Bacterial degradation of hydrocarbons in contaminated soil in the coastal zone of Moa-Holguín by means of biomagnificacion.

Guilarte G., Van Caneghem J.*, Holsbeeks I.*,

*** KU Leuven, Campus Group T, Andreas Vesaliusstraat 13, 3000 Leuven, Belgium**

Abstract:

Oil pollution and industrial waste in coastal areas linked to the socioeconomic activities of port companies becomes an environmental problem affecting mangrove ecosystems and associated fauna. In this work soil samples were collected from areas contaminated with hydrocarbons and near storage tanks crude oil for insulating degradative strains. Four morphologically different bacterial strains were isolated (1A, 2B, 3C and 4D) and their ability hydrocarbon degradation was evaluated in microcosm. Using biomagnification technic in trials in microcosm, it was found that the 4D strain growth was better compared whit the other strains, and showed better rates of biodegradation and 3C. It was found that using bioaugmentation with native isolates can be achieved high rates of biodegradation.

Keywords: Microcosm, Bacteria, Microbial degradation

1. Introduction

The widespread use of fuels and products derived from crude oil, has increased soil contamination due to spills of this complex mixture of organic compounds, causing an imbalance in the ecosystem that negatively impacts the ecological environment (Garcia *et al*., 2013). Bioremediation by microbial degradation is considered as an effective technique in soil decontamination using strategies such as biostimulation of native microorganisms, and biomagnification (Windevoxhel *et al.,* 2013) based on inoculating strains or microbial consortia laboratory that were first grown in a laboratory.

The tools used in bioremediation to optimize the degradation rate are biostimulation and bioaugmentation. Biostimulation aims at minimizing factors limiting the growth of microorganisms in this way enhancing pollutant degradation. Typical factors limiting the effectiveness of the biodegradation process are the availability of nutrients such as nitrogen and phosphorus, the water content, and oxygen and contaminant bioavailability. On the other hand, bioaugmentation is the inoculation of specific microorganisms that are generally microorganisms native to the ecosystem that degrade the aimed contaminant in the habitat (Cheung *et al.,* 2001).

Due to the undoubted advantages that it brings, the bioaugmentation technique was used during the experiments. Several studies have shown that environmental biotic and abiotic factors influence the effectiveness of bioaugmentation (Margesin *et al.,* 2011) therefore, it is to use microorganisms indigenous to the contaminated site for bioaugmentation. (Nozari *et* al. (2014) indicated that both the inoculation strategy and the fate of the inoculated microorganisms in the soil are important factors to take into consideration for the success of bioaugmentation strategies. Bioaugmentation ensures that specific microorganisms capable of degrading the contaminant to its basic molecules are present. Bacteria are the most commonly used for bioaugmentation microorganisms.

The biodegradability of a hydrocarbon mixture present in contaminated soil depends on several factors including the presence of a potentially active degrading microbial population, the molecular structure of the contaminant, its concentration and bioavailability and environmental factors such as pH,

temperature, soil moisture, presence of electron acceptors available as well as the existence of inorganic nutrients (source of N and P) available (Di Martino., 2015).

It is important to state the environmental and socioeconomic role of this industry. On an environmental level, its activities might negatively influence the floristic species which play a vital role in environmental conservation of the coastline and the existing animal species. On a socio-economic level, however, the port company provides significant permanent jobs in both production and administration, reflecting in a significant increase in the life quality of its staff and it is fundamental for economic growth of the region. (Garcia *et al.*, 2013).

The port company located in the Moa municipality of Holguin province is a key link in the infrastructure of the Business Group "Cubaníquel". Its primary function is reception and storage of products or raw materials imported for the local nickel industry and the export of its produced finished products.

Nevertheless, the port's maritime activity causes a negative impact on the coastal ecosystem, which is associated not only to the absence of treatment systems, but also to dredging activities, to the absence of a proper sewerage system and to the synergy of the environmental impacts of all economic activity, leading to, amongst others, contamination of soil with hydrocarbons in large areas around the port.

The overall objective of this study was to isolate native microorganisms in the study area and check their ability to grow in soils contaminated with hydrocarbons. Furthermore, the degradation of the microcosm in soils polluted with hydrocarbon that were treated with the selected strains was assessed.

2. Materials and methods

Figure 1. Aerial image of the area contaminated with hydrocarbons (*Polluting sources in coastal and Zone of isolation of microorganisms)

2.1 Description of the area and sampling:

Located on the north eastern coast of the island of Cuba, in Holguin province, the port company is on an artificial dock south of Yaguasey Cove, Bay Cayo Moa (20 ° 39 "30 north latitude and 74 ° 55" 40' west longitude).

Three sampling point were chosen from soils contaminated with hydrocarbons exactly in the coastal line nearby at the mangrove (Figure 1) located in Norwest and Southwest from the port company. The samples for isolation was taken from to the areas nearby the pollution source i.e. the receiving and storage of fuel area, where hydrocarbon containing effluents from the washing and systematic maintenance of storage tanks are discharged.

The soil samples were transported in wide mouth glass flasks, with lid and seal made of Teflon.

The sampling was carried out between 9:00 and 11:00 am, at a depth of about 5.0 cm according to the technical manual survey (Guide for soil sampling 2014). The samples were sieved to 12 mm.

2.2 Determination of the hydrocarbon content

The hydrocarbons content was determined as extractable organic matter (EOM) by extraction in a Soxhlet with dichloromethane, according to the equation:

$$\%EOM = \frac{W_{b+m} - W_{eb}}{W_{soil}} x100$$

Where:

W_{b+m} is the mass of the round-bottom flask with organic matter after distillation (g)

W_{eb} is the mass of the empty round-bottom flask (g)

W_{soil} is the mass of soil with material organic.

This technique is key in the work because of the three types of contaminated soils on the coastal zone only the most contaminated soil (with more concentration of hydrocarbons) will be used for the biodegradation process.

2.3 Isolation of bacteria from soil contaminated with hydrocarbon

The bacteria were isolated from soils contaminated with hydrocarbon sampled distant from the sampling points of soils used for the hydrocarbon degradation analysis. Between the area used to isolate the microorganisms near the hydrocarbon storage tanks and the area where the soil samples were taken in the coastal zone indicated on the map there is a distance of approximately 1.5 kilometers. Samples were stored in sterile flasks.

From each of these samples, 1 g of soil was taken, which was dissolved, with the aid of a magnetic stirrer, into 10 ml of sterile distilled water for 15 min. Subsequently, 1 mL of this solution was added to 99 mL of Tryptone Soya Broth (CTS). Decreasing serial dilutions were made (10^{-1}-10^{-10}) in 0.85% sterile physiological saline water and dilutions were plated using spread chosen in petri dishes containing Tryptone Soya Agar (ATS), using a Drigalsky spatula. The plates were incubated at 30 ± 5 °C, inverted, during 16-24h. After this time the Colony Forming Units (CFU) were counted and the result was expressed as CFU / mL.

The colonies were chosen according to their macroscopic morphological differences, color, elevation, shape, size and borders. They were purified by depletion on plates containing ATS; the morphology of the colonies by

observing the stereo microscope and the cells by Gram stain and subsequent observation the microscope described.

2.4 Determination of growth of heterotrophic population hydrocarbon source (Vela Trial).

For the determination of the growth capacity in a medium with hydrocarbon as sole carbon source, the isolated bacteria were grown in prepared medium plates candle with oil. The average (Vela & Ralston, 1978) was composed by **Solution A**: $Ca(NO_3)_2$, 30 g; KNO_3, 3.5 g; $NaHCO_3$, 62.5 g; NH_4Cl, 35 g; Distilled H_2O, 2 liters. **Solution B**: $FeSO_4.7H_2O$, 5g; $MnCl_2.H_2O$, 3.5 g; $ZnSO_4$, 0.75 g; Distilled H_2O, 1 liter. **Solution C:** KH_2PO_4,10 g; Distilled H_2O 150 mL.
The working medium was formulated by mixing 0.8 ml of solution A, 0.4 ml of solution B and 0.2 mL of the solution will C in 180 mL of distilled water.after which 4 g of bacteriological agar was added.

An oil emulsion was prepared consisting of 18 mL of Distilled H_2O, 1 small drop of TWEEN 20 and 2 g of crude oil. The emulsion was allowed to stir for 4 hours. The emulsion was then added to the formulated working medium. The mixture was sterilized at 121 °C for 15 min. Subsequently, the obtainded medium was distributed aseptically in petri dishes and after 24 h the isolated bacteria were seeded in a groove in the center of the plate. Plates were incubated for 7 days at 30 °C, observing the bacterial growth daily.

2.5 Hydrocarbon degradation by isolates using microcosms.

The effect on hydrocarbon degradation of the isolated micro-organsims was assessed on the more contaminated soil in the vicinity of the coastal mangrove ecosystem area (Figure 1). 400 g of soil contaminated with hydrocarbons (previously sterilized) was placed in aluminum pans of 80 cm x 40 cm and later traslated to the laboratory (ex-situ). The soil in the pans was homogenized before inoculation with the isolated strains (Attachments 1).

The isolated bacteria were CTS grown overnight (overnight) stirring at room temperature. The concentration of inoculum of each selected strain was adjusted to 10^6 CFU / mL, using Pattern 5 of Mc Farland using sterile physiological saline to 0.85%. Cultures were homogeneously dispersed on the soil contained in the trays, using an atomizer.

For 8 weeks soil samples (1g) were taken weekly from the pans to determine bacterial concentration CFU (colony forming units) and EOM (extractable organic matter). The determination of CFU / mL was performed according to the procedure described above. Assays were performed in triplicate (Attachments 1).

3. Results and Discussion:

Table 1 gives the results of the analysis of the three soil samples taken in the study area. Of the three samples analyzed, the soil with the highest value of extractable organic material (EOM) i.e. soil 3 (81.6%) was selected to represent a worst case scenario. Soils 1 and 2 presented a EOM content of 59.8% and 72.4%, respectively, which, although lower, remain very high values, evidencing a high level of contamination in the study area.

Table.1. Chemical characterization of soils.

	Soil 1	**Soil 2**	**Soil 3**
EOM (%)	59.8	72.4	81.6
Total Solids (%)	62	73	86
Fixed Solids (%)	31	44	57
Volatile Solids (%)	24	32	42

3.1 Isolation of microorganisms native to the study area

A total of 25.0 ± 2.0 x 10^6 CFU / mL were isolated. The number of bacterial communities in contaminated sites is usually lower than in unpolluted systems, approximately 0.1% of the total microbial population, and may vary according to the time of sampling or the extent of hydrocarbon pollution, climatic conditions and soil type (Echeverri, 2011)

From the characterization of the colonies under the stereomicroscope four bacterial strains were selected with different morphology. The morphological characteristics of the selected bacterial strains are given in (Table 2).

Table 2. Morphological characteristics of the colonies and cells of the bacterial strains isolated from the soils contaminated with hydrocarbon in the Puerto Moa Company.

Strains isolated	Colony characteristics	Characteristics of cells
1A	Circular and dotted with flat elevation and smooth edges.	Cocos Gram +
2B	Filamentous form with convex elevation and wavy edges.	Bacillus Gram +, some forming long filaments together
3C	Filamentous form with convex edges lifting and also filamentary.	Bacillus Gram +, sporulated (Central endospora),
4D	Convex shape filamentary with wavy edges and lift	Bacillus Gram +, some forming long filaments together

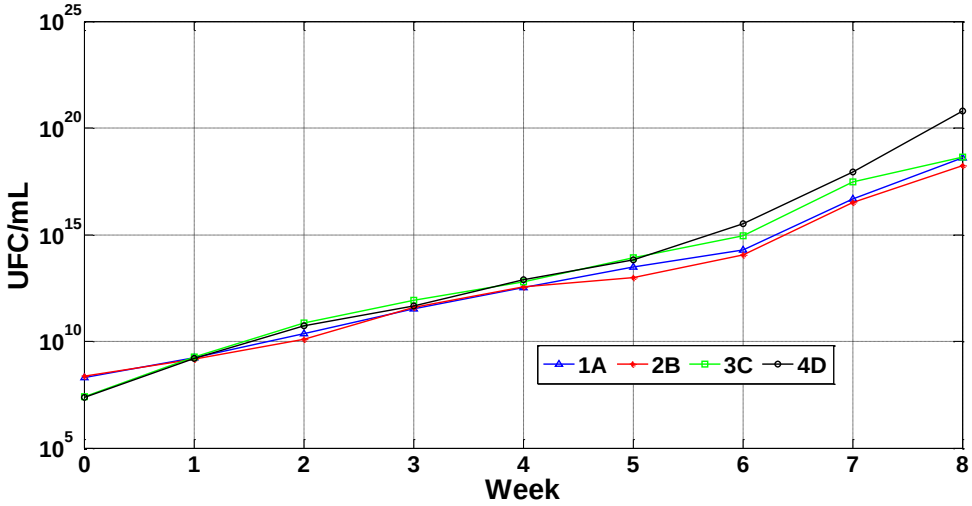
3.2 Test of microbial growth in microcosm

Generally, once inoculated the 4 bacterial strains in trays containing the test microcosm, it could be seen the ability of degradation of these organisms against the contaminant compound by carrying out the different tests indicating that microbial activity exists. Strains 3C and 4D were those that showed growth in the Vela trial (Attachment 2), whereas strains 1A and 2B still did not show activity. This could be another indicator that we are working with metabolically differentiated strains.

Table 3 shows the growth of strains 1A, 2B, 3C and 4D during the 8 weeks of treatment in the microcosm, expressed in CFU / mL. The growth trend of the bacterial population concerning the four strains under study is shown. Over the last two weeks, the microbial density for each of the strains increased rapidly

towards values in the order of 17 x 10^{15}, 39 x 10^{15}, 45 x 10^{15}, 65 x 10^{16} UFC / mL for 2B, 1A, 3C and 4D, respectively.

Figure 2: Microbial growth of strains 1A, 2B, 3C and 4D within 8 weeks of treatment microcosms.



Remarkably, there was no appreciable latency phase in the growth of these microorganisms in the test microcosms, however in the first week of experiment the increase in microbial population was slower than over the following weeks. The absence of a latency phase is most likely because the applied bacteria were isolated from a hydrocarbon contaminated area, so their biosynthetic machinery was already adapted to biodegradation of these compounds for their growth and development.

Using the technique of bioaugmentation as strategy for treating soils polluted with hydrocarbon showed good results in the field test. In order to sanitize the soils in the considered polluted area by bioremediation, it is undoubtedly necessary to upscale these field tests that took place under rather controlled conditions and include new parameters in the selection of experimental strains showing the best performance. Specific biochemical tests could provide more information about them and the possibility of optimization of the decontamination process / technique by including microbial surfactants or other aggregates that can optimize the degradation process.

3.3 Degradation of hydrocarbon in microcosm by the selected strains.

The above explained microbial growth (Section 3.2) demonstrates the ability of the four selected strains to grow from the hydrocarbons present in the soil samples. This ability is also evidenced by a decreased percent of Extractable Organic Matter (EOM) in the treated soil samples over time. Figure 3 gives the hydrocarbon degradation, expressed as $EOM_t/EOM_{t=0}$ in %, as a function of the treatment time for the four selected bacterial strains. The experiment with the 4D strain showed 83.9% degradation of hydrocarbons after eight weeks, similar to the degradation obtained with the 3C (83.1%) strain. However, 2B and 1A strains showed lower degradation values with 73.9 and 38.9%, respectively. However, it should be noted that 2B and 3C strains achieve a higher degradation in the first weeks reaching at week 6 a value of 70% and 54.1% respectively. This might happen because these strains required a shorter time to assimilate the carbon source used.

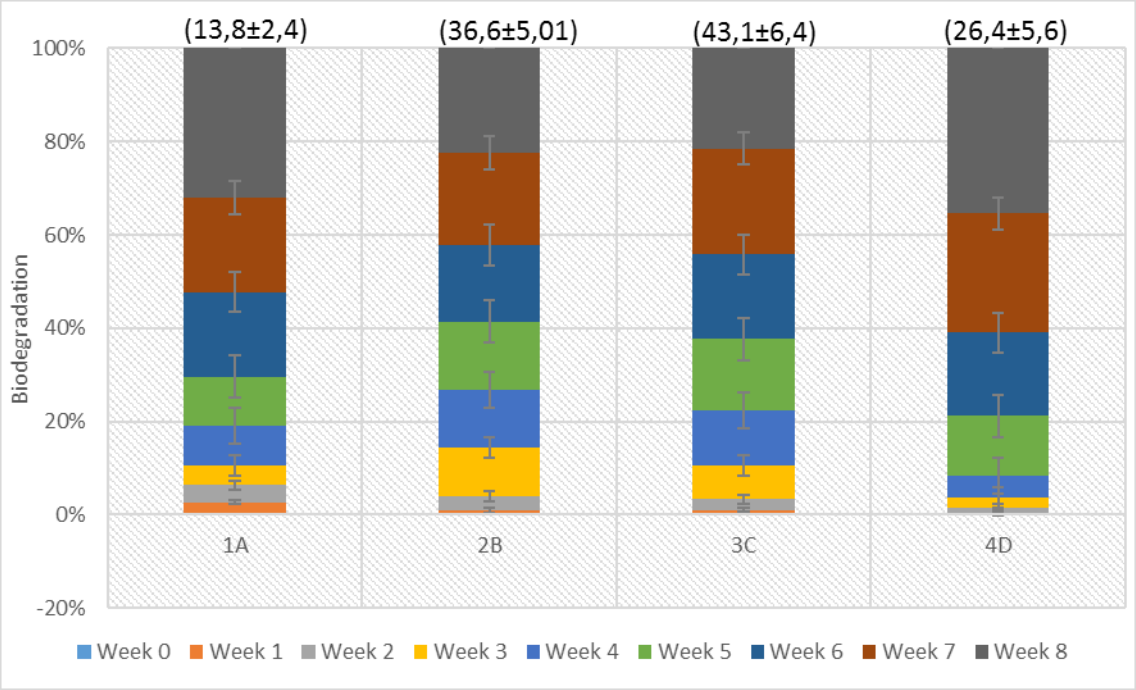
Nozari *et al*. (2014) used a consortium of bacteria *(Acinetobacter radioresistens, Bacillussubtilis, and Pseudomonas aeruginosa*) and managed to establish, by means of bioaugmentation in the microcosm, values of hydrocarbon removal of 45.95% within three days in contaminated soils, modified with essential nutrients and solid mineral resources.

Prakash*et (*2015) used a bacterial consortium (bioaugmentation) of the genus *Kocuria* (Gram +) for degradation of PAHs in microcosm in experimental conditions very similar to those applied in this study in terms of pH, nitrate, phosphate and field capacity. However, they used differnent inoculum culture conditions (1. solid waste pretreated medium + corn steep liquor and 2. standard nutrient medium.), and obtained values of 68% degradation (third week) to 70% (seventh week). These results are very similar to those obtained in this work. The experiment made by these authors showed that for Gram + bacteria the composition and management of the culture medium is vital to PAH degradation processes. Windevoxhel *et al. (*2011) also obtained high rates of hydrocarbons biodegradation by using native strains, reaching removal percentages of 97.2%, 96.0% and 93.1% for three soils tested at different concentrations of hydrocarbons and using the bioaugmentation technique.

It is particularly interesting to assess the growth of bacteria that degrade hydrocarbons isolated from the same soil as a function of to the type of contaminant that is present in the soil as determining factor. The results of Turner *et al.* (2014) show that growth and the percentage of extractable organic matter are closely related and not only correspond to microbial diversity but are also especially related to taxa degradation capabilities of hydrocarbons in a same soil. They used the same soil in three microcosms contaminated with different hydrocarbons (benzene, ethylbenzene and xylene) and a consortium of bacteria previously isolated from the soil. Indeed, differential results were shown for growth and degradation against different types of hydrocarbons. In general, these differences observed in an isolated consortium of the same soil are attributable to the direct effects of the chemical properties of each pollutant on the bacterial indigenous community. Therefore, we could assume that in our case the 3C strain and mainly 4D are better adapted to the most common contaminant (hydrocarbon) present in the soils in and around the port company.

Since the port company is located in a coastal area is important to take into account the work of Paniagua *et al* (2015) as they showed that PAH degrading bacteria can be isolated from a contaminated coastal environment and have a good ability to degradation thereof. Their results showed that there is a positive correlation between the genes of these bacteria and degradability of pollutants such as pyrene and fluoranthene.

Figure 3: *Percentage of hydrocarbon biodegradation strains 1A, 2B, 3C and 4D during the 8 weeks of treatment.*



Conclusions:

To assess the possibility of bioremediation of soils contaminated with oil in and around the Port Company in Moa, Cuba, bioaugmentation experiments were conducted. First, four bacterial strains were isolated from the conatiminated soils based on their capacity to grow in soil contaminated with mineral oil. Then the selected strains were inoculated in sterilized contaminated soils samples that were placed in microcosm (ex-situ) coming of the coastal zone and mangrove swamp. After 8 weeks, EOM degradation of up to 84% was obtained showing biodegradation of hydrocarbons from mineral oil in coastal soils by means of indigenous bacterial strains is a promising technique to sanitize the contaminated soils.

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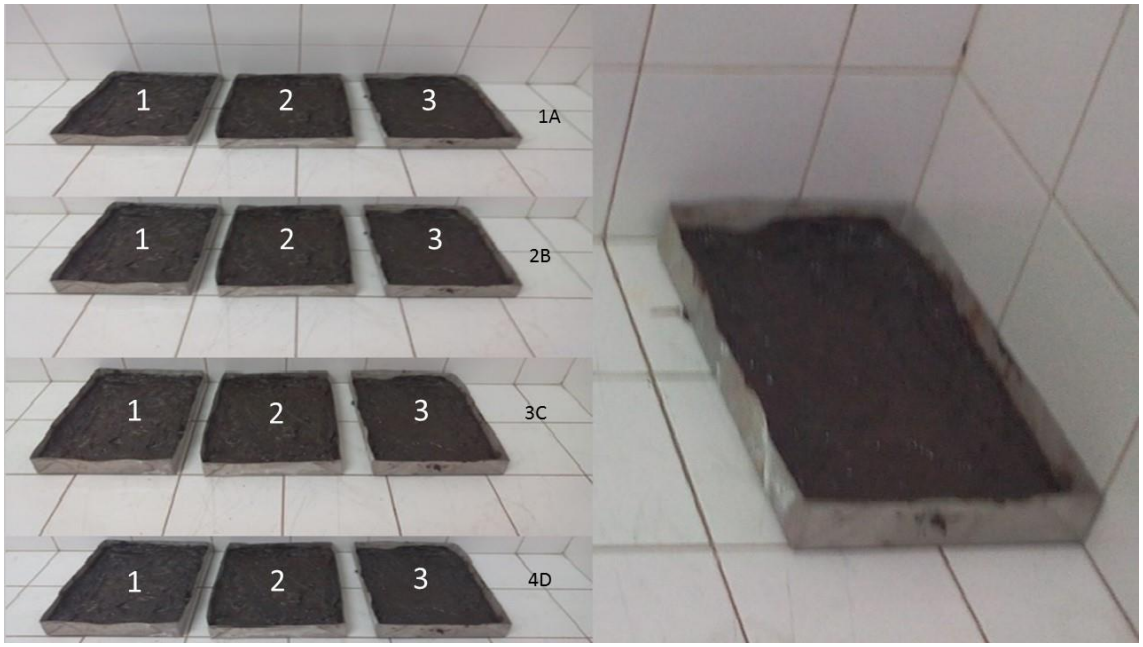
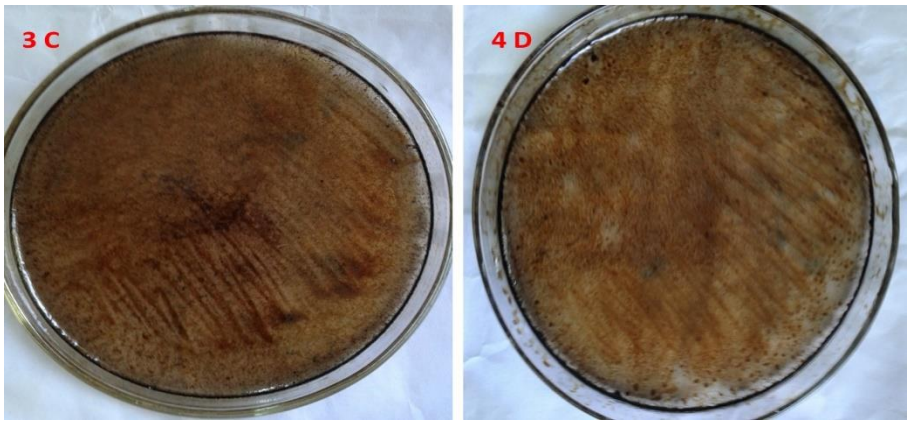


(Attachments 1). Homogenized microcosmos in the laboratory (400 g of soil + hydrocarbon)



(Attachments 2). Growth in Vela trial of strains 3C and 4D