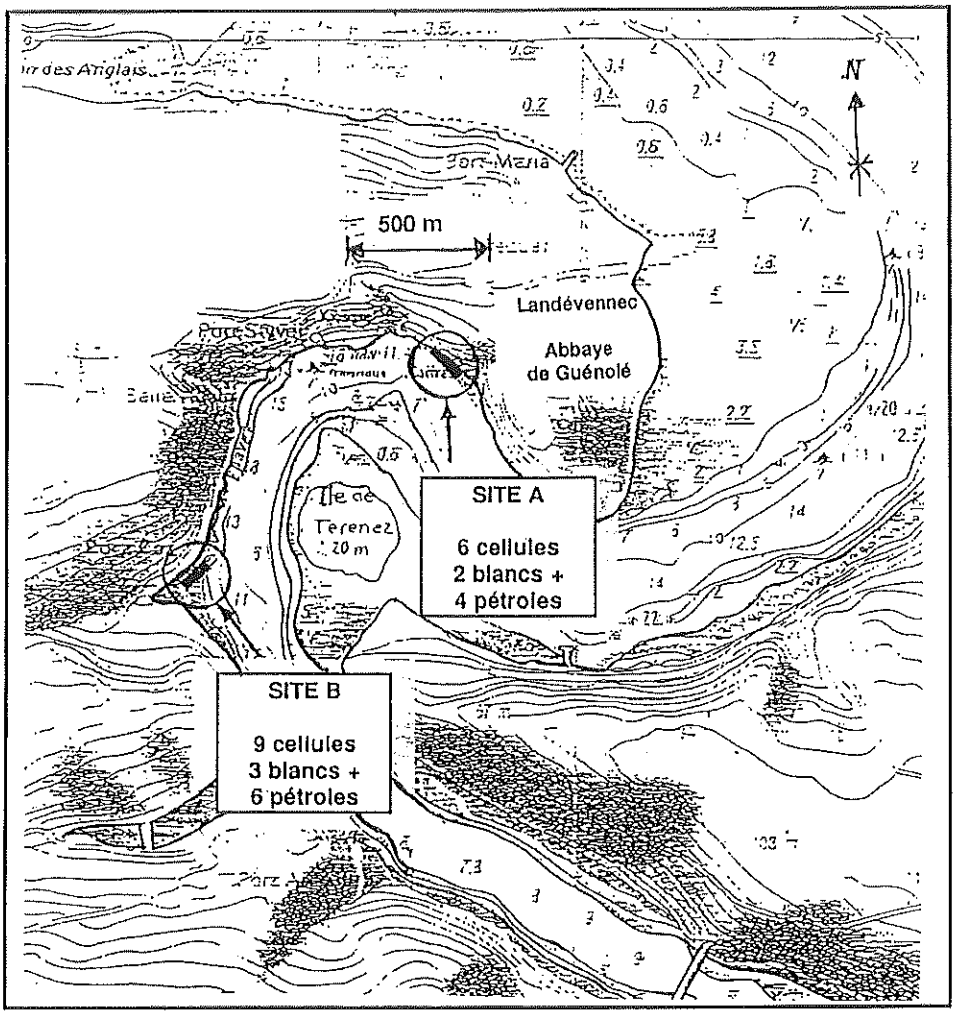

Figure 3 - Sites location

3.2. Oil

The oil pollutant used for the experiment is a light Arabian crude which has been allowed to evaporate to a chemical state equivalent to a topping at 150°C, and emulsified with 25 % sea water. to simulate the composition of a typical oil slick reaching the shoreline. 3.8 l/m^2 of this mixture is spread on the oiled-plots.

3.3. Nutrient Addition

Nitrogen enrichment is accomplished by spreading a slow released inorganic fertilizer (NO_3, NH_4, P_2O_5 coated with an organic resin material) at regular time intervals (approximately every month).

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3.4. Measurements and analyses

3.4.1. Chemical Analyses of the Oil

Chemical analyses are made by the CEDRE, EPSHOM, IFP, IFREMER, and MNHN laboratories,.

The oil is extracted from the sediment samples using the Soxhlet method with $CHCL_3$.
The total concentration of extractable hydrocarbon is assessed by gravimetry, and by infrared spectrometry after purification on Florisil column.
After the precipitation of the Asphalt fractions which is then filtrated on a glass microfiber filter part of the extract, is passed though a silica gel column using hexane, followed by a mixture of hexane-dichloro-methane, and then methanol, in order to separate the asphalt fractions, the alcanes (F1), the aromatics (F2) and the resins, which are subsequently weighed.
The F1 and F2 fractions are analysed using GC-FID.
Another part of the original extract is analysed using the mass spectrometry, Fisher method [13], in order to determine the relative composition of the oil according to the following groups: n-alkanes, iso-alkanes, cyclo-alkanes, aromatics (cycles 1, 2, 3, 4, and 5), the benzo-thiophenes and their C1, C2, and C3 derivatives, as well as the di-sulphur compounds.

In addition, representative samples are analysed by GC-MS in order to evaluate the biodegradation of the alkanes in relation to the conservative biomarkers (triterpanes).
Finally, part of the extracts are analysed using GC-FPD in order to analyse the sulphur compounds.

3.4.2. Bacteriological Analyses of the Sediment

The micro bacteriological analyses are performed by CEDRE, DFO, ELF and the WSL.

3 different methods are performed

- Counting of heterotrophic marine and oil-degrading bacteria by the most probable number method (MPN)
- Activity measurements on traced hexadecan and phenantrene.
- In situ respiro-metric measurements.

3.4.3. Analysis of Interstitial Water

For each plot the temperature and concentration of dissolved

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oxygen and nitrogen are determined.

3.4.4. Additional Analyses

The total organic carbon content of the sediment is regularly analysed on each plot.
The sediment granulometry is assessed.

4. EXPERIMENTAL PHASE

The experiment began on October 6, 1993 for a time period of 9 months, and will last for 9 months.

The table 1 defines the sampling, measurements and analyses dates, already completed and scheduled.

Time (weeks) sampling schedule and type of measurement	0	1	2	3	4	6	8	10	16	20	27	36
Oil concentration		x	x	x	x	x	x		x		x	x
Oil analysis GC-FID	x		x		x		x		x		x	x
GC-FPD	x		x		x		x		x			x
MS-Fischer	x		x		x		x		x			x
GC-MS	x		x				x		x			x
Interstitial water, O2, nutrient	x	x	x	x	x	x	twice a month					
Sediment TOC	x		x		x		x		x		x	x
Sediment granulometry	x								x			x
Micro-biologic MNP&Activity	x		x		x	x	x		x		x	x
in situ respirometry						x	x	x	x	x		

Table 1- Sampling and measurements schedule

5. INITIAL OBSERVATIONS

5.1. On the test plots

The use of a frame covered with a fine mesh is effective in maintaining the sediment as well as the fertiliser in place. This also enabled us to avoid cross-contamination between the different plots.

However, this fine mesh has the disadvantage of trapping fine grained sediment particles ; in some instances, there are some muddy deposits at the plot's lower end (covering several millimetres); up to now, these deposits do not seem to interfered in the sampling procedure since they are located within the non-sampled buffer zone. Also, due to the frame and cover set-up, some dead leaves and drifting seaweed can be trapped within the plots ; during visits to the site, this debris are systematically removed by hand.

The oil seems to have moved to the lower end of the oiled plots

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under the effect of the slope of the beach : the oil concentrations are visibly greater in these areas.

5.2. Interstitial Water

Interstitial water sampling is one of the most delicate aspects of the experiment. When the tide level drops, and once the plots are dry, the sampling tubes for the interstitial water quickly empty. A simultaneous water sampling from the 15 plots which are nearly located at the same tide level and in two different areas, requires at least 5 people divided in two teams.
In addition, the sampled water often contains fine sedimentary particles which may interfere with the Chemical analysis A filtering operation before bottling the samples is thus necessary.

Figure 4 reveals an evolution in the amount of nitrates in the various plots (on the graphs, small arrows indicate the times of fertiliser additions).
Generally, the amount of nitrates in the interstitial water increases 8 to 12 weeks after the first application, while the nitrogen concentrations between the various plots is highly variable.

Besides, it appears that the plots only with oil have a lower nitrogen concentration than the blank plots (and even that of the Aulne River), which suggests that the in-situ existing nitrogen is being consumed by the micro-organisms responsible for oil bio-degradation.

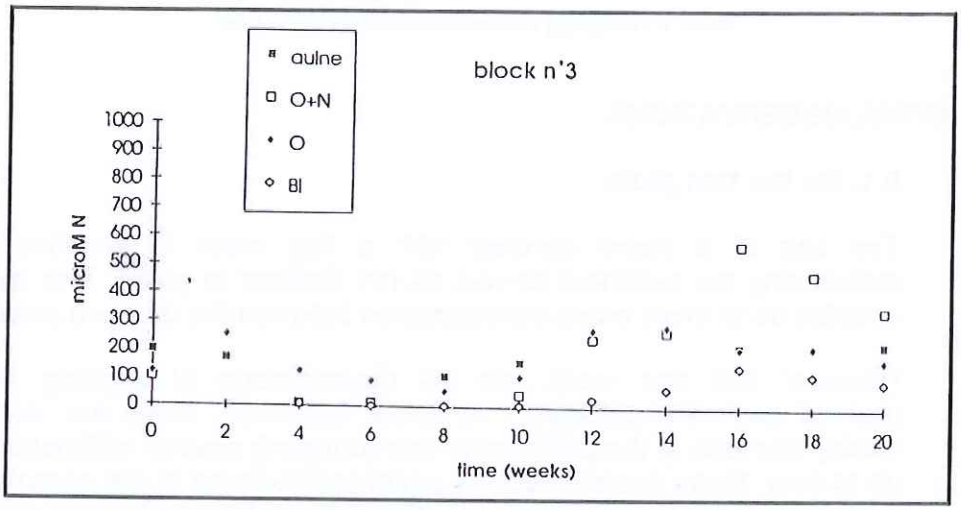

Figure 4 - Nitrate concentration of interstitial water versus time

On the contrary, the oiled and fertilised plots show higher concentrations of nitrogen than that found in the control plots or in the Aulne River.

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5.3. Oil

5.3.1. Oil Concentrations in the sediment

These concentrations decrease rapidly from approximately 6000-8000 ppm to 1000 - 2000 ppm (figure 5).

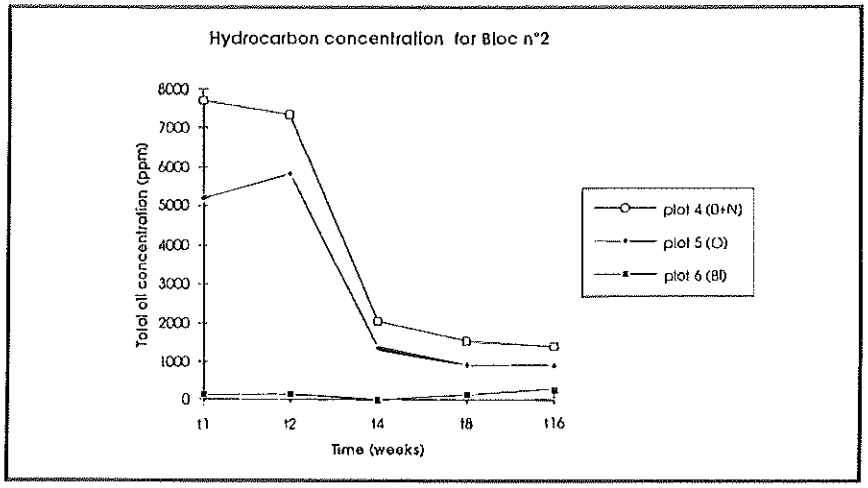

Figure 5 : Oil concentration versus time

5.3.2. Oil Composition Analyses

At the time of writing, only the GC-FID analyses on samples collected at 2, 4, 8 and 16 weeks have been done and show the following observations (See Figure 6):

- The concentration of the norhopane naturally contained in the oil used in the experiment is high enough to be detected by GC-FID and to use this compound as internal standard.
- The first un-oiled, untreated control plot reveals a prior oil contamination on the order of 300 ppm.
- The other un-oiled control plots are clean.(Figure 6.A)
- The analyses of the 2 and 4 weeks samples seems to reveal some degradation in the oiled plots of the first group, (nC17 and nC18 are in the same order as those of phytane and the pristane); this observation is not confirmed by the analyses of 8 and 16 weeks which means that we can record significant variations in the same plot (Figure 6.B).

So far, due to the short period of time elapsed since the study begins, it is not possible to make conclusions on the efficiency of bioremediation treatment.

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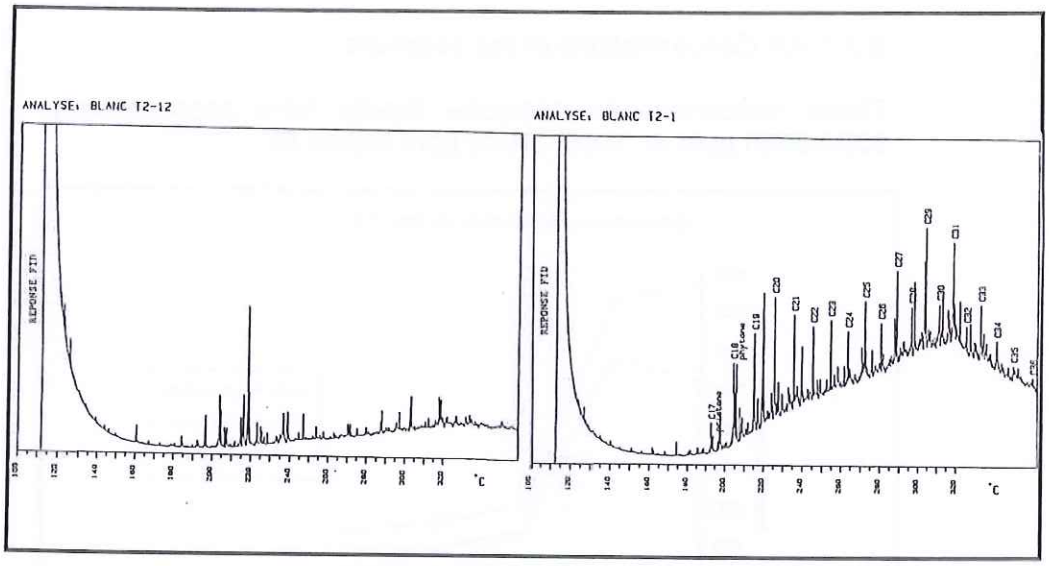

Figure 6.A - GC-FID on some of the control plots

6. CONCLUSIONS

This field trial to validate the protocol is still on the way as of may 1994. Samples have yet to be collected and analysis have to be completed before making significant conclusions concerning the following points :

6.1. In terms of the site chosen (that is, a coarse sand beach in an estuary, in a temperate climate) :

6.1.1. What are the kinetics of oil decontamination in sediment?

6.1.2. What are the related kinetics for the degradation of the various compounds found in oil ?

6.1.3. Can a massive addition of nitrogen accelerate the biodegradation of oil in sediment ?

6.2. in terms of the protocol, this study will enable us to validate, to complete and to improve the methodology originally defined by the working group. It will also enable us, by comparing the results obtained from the different analytical techniques, to define a list of objective elements and Chemical criteria which can be used to quantify the biodegradation of oil.

The test will terminate in June, 1994, and the results will be studied during the Summer. The totality of the results and measurements will be presented and discussed at the end of September 1994 in Brest (France) during a technical seminar, to finalise the experimental protocol in light of the knowledges gained from the study.

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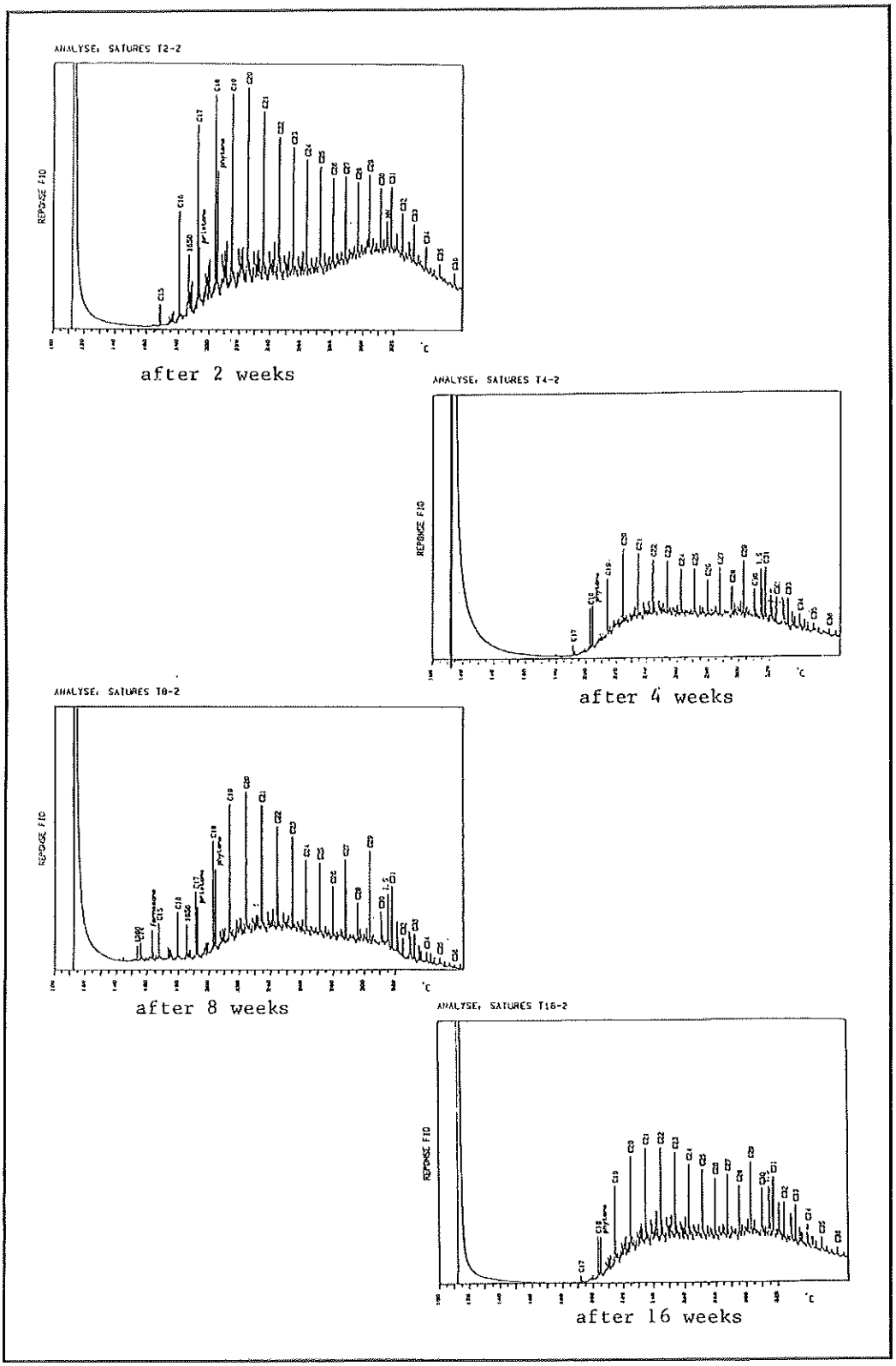

Figure 6.B - GC-FID of the satured fraction

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